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Characterization of Salivary Metabolic Profiles in Early Childhood Caries (ECC): Diagnostic, Preventive and Therapeutic Implications

Tese no âmbito do Doutoramento em Ciências da Saúde, ramo de Medicina Dentária, orientada pela Professora Doutora Ana Luísa Moreira Costa e Professora Doutora Ana Maria Pissarra Coelho Gil, apresentada à Faculdade de Medicina da Universidade de Coimbra.

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CHARACTERIZATION OF SALIVARY METABOLIC PROFILES IN EARLY CHILDHOOD CARIES (ECC): Diagnostic, Preventive and Therapeutic Implications

Joana Leonor de Sousa e Almeida Pereira

Doctoral Thesis in the Doctoral Program in Health Sciences, branch of Dentistry, under the supervision of Professor Ana Luísa Moreira Costa and Professor Ana Maria Pissarra Coelho Gil, presented to the Faculty of Medicine of the University of Coimbra.

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universidade de aveiro

"We can be heroes
We can be heroes
We can be heroes just for one day
We can be heroes"

Bowie D. & Eno B. (1977). Heroes. On ***Heroes*** [CD]. Berlin: Warner.

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Resumo

Introdução: A cárie precoce da infância (ECC), doença de elevada prevalência com consequências significativas em crianças de idade pré-escolar, é atualmente considerada um problema de saúde pública mundial. A inexistência de preditores de risco consistentes, bem como os desafios inerentes ao tratamento, constituem claras limitações em termos de abordagem e geram uma demanda eminente na definição de novas estratégias que permitam a sua predição e/ou diagnóstico precoce. Ainda que as potencialidades diagnósticas do metaboloma salivar tenham conduzido a relevantes progressos médicos, a aplicação desta abordagem no contexto da investigação em ECC permanece ainda parcamente explorada. O presente trabalho teve como objetivo primário pesquisar o impacto metabólico da ECC na saliva de crianças através de um estudo observacional longitudinal de larga escala, visando a identificação de perfis metabolómicos associados à presença ou progressão da ECC. Com este intuito realizou-se um estudo piloto prévio objetivando estabelecer um protocolo de colheita salivar adequado a estudos metabolómicos e a avaliar preliminarmente o impacto relativo do género, estadio de dentição e cárie dentária no metaboloma salivar de uma coorte pequena de crianças. Em paralelo, procedeu-se à caracterização da prevalência e gravidade de ECC na coorte principal e explorou-se a associação da doença com características sociodemográficas e de comportamentos relativos à saúde oral dos participantes.

Materiais e métodos: Conduziu-se inicialmente um estudo piloto transversal numa amostra de crianças saudáveis (4-16 anos, *n*=38) com o intuito de comparar os efeitos de: (a) estimulação e ausência de estimulação salivar, e (b) colheita através de salivação passiva (PD) e de um dispositivo absorvente no metaboloma salivar, usando espetroscopia de ressonância magnética nuclear de protão (^{1}H-RMN, 500 MHz). No estudo principal procedeu-se à observação oral e à colheita de amostras de saliva não estimulada (USS) de uma coorte de crianças saudáveis (5 anos, *n*=77 controlos e *n*=45 participantes com ECC) de jardins de infância do distrito de Coimbra, em dois períodos de avaliação (aos 0 e 6 meses). Os estudos cumpriram os requisitos éticos e legais exigidos. A colheita e processamento das amostras salivares realizou-se sob condições estandardizadas. A avaliação clínica oral foi efetuada por uma Médica dentista experiente, sujeita a calibração prévia, e seguiu os critérios de diagnóstico ICDAS-II, bem como práticas de prevenção de contaminação cruzada. Procedeu-se ainda à colheita de dados sociodemográficos e de comportamentos relacionados com a saúde oral. As amostras salivares foram analisadas por ^{1}H-RMN, sendo que, atendendo a restrições temporais do presente estudo, apenas os dados de ^{1}H-RMN da avaliação inicial foram sujeitos a análise estatística multivariada e univariada, nesta dissertação. Os dados clínicos, sociodemográficos e comportamentais foram analisados por métodos univariados.

Resultados: Não se detetaram diferenças significativas entre as composições da USS e da saliva estimulada (SS), e a USS foi escolhida para os estudos seguintes. O uso do dispositivo absorvente induziu alterações composicionais significativas, tendo-se considerado o PD como o método preferencial de colheita. No estudo piloto, os potenciais efeitos de confusão do género e estádio de dentição foram residuais em comparação ao impacto da cárie, que induziu variações nos níveis de 21 metabolitos, sobretudo aminoácidos e monossacarídeos. Na coorte principal, as possíveis variáveis de confusão não produziram efeitos detetáveis no metaboloma salivar. Nenhuma das alterações significativas associadas a cárie verificadas no estudo piloto foi confirmada nas amostras da primeira colheita nesta coorte. Surpreendentemente, verificou-se uma variação significativa nos níveis de alanina entre controlos e

crianças com ECC. Crianças do sexo masculino na fase mista da dentição com ECC apresentaram níveis diminuídos de alanina, enquanto que participantes do sexo feminino com lesões em dentina na mesma fase da dentição exibiram níveis aumentados deste metabolito. Detetaram-se ainda tendências qualitativas envolvendo alguns metabolitos, um dos quais a dimetilsulfona, que havia apresentado uma variação significativa no estudo piloto. Na avaliação inicial, a prevalência de ECC e o valor médio do índice de dentes cariados, perdidos e obturados (d_{A-6}mft) foram 40.10% e 1.62 ± 2.52 (*n*=142), enquanto que aos seis meses foram 46.50% e 1.54 ± 2.54 respetivamente (*n*=114). O componente cariado contribuiu 80.40% e 63.40% para o d_{A-6}mft na primeira e na segunda avaliações, respetivamente. A implementação de hábitos de escovagem dentária e a escovagem bi-diária encontraram-se inversamente relacionados com a ECC, e verificou-se uma associação significativa entre a ingestão diária de doces e a doença nesta coorte.

Conclusões: O presente estudo inclui a caracterização mais completa do metaboloma da USS de crianças já realizada por NMR e representa um válido contributo para o conhecimento acerca dos protocolos de colheita de saliva para estudos de NMR. Um perfil composto por 21 metabolitos, indicativo de hidrólise proteica e de desglicosilação foi, pela primeira vez associado à presença de cárie no metaboloma de USS de crianças, apesar de este achado não ter sido confirmado na coorte de maiores dimensões, por possíveis razões que serão nesta tese discutidas. Interessantemente, verificou-se uma variação de alanina, estando a direção da mesma aparentemente associada ao género, por motivos não inteiramente conhecidos. Apesar das limitações do trabalho no que respeita as dificuldades inerentes ao desenvolvimento de um estudo observacional numa amostra de crianças simultaneamente de grandes dimensões e bem definida, sob condições bem controladas, os resultados obtidos consubstanciam as potencialidades da metabolómica salivar no contexto da investigação em ECC, sendo que a realização adicional de estudos longitudinais em amostras de grandes dimensões se assume fundamental.

PALAVRAS-CHAVE: cárie precoce da infância, saliva, metabolómica, crianças, biomarcadores.

Abstract

Introduction: Early childhood caries (ECC), a widely prevalent disease with devastating consequences on young children, is currently considered a worldwide public health burden. The lack of consistent ECC risk predictors, along with the challenges regarding its care, has hindered ECC's management and generated a clear demand for new strategies enabling disease prediction and/or earlier diagnosis. Although the knowledge that saliva's metabolome is a potentially informative mirror of homeostasis has led to major developments in many medical fields, the application of this approach regarding ECC still remains largely unexplored. The present work sought to characterize the metabolic impact of ECC in children's saliva through the development of a large-scale longitudinal observational study, aiming to search for a metabolomic profile specifically associated to ECC's presence or clinical stages. To achieve this, a pilot study was previously conducted to establish a saliva collection protocol suitable for metabolomic research and to preliminary gauge the relative impacts of gender, dentition stage, and caries on the saliva metabolome in a small children cohort. Additionally, ECC's prevalence and severity were determined in the main study participants and the association between the disease and sociodemographic and oral health related behavior characteristics of the subjects was studied.

Material and methods: A cross-sectional pilot study of saliva from healthy children (4-16 years old, *n*=38) was firstly conducted to compared the effects of: (a) stimulation and unstimulation conditions, and (b) collection through passive drool (PD) and using an absorbing device in the salivary metabolome of children, using a nuclear magnetic resonance (^{1}H-NMR, 500 MHz) metabolomics approach. In the main study, oral examination and collection of unstimulated saliva (USS) were performed in a five-year-old healthy cohort (*n*=77 controls and *n*=45 ECC-afflicted) from preschools in Coimbra, in two evaluation periods (initially and after 6 months). Both studies met all ethical and legal requirements for human research. Saliva samples collection and handling were performed under strictly standardized conditions. A calibrated and experienced dentist performed oral examinations according to ICDAS-II diagnostic criteria and all recommendations regarding prevention of contamination and cross-contamination were followed. Metadata on sociodemographic and oral health related behavior variables of participants were also collected. Salivary samples were analyzed through ^{1}H-NMR, for only data from baseline evaluation was subjected to multivariate and univariate analysis in the present dissertation, due to time constraints of this research. Metadata was assessed by univariate statistical methodologies.

Main results: No significant compositional differences were found between USS and stimulated saliva (SS), and the former was used for subsequent studies. Swab collection induced significant changes in sample composition, indicating PD as preferential. In the pilot cohort, the confounding effects of gender and dentition stage were found to be minimal when compared to that of the dental caries, which induced variations in the levels of 21 metabolites, mostly amino acids and monosaccharides. In the main study, possible confounding variables were not shown to produce detectable compositional trends. None of the caries-related significant variations detected in the pilot study could be confirmed in the baseline samples from the larger cohort. Interestingly, alanine was found varying significantly between controls and ECC cases. ECC-afflicted males in mixed dentition exhibited lower alanine levels, whilst females with ECC lesions in dentin in the same dentition phase presented increased amounts of this metabolite. Qualitative tendencies regarding increased levels of some metabolites in ECC were also detected and these trends included a dimethylsulfone variation, previously seen varying significantly in our pilot study. At baseline, ECC prevalence and average decayed/missing/filled teeth score ($d_{A\text{-}6}$mft)

were 40.10% and 1.62 ± 2.52 (*n*=142), whilst at the six-month evaluation rates of 46.50% and 1.54 ± 2.54 were found (*n*=114). The decayed component contributed for 80.40% and 63.40% of the dA-6mft in the first and second evaluation, respectively. Establishment of toothbrushing habits before one year of age and twice daily toothbrushing were inversely associated to ECC and a significant association between daily ingestion of sweets and the disease was also detected within the cohort.

Conclusions: This study presents the most comprehensive characterization of children's USS metabolome by ^{1}H-NMR to date, and to the best of our knowledge, and contributes to fill in relevant gaps regarding saliva collection protocols for ^{1}H-NMR-based studies in pediatric populations. A 21-metabolite profile indicative of protein hydrolysis and deglycosylation was, for the first time to our knowledge, found in children's USS metabolome associated to caries, although the putative hypotheses advanced were not further confirmed in the larger cohort, due to possible reasons discussed later in this thesis. Strikingly, a novel observation regarding alanine variation was seen and the direction of which appeared to be related to gender, for reasons not entirely understood. Despite the limitations of this work, in particular the difficulties inherent to conduct an observational study with a large yet strictly defined cohort of children under well-controlled conditions, our findings confirm the potentialities of saliva metabolomics in ECC research and further longitudinal investigations with large cohorts is warranted.

KEYWORDS: early childhood caries, saliva, metabolomics, children, biomarkers.

List of abbreviations

1D	One dimensional
^{1}H-NMR	Proton nuclear magnetic resonance
2D	Two dimensional
ACeS	Baixo Mondego Health Centers Group *(Agrupamento de Centros de Saúde do Baixo Mondego – AceS Baixo Mondego)*
ANOVA	Analysis of variance
ARSC	Regional Health Administration of the Centre Region *(Administração Regional de Saúde do Centro - ARS Centro)*
br	Broad
CA	Caries-afflicted
CE-MS	Capillary electrophoresis mass spectrometry
CE-TOF-MS	Capillary electrophoresis and time-of-flight mass spectrometry
CI	Confidence interval
CF	Caries-free
CICECO	Centre for Research in Ceramics and Composite Materials *(Centro de Investigação em Materiais Cerâmicos e Compósitos – CICECO)*
CPMG	Carr-Purcell-Meiboom-Gill
CPP-ACP	Amorphous calcium phosphates stabilized by casein proteins
CR	Classification rate
d	Doublet
d value	Number of teeth with untreated carious lesions per child (Decayed component of dmft score)
dd	Doublet of doublets
DM	Diabetes mellitus
dt	Doublet of triplets
dmft	Decayed, missing, or filled teeth score
ECC	Early childhood caries
EMP pathway	Embden-Meyerhof-Parnas pathway
ES	Effect size
FID	Free Induction Decay
FMUC	Faculty of Medicine of the University of Coimbra *(Faculdade de Medicina da Universidade de Coimbra – FMUC)*
FN	False negative
FP	False positive
FPR	False positive rate
FT	Fourier transformation
FQM	Feature quantification matrix
H_0	Null hypothesis
HAP	Hydroxyapatite
HMDB	Human metabolome database
HSQC	Heteronuclear single quantum correlation
ICDAS-II	International Caries Detection and Assessment System
Igs	Immunoglobulins
IPSS	Private Institution of Social Solidarity *(Instituição Particular de Solidariedade Social – IPSS)*
LC-MS	Liquid chromatography mass spectrometry
LV	Latent variable
m	Multiplet
max.	Maximum
MCCV	Monte Carlo cross-validation
min.	Minimum
MS	Mass spectrometry
MVA	Multivariate analysis

M_w	Molecular weight
m/z	Mass-to-charge ratio
NMR	Nuclear magnetic resonance
OHRQoL	Oral health-related quality of life
OSCC	Oral squamous cell carcinoma
p	p-value
PC	Principal component
PCA	Principal component analysis
PD	Passive drool
PLS-DA	Partial least squares-discriminant analysis
PNSE	National School Health Programme *(Programa Nacional de Saúde Escolar - PNSE)*
PRPs	Proline-rich proteins
q	Quartet
Q^2	Goodness of prediction or prediction power
r	Correlation coefficient
ROC	Receiver operating characteristic
s	Singlet
SCS	SalivaBio's Children's Swab® (Salimetrics, State College, PA, USA)
SD	Standard deviation
S-ECC	Severe early childhood caries
Sens.	Sensitivity
Spec.	Specificity
SS	Stimulated saliva
STOCSY	Statistical total correlation spectroscopy
SW	Spectral width
t	Triplet
TCA cycle	Tricarboxylic acid cycle
TN	True negative
TOCSY	Total correlation spectroscopy
TP	True positive
TPPI	Time proportional phase incrementation
TPR	True positive rate
TSP	3-trimethylsilylpropionic acid
UA	University of Aveiro *(Universidade de Aveiro – UA)*
U_i	Unassigned spin system
USS	Unstimulated saliva
UV	Unit Variance
VIP	Variable importance to the projection
VS	Variable selection
WHO	World Health Organization

List of publications and oral presentations including the work presented in this thesis

Pereira J. L., Duarte D., Carneiro T. J., Ferreira S., Cunha B., Soares D., Costa A. L., Gil A. M. (2019). Saliva NMR metabolomics: Analytical issues in pediatric oral health research. *Oral Diseases,* 00,1–10. https://doi. org/10.1111/odi.13117

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Santos F. M., Pereira J. L., Soares A. D., Rosa, S., Xavier M.T., Costa A. L. (2016). #045 Colheita salivar não estimulada em crianças: estudo piloto. *Revista Portuguesa de Estomatologia, Medicina Dentária e Cirurgia Maxilofacial.* 57, 18. http://dx.doi.org/10.1016/j.rpemd.2016.10.044. 10.1016/ j.rpemd.2016.10.044. *Abstract publication*

Oral presentations

"Cárie precoce da infância: uma questão de saúde pública" (Conference; invited speaker) - 1º Congresso Ibérico de Saúde Oral Preventiva e Comunitária – Montijo, 5th May 2017;

"Potenciais fatores de influência no metaboloma salivar de crianças – estudo piloto" (Oral communication). Pereira J. L., Ferreira S. C., Soares A. D., Gil A. M., Costa A.L. - XXIV Congresso da Ordem dos Médicos Dentistas - Lisbon, 12-14th November 2015;

Posters presentation

"Application of NMR metabolomics to the study of saliva stability". Castro B., Duarte D., Pereira J. L., Costa A. L., Gil A. M. – XI Dia da Bioquímica da Universidade de Aveiro - Aveiro, 15th May 2019;

"Colheita salivar não estimulada em crianças: relevância e descrição clínica". Santos F. M., Soares A. D., Xavier M.T., Pereira J. L., Costa A. L. - XV Reunião Anual de Medicina Dentária e Estomatologia de Coimbra - Coimbra, 2-9th April 2016;

"Analysis of potential influence factors on children´s salivary metabolome – pilot study". Pereira J. L., Ferreira S. C., Gil A. M., Costa A. L. - 9th European Academy of Paediatric Dentistry Interim Seminar and Workshop - Brussels, Belgium, 8-9th May 2015.

Table of contents

Acknowledgements ix

Resumo xi

Abstract xiii

List of abbreviations xv

List of publications and oral presentations including the work presented in this thesis xvii

Chapter 1. Background 1
1.1. Early Childhood Caries 3
1.1.1. Disease definition, clinical characteristics and etiology 3
1.1.2. ECC as a public health problem: epidemiology and impact on life quality 10
1.1.3. ECC management: challenges in standard care and disease prediction 13
1.2. Saliva: biological aspects and potential for disease diagnosis 15
1.2.1. Salivary characteristics and principles of secretion mechanisms 15
1.2.2. The role of saliva in the oral environment and in dental caries 17
1.2.3. Diagnostic potential of saliva: salivary sample advantages and current applications 19
1.3. Saliva metabolomics in oral health research 21
1.3.1. Metabolomics: concepts and strategies 21
1.3.2. Analytical techniques: Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry 23
1.3.2.1. Nuclear Magnetic Resonance Spectroscopy 23
1.3.2.2. Mass Spectrometry 24
1.3.3. Statistical tools in metabolomics 25
1.3.4. State of art of saliva metabolomics 31
1.3.4.1. Salivary metabolomics in the context of oral diseases research in adults 32
1.3.4.1.1. Saliva metabolomics and dental caries 33
1.3.4.2. Potentialities of salivary metabolomics in pediatrics 35
1.3.4.2.1. Metabolomics of children's saliva and dental caries research 36
1.3.4.3. Salivary metabolome under physiological conditions 39
1.4. Dissertation topic choice 45
1.5. Aims of this thesis 46
1.6. Outline of this thesis 47

Chapter 2. Experimental procedures 49
2.1. Definition of study population 51
2.1.1. Pilot study (small cohort) 51
2.1.2. Observational study (large cohort) 54
2.2. Collection of saliva and metadata 57
2.2.1. Saliva collection 57
2.2.2. Clinical oral examination 58
2.2.3. Collection of medical, sociodemographic and oral health related behavior metadata 61
2.3. NMR metabolomics 62
2.3.1. Sample preparation for NMR analysis 62
2.3.2. Analysis by NMR spectroscopy 62
2.3.3. Pre-processing and multivariate analysis of NMR data 63
2.3.4. Variable description and operationalization 64
2.3.4.1. Participants' identification and medical status data 64
2.3.4.2. Sociodemographic and oral health related behavior data 64
2.3.4.3. Clinical data from oral examination 65
2.3.5. Metadata statistical analysis 67

Chapter 3. Statistical analysis of metadata from the observational study ... 69
3.1. Definition of study population ... 71
3.2. ECC experience and sociodemographic characteristics ... 72
3.3. ECC experience and oral health related behavior factors ... 78
3.3.1. Oral hygiene habits ... 78
3.3.2. Dietary habits ... 80

Chapter 4. Characterization of saliva by NMR spectroscopy ... 85
4.1. Basic principles of NMR spectroscopy ... 87
4.2. Typical 1D 1H NMR spectrum of saliva ... 90
4.3. Complementary NMR assignment strategies (2D experiments) ... 94

Chapter 5. Optimization of saliva collection protocol for metabolomics ... 99
5.1. Effects of stimulation ... 102
5.2. Effects of using an absorbing/absorbent device ... 107

Chapter 6. Pilot study: preliminary evaluation of relative impact of dental caries on saliva metabolome 113
6.1. Impact of gender ... 115
6.2. Impact of dentition stage ... 118
6.3. Impact of dental caries ... 120

Chapter 7. Observational study: impact of ECC on saliva metabolome in a large cohort ... 129
7.1. Impact of gender ... 131
7.2. Impact of dentition stage ... 134
7.3. Impact of ECC ... 136
7.3.1 Full unmatched cohorts ... 136
7.3.2 Cohorts matched for gender and dentition stage ... 141

Chapter 8. Conclusions and future directions ... 149

References ... 153

List of figures ... 181

Annexes ... 185
Annex I: Ethical approval from the FMUC ethics committee ... 187
Annex II: Ethical approval from the ARSC ... 188
Annex III: Ethical approval from ACeS Baixo Mondego ... 190
Annex IV: Informed consent used in this study ... 192
Annex V: Assent for children's participation in research ... 198
Annex VI: Communication letter to inform parents on child's oral health status ... 199
Annex VII: Study presentation letter ... 200
Annex VIII: Participant information sheet regarding medical, sociodemographic and oral health related behavior metadata ... 202
Annex IX: Oral examination assessment form ... 206

Chapter 1. Background

Chapter 1. Background

1.1. Early Childhood Caries

1.1.1. Disease definition, clinical characteristics and etiology

Despite the overall valuable improvements and breakthroughs in dentistry practice and the decrease in dental caries prevalence in various countries worldwide in recent decades (Çolak, 2013; Dye et al., 2007; Edem, 2018; Kassebaum et al., 2017), dental caries among infants and toddlers still remains a global public health challenge (Edem, 2018; Kaya, Mandaci, & Kargul, 2018; Phantumvanit et al., 2018). It is estimated 60 to 90% of schoolchildren worldwide are affected by dental caries (Petersen, 2003) and, in fact, this condition is considered the most common chronic disease of childhood (K. J. Chen, Gao, Duangthip, Lo, & Chu, 2019; J. Xiao et al., 2019), with a rate five times greater than asthma, the next most prevalent disorder (Becker et al., 2002; Edem, 2018; Kagihara, Niederhauser, & Stark, 2009; United States Department of Health and Human Services (HSS), 2000) and represents the 12th most prevalent disease in all age groups combined (GBD 2015 Disease and Injury Incidence and Prevalent Collaborators., 2016; Kassebaum et al., 2017; Phantumvanit et al., 2018). According to data reported by the 2015 Global Burden of Diseases study, over 560 million children internationally experience dental caries specifically in primary dentition (GBD 2015 Disease and Injury Incidence and Prevalent Collaborators., 2016; Kassebaum et al., 2017).

Early childhood caries (ECC), a particularly severe and rampant form of dental caries in children younger than 71 months, substantially impacts the life of children, families and society, as it comprises several medical, economic and social consequences (American Academy of Pediatric Dentistry (AAPD), 2017c; Phantumvanit et al., 2018; Rai & Tiwari, 2018; Twetman & Dhar, 2015). Though having been long recognized as a unique entity with distinctive clinical features, the condition was comprehensively described in international literature for the first time as 'nursing bottle mouth' in 1962 by Elias Fass (Fass, 1962; Kumar, Pandey, Agrawal, & Agrawal, 2011; Vadiakas, 2008). This initial term arose from the link Fass inferred between ECC and nocturnal milk bottle habits through patients' history taking and, over the years, multiple terms have been progressively used, including 'baby bottle caries', 'baby bottle tooth decay', 'nursing bottle caries', 'nursing caries', 'rampant caries', 'milk bottle syndrome' or 'prolonged nursing habit caries' (Anil & Anand, 2017; Edem, 2018; Grauwe, Aps, & Martens, 2004; Kawashita, Kitamura, & Saito, 2011; N. Tinanoff, 1998; Wyne, 1999). Only more recently the adoption of a less specific terminology was recommended, since former designations reflected and focused on a narrow concept of the disease etiology, presently recognized as of complex and multi-factorial nature, and the term ECC was widely accepted (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c; Çolak, 2013; Drury et al., 1999; Grauwe et al., 2004; N. Tinanoff, 1998).

Currently, ECC is defined as the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled tooth surfaces in any primary tooth in a child under the age of six (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c; Drury et al., 1999). Based on the child's age and the location and severity of the disease (that is, the number of decayed, filling or missing teeth per child, the dmft score), the disease can be further classified as severe ECC (S-ECC) (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c). S-ECC consists on any sign of

smooth-surface caries in a child younger than three years of age, and from ages three through five, one or more cavitated, missing (due to caries), or filled smooth surfaces in primary maxillary anterior teeth or a dmft score greater than or equal to four (at three years of age), greater than or equal to five (at four years of age), or greater than or equal to six (at five years of age) (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c).

ECC comprehends unique characteristics regarding clinical appearance and development, its lesions present early onset, affecting multiple teeth soon after their eruption, and progress through a rapid and atypical pattern of dental caries (Anil & Anand, 2017; Edem, 2018; Kawashita et al., 2011; Vadiakas, 2008). The disease affects tooth surfaces usually less prone to caries risk, including smooth surfaces such as labial surfaces of incisors and molars' lingual and buccal surfaces (Anil & Anand, 2017; Edem, 2018; Harris, Nicoll, Adair, & Pine, 2004; Vadiakas, 2008). Maxillary primary incisors are more vulnerable to the disease and are typically the first teeth to be affected (Edem, 2018), whereas mandibular incisors are usually more protected by tongue and by saliva flow from submandibular and sublingual salivary glands (Edem, 2018; Kawashita et al., 2011) After maxillary incisors have been involved, as the disease develops, buccal and occlusal surfaces of primary maxillary molars are affected, followed by the buccal surfaces of the primary canines and then by mandibular molars, in a pattern that resembles the sequence of dental eruption (Anil & Anand, 2017; Edem, 2018; Kawashita et al., 2011). In general, the longer teeth have erupted, the more extensively they are affected by ECC (Hallett & O'Rourke, 2006; Kawashita et al., 2011) and, therefore, primary molars usually exhibit more extensive lesions compared with second molars, which emerge approximately one year after (Vadiakas, 2008).

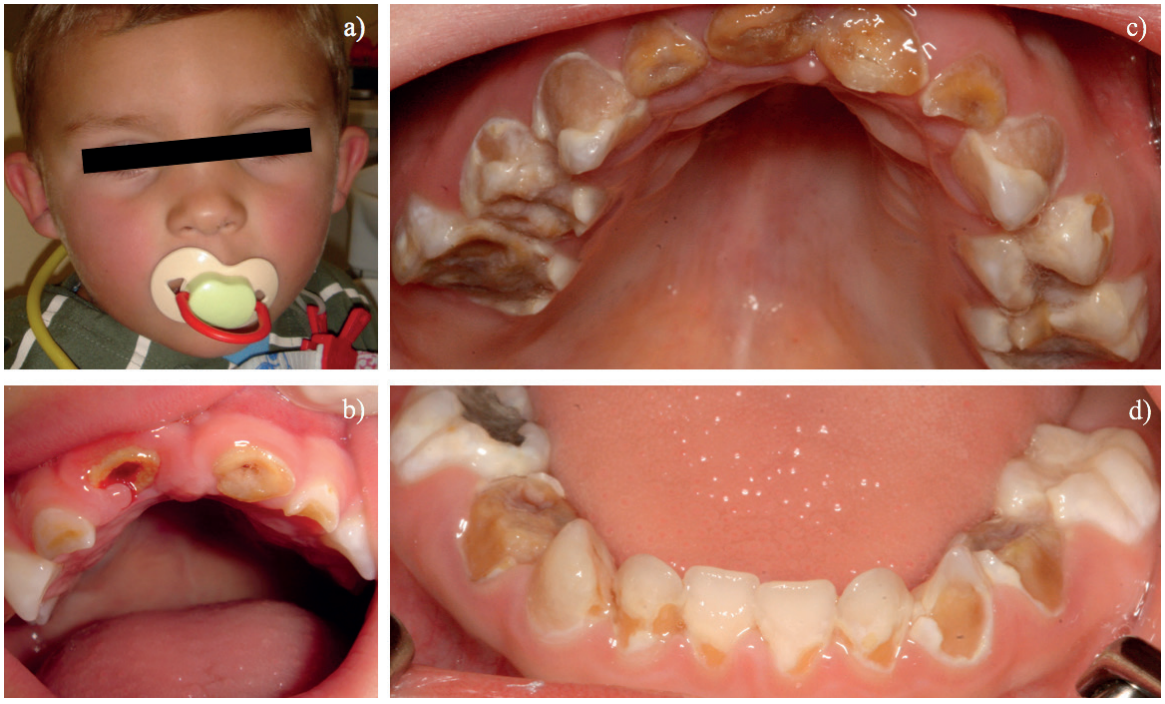

Figure 1.1. a) and b) Two-year-old boy exhibiting ECC cavitated lesions in the maxillary incisors. c) and d) Intraoral photographs of a three-year-old girl presenting ECC lesions in all primary dentition. Distinct clinical stages of the disease can be observed in the affected teeth. Photographs courtesy of Professor Ana Luísa Costa.

The initial clinical presentation of this condition generally involves opaque white-spot demineralization lesions on maxillary primary incisors, mainly along the gingival margin (Anil & Anand, 2017; Çolak, 2013; Edem, 2018; Kawashita et al., 2011) and lesions often extend to multiple surfaces in each of the affected teeth (Edem, 2018). If the disease continues to develop, demineralization lesions in teeth enamel surface undergo cavitation, usually assuming a yellow, brownish and/or black clinical appearance (Çolak, 2013; Kawashita et al., 2011; Kumar et al., 2011) and even though the disease is asymptomatic in early stages, as ECC lesions further extend into dentin or into dental pulp, severe discomfort and pain may be experienced (K. J. Chen et al., 2019). Other ECC signs and symptoms include teeth sensitivity associated to thermal stimuli, difficulty in brushing and chewing and abscesses of dental origin (Ganesh, Muthu, Mohan, & Kirubakaran, 2019; Kühnisch et al., 2016). ECC progress often causes fracture of dental crowns, leading to root stumps (Çolak, 2013; Kawashita et al., 2011; Kumar et al., 2011) and, in severe cases, this rampant break down process can occur in anterior teeth while they are still erupting (Anil & Anand, 2017; Edem, 2018). In fact, if left untreated, the carious process continues to develop and spread, resulting in eventual complete destruction of primary dentition in infants and toddlers in very short span of time (Berkowitz, 2003; Hallett & O'Rourke, 2006; Wulaerhan, Abudureyimu, Bao, & Zhao, 2014).

ECC etiology

Similarly to any type of dental caries, ECC's basic biological mechanism relies on the progressive dental plaque-induced demineralization of dental tissues, resulting from an imbalance of several protective and risk factors over time (American Academy of Pediatric Dentistry (AAPD), 2017c; Gussy, Waters, Walsh, & Kilpatrick, 2006; Kagihara et al., 2009). ECC is a multifactorial disease whose etiology involves bacterial, dietary and host determinants that need to be simultaneously present for the disease's initiation and progression, and are influenced by the interaction of a multitude of sociological, behavioral and environmental aspects (Anil & Anand, 2017; Gussy et al., 2006; Slayton, 2015; Taneja & Singh, 2019; Wulaerhan et al., 2014).

Even though tooth-adherent specific cariogenic bacteria, primarily Mutans Streptococci, are required to experience ECC, their presence is not sufficient for developing disease (Fakhruddin, Ngo, & Samaranayake, 2019; Milgrom et al., 2000). Cariogenic microorganisms in dental plaque, a polymicrobial biofilm, metabolize fermentable carbohydrates, producing acidic substrates as end-products that, over time, can cause demineralization of a susceptible tooth surface or host (Fakhruddin et al., 2019; Gussy et al., 2006; Lenander-Lumikari & Loimaranta, 2000; Meyer & Enax, 2018). These processes are, however, mediated by multiple factors that either protect tooth structure, like saliva's characteristics, host immunity and adequate fluoride exposure, or that increase the risk of experiencing demineralization, such as poor oral hygiene, oral colonization with high levels of cariogenic bacteria, susceptible teeth surfaces due to enamel hypoplasia and ingestion of dietary carbohydrates (American Academy of Pediatric Dentistry (AAPD), 2017c; Slayton, 2015).

Fermentable carbohydrates support bacterial metabolism in biofilms, subsequently leading to the production of acids, such as lactic, formic and acetic acids, whose hydrogen ions lower the dental plaque's pH (Balakrishnan, Simmonds, & Tagg, 2000; Dawes, 2003; Gussy et al., 2006; Lenander-Lumikari & Loimaranta, 2000). Following carbohydrates' ingestion, a sudden drop in pH (generally from a slightly acidic basal value of 7,0 to values ranging from 3,0 to 5,0, approximately) occurs within a five-minute period (Bowen, 2013; Dawes, 2003; Lenander-Lumikari & Loimaranta, 2000). Depending on the child's protective factors, including the amount of remineralizing agents and buffer capacity of saliva, the pH

can gradually return to physiological values after twenty minutes (Bowen, 2013; Dawes, 2003). However, if pH values remain diminished for considerably longer time periods, demineralization of dental tissues will be fostered (Balakrishnan et al., 2000; Bowen, 2013; Dawes, 2003; Gussy et al., 2006). The tendency for loss of minerals in such conditions can be explained by the composition of the tooth structure and the fundamental principles inherent to the critical pH, that is, the pH at which a solution is saturated with respect to a particular mineral (Dawes, 2003). Dental enamel is predominantly constituted by hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$, and, when in contact with water, small amounts of HAP can dissolve, releasing calcium, phosphate and hydroxyl ions (Dawes, 2003; Dawes et al., 2015; Meyer & Enax, 2018), as the following reaction takes place:

$$Ca_{10}(PO_4)_6(OH)_2 \rightleftharpoons 10\ Ca^{2+} + 6\ PO_4^{3-} + 2\ OH^-$$
$$\text{Precipitation} \rightleftharpoons \text{Dissolution}$$

Whenever pH values are below the critical pH the solution is unsaturated in relation to the mineral (enamel, in this case) and, to compensate for lower concentrations of hydroxyl ions, calcium and phosphate ions will tend to dissolve until saturation is reached (Dawes, 2003; Dawes et al., 2015). On the other hand, if the pH of the solution is higher than the critical pH, the solution is supersaturated and minerals will tend to precipitate (remineralization) (Bowen, 2013; Dawes, 2003). Despite frequently referred to as 5.5 (regarding enamel), the critical pH should not be viewed as a fixed value, but rather as a range of values, since it depends on aspects such as the levels of carbonate and fluoride in HAP (that influence enamel's solubility) and on the amounts of salivary and dental plaque's hydroxyl, calcium and phosphate ions (Bowen, 2013; Dawes, 2003). When in homeostasis, salivary and dental plaque's pH are usually higher than the critical pH and consequently they are considered supersaturated in relation to enamel, hence explaining why tooth structure does not dissolve in saliva or under plaque, in physiological circumstances (Bowen, 2013; Dawes, 2003). The cycle of demineralization and remineralization processes is continuous and any significant shifts in the oral environment can affect whether carious lesions are initiated, developed or eventually reverted (Balakrishnan et al., 2000; Bowen, 2013; Dawes, 2003; Gussy et al., 2006). Whenever the subject's protective and caries-inducing factors are not balanced over time and risk factors (as frequent exposure to sugars) prevail, the rate of demineralization will exceed that of remineralization, thereby promoting loss of minerals (Balakrishnan et al., 2000; Bowen, 2013; Dawes, 2003; Gussy et al., 2006).

As progressive and continued demineralization of tooth structures leads to cavitation, biofilm removal from rough and cavitated dental surfaces becomes more difficult and, consequently, bacterial growth and disease progress is fostered (Kagihara et al., 2009). In children in young ages this process is particularly aggravated due to the characteristics of primary teeth composition (American Academy of Pediatric Dentistry (AAPD), 2017c; Kagihara et al., 2009; Meyer & Enax, 2018). Enamel, the hardest tissue in the human body, is a highly mineralized structure, whereas dentin, the subjacent layer, presents a highly organic composition of about 70% HAP, 10% water and 20% proteins, the majority of which collagen (Meyer & Enax, 2018). Enamel production by ameloblasts is restricted to a specific time period in which these cells produce a variety of proteins and attract calcium and phosphate ions to enable their mineralization (Meyer & Enax, 2018). Since the enamel of primary teeth is generated in considerably less time than permanent's teeth enamel, *i.e.* twenty-four months in comparison to up to sixteen years, respectively, as a consequence the former exhibits half the thickness of the latter (Meyer & Enax, 2018). Accordingly, young children are more at risk of developing carious lesions, as their recently erupted primary teeth present immature enamel (American Academy of Pediatric Dentistry (AAPD), 2017c; Vadiakas, 2008) that can be faster demineralized by acids than that of permanent teeth (Kagihara et

al., 2009; Meyer & Enax, 2018). After demineralization has progressed past the enamel layer, the highly organic dentin layer can be rapidly affected, thus partially justifying the rapid progress of dental caries in primary dentition (Kagihara et al., 2009). Besides from susceptibility of the newly erupted primary teeth, other factors that explain young children's substantial high ECC risk relate to the particularities of their dietary habits and to the fact that the establishment of both oral flora and host defence systems is still in progress (American Academy of Pediatric Dentistry (AAPD), 2017a; Vadiakas, 2008).

Several microbiological studies have illustrated the key etiological role of Mutans Streptococci in dental caries, and specifically in ECC (Bachtiar, Gultom, Rahmasari, & Bachtiar, 2018; Balakrishnan et al., 2000; Berkowitz, 2003; Fakhruddin et al., 2019; Lenander-Lumikari & Loimaranta, 2000; Vadiakas, 2008). Investigations have reported that typically ECC-afflicted children's saliva and cultivable dental plaque flora comprise much higher levels of Mutans Streptococci in comparison to ECC-free children (Anil & Anand, 2017; Bachtiar et al., 2018; Berkowitz, 2003; Vadiakas, 2008). Until recently studies had shown that Mutans Streptococci play a crucial part in lesions' onset and cavitation required for secondary colonization by microbes such as lactobacilli and *Candida albicans (Anil & Anand, 2017; Fakhruddin et al., 2019)*. However, and even though current ECC microbial risk markers comprise Mutans Streptococci and Lactobacillus species, more recent studies have indicated the cariogenic microbiome in young children is very diverse and revealed further bacterial species may also be associated with ECC's initiation and progresso (American Academy of Pediatric Dentistry (AAPD), 2017c; Fakhruddin et al., 2019; Vadiakas, 2008).

Mutans Streptococci possess a variety of caries-inducing properties and capacity to predominate in dental plaque (Balakrishnan et al., 2000; Fakhruddin et al., 2019; Lenander-Lumikari & Loimaranta, 2000). Acidogenicity and aciduricity are among those properties and refer to the capacity of metabolizing substrates into organic acids and then surviving and proliferating in such acidic environments, respectively (Anil & Anand, 2017; Balakrishnan et al., 2000; Fakhruddin et al., 2019; Lenander-Lumikari & Loimaranta, 2000). Other properties comprise synthesis of extra- and intracellular polysaccharides (Azevedo, van de Sande, Romano, & Cenci, 2011; Balakrishnan et al., 2000; Fakhruddin et al., 2019). The synthesis of extracellular polysaccharides occurs through the metabolism of sucrose and the production of these molecules increases the porosity of the dental plaque matrix, consequently promoting diffusibility of substrate, increasing the acidogenicity of the biofilm and enhancing bacterial adhesion to dental surfaces (Azevedo et al., 2011; Balakrishnan et al., 2000; Ccahuana-Vásquez et al., 2007; Fakhruddin et al., 2019). Intracellular polysaccharides, on the other hand, are synthesized from sucrose and other carbohydrates and allow Mutans Streptococci survival and continued acid production during periods of nutrient deprivation (Azevedo et al., 2011; Balakrishnan et al., 2000; Ccahuana-Vásquez et al., 2007; Fakhruddin et al., 2019).

Early acquisition of Mutans Streptococci has been proven to be a key risk factor for developing ECC (Bastos et al., 2015; Damle et al., 2016; Doméjean et al., 2010; Kagihara et al., 2009; Lenander-Lumikari & Loimaranta, 2000; Slayton, 2015). Mother- (or caregiver-) to-child transmission is known to be the major pathway for Mutans Streptococci initial acquisition, through a process designated vertical transmission, with saliva being the principle vehicle of transfer (Bastos et al., 2015; B.W. Chaffee, Gansky, Weintraub, Featherstone, & Ramos-Gomez, 2014; Damle et al., 2016; Doméjean et al., 2010). Although most initial studies have reported that oral colonization occurred between the age of 19 and 33 months, during the 'first window of infectivity' period, it has been shown Mutans Streptococci acquisition can occur since shortly after birth, as pre-dentate oral cavities have been found colonized by Mutans Streptococci (Bastos et al., 2015; Berkowitz, 2003; B.W. Chaffee et al., 2014; Damle et al., 2016; Doméjean et al.,

2010; Slayton, 2015; Vadiakas, 2008). The extent of Mutans Streptococci transmission depends upon the frequency and amount of exposure to Mutans Streptococci through saliva-sharing activities (American Academy of Pediatric Dentistry (AAPD), 2017c) and research has shown that children whose mothers present elevated levels of Mutans Streptococci as a result of untreated caries lesions exhibit higher ECC risk (American Academy of Pediatric Dentistry (AAPD), 2017c; J. Xiao et al., 2019). On the other hand, horizontal transmission, which relates to transmission between other family members or between unrelated infants in preschools, is also a known source of Mutans Streptococci acquisition (American Academy of Pediatric Dentistry (AAPD), 2017c; Anil & Anand, 2017; Doméjean et al., 2010; Vadiakas, 2008). Accordingly, late initiation of oral hygiene habits, as well as inappropriate hygiene practices like poor toothbrushing regularity and/or technique and toothbrushing without supervision by caregivers constitute important factors increasing risk of ECC (Anil & Anand, 2017; Meyer & Enax, 2018; Paglia et al., 2016). Ineffective mechanical dental plaque removal results in higher deposits of plaque, affecting microbial colonization and increasing acid challenges experienced by dental surfaces (Anil & Anand, 2017; Meyer & Enax, 2018; Paglia et al., 2016; Norman Tinanoff & Reisine, 2009).

As previously stated, infant feeding practices have been long implicated as causative factors of ECC (American Academy of Pediatric Dentistry (AAPD), 2017c; Drury et al., 1999; Grauwe et al., 2004; N. Tinanoff, 1998). Firstly, ECC's etiology was solely attributed to bottle usage after the first year of life and only later *ad libitum* (*i.e.* on demand) breastfeeding was also associated to the disease (American Academy of Pediatric Dentistry (AAPD), 2017c; Grauwe et al., 2004). Since, in the last decades, the disease was reckoned as a multifactorial condition, the significance of overall fermentable carbohydrates metabolism by tooth-adherent bacteria in ECC's etiology was highlighted (American Academy of Pediatric Dentistry (AAPD), 2017c; Anil & Anand, 2017; Berkowitz, 2003). Currently, available evidence on the role of sugars (like sucrose, fructose and glucose) and other fermentable carbohydrates (such as highly refine flour or cooked starch) in ECC's initiation and development is solid and overwhelming (Benjamin W. Chaffee, Feldens, Rodrigues, & Vítolo, 2015; Gussy et al., 2006; P. J. Moynihan & Kelly, 2014; Phantumvanit et al., 2018; Vadiakas, 2008). High and frequent consumption of sugar-sweetened foods and beverages constitutes a known associated risk factor and, in particular, sucrose is considered the most cariogenic substrate, attending to the fact that it is metabolized to acids and also to intracellular and extracellular polysaccharides by dental plaque microbiota, as aforementioned, promotes improved bacterial adhesion to dental structures and favors the growth and selection of cariogenic bacteria (Azevedo et al., 2011; Ccahuana-Vásquez et al., 2007; Gussy et al., 2006; Naidoo & Myburgh, 2007; Vadiakas, 2008). Specific infant feeding practices have also been reported as risk factors for ECC (American Academy of Pediatric Dentistry (AAPD), 2017c; B.W. Chaffee et al., 2014; Meyer & Enax, 2018). Children who regularly are bottle-fed or use sippy cups with any liquid other than water during nighttime are at high risk for ECC and, moreover, concerns have been expressed on the potential impact prolonged and *ad libitum* breastfeeding might exert on ECC's development (American Academy of Pediatric Dentistry (AAPD), 2017c; Anil & Anand, 2017; Meyer & Enax, 2018; Phantumvanit et al., 2018; Tham et al., 2015).

Host-related factors, mostly associated with tooth structure and salivary characteristics, can also predispose a child or a particular tooth/dental surface to experience ECC (Gussy et al., 2006; Norman Tinanoff & Reisine, 2009; Vadiakas, 2008). Besides from the aforementioned immaturity of enamel generally increasing susceptibility to caries processes until final maturation is achieved, specific developmental defects of the tooth structure may be related to ECC occurrence (Gussy et al., 2006; Vadiakas, 2008). Some studies have reported a significant relationship between caries development and hypoplastic or hypomineralized defects, lesions which may occur as consequence of developmental disturbances to tooth germs during embryological or perinatal periods (Caufield, Li, & Bromage, 2012; Gussy et al.,

2006; Vadiakas, 2008). In particular, association between enamel hypoplasia and ECC prevalence has been evidenced in many investigations (American Academy of Pediatric Dentistry (AAPD), 2017c; Gussy et al., 2006; Vadiakas, 2008). This probable higher susceptibility has been ascribed to the fact that conditions including pre- or perinatal malnutrition, illnesses or infections and premature birth or low-birth weight may disturb dental development, resulting in loss of integrity of the enamel's surface and thus enabling increased plaque accumulation in such porous and irregular surfaces (Caufield et al., 2012; Gussy et al., 2006; Vadiakas, 2008).

Saliva is considered the host's leading defense system and exerts a protective role against the development of ECC, mainly through buffering plaque's organic acids, aiding in oral clearance, acting as a mineral's reservoir to support remineralization processes and through its antimicrobial activity (Gussy et al., 2006; Kubala et al., 2018; Pedersen, Sørensen, Proctor, Carpenter, & Ekström, 2018; Vadiakas, 2008). Multiple salivary components interplay in these biological mechanisms, including calcium, phosphates, and pellicle-forming proteins, proteins able to maintain mineral supersaturation in both saliva and dental plaque and compounds with antimicrobial properties (A.V. N. Amerongen, Bolscher, & Veerman, 2004; Gussy et al., 2006; Humphrey & Williamson, 2001; Vadiakas, 2008). All of these functions largely depend upon saliva's flow and composition, which will be addressed in detail in section 1.2 of this chapter (*'Saliva: biological aspects and potential for disease diagnosis'*).

Lastly, in addition to the biological variables involved in ECC's etiology, awareness on the relevance of multiple behavioral, social, cultural and environmental determinants of the disease has been raised (Gussy et al., 2006; Vadiakas, 2008). Consistent evidence has shown low socioeconomic status and family income level may be important determinants of ECC (American Academy of Pediatric Dentistry (AAPD), 2016; Gussy et al., 2006; Norman Tinanoff & Reisine, 2009; Vadiakas, 2008). Indeed, children from low-income families not only are more frequently afflicted by ECC, but also more likely present untreated disease (Kagihara et al., 2009; Norman Tinanoff & Reisine, 2009). Research has demonstrated ECC is particularly prevalent among socially disadvantaged and ethnic minority communities and specific social and behavioral determinants have been held responsible for this, including caregiver's education level and behavioral patterns related to feeding and hygiene practices and to parental caries experience / regularity of dental visits (Çolak, 2013; Kawashita et al., 2011; P. Moynihan et al., 2019; Phantumvanit et al., 2018; Seow, 2012; Vadiakas, 2008). An association between low levels of parental (especially maternal) education and higher rates of ECC has also been established (American Academy of Pediatric Dentistry (AAPD), 2016; Kagihara et al., 2009; Phantumvanit et al., 2018).

1.1.2. ECC as a public health problem: epidemiology and impact on life quality

ECC poses a significant public health burden, owing to its global high prevalence and devastating effects for both preschool children and their families and financial impact on society (Meyer & Enax, 2018; Phantumvanit et al., 2018; Slayton, 2015). Currently considered a global pandemic, especially in developing countries (Çolak, 2013; Kumar et al., 2011), with nearly 1.8 billion new cases per year worldwide (GBD 2015 Disease and Injury Incidence and Prevalent Collaborators., 2016; J. Xiao et al., 2019), ECC is additionally characterized by a large proportion of untreated lesions, regardless of the country's socioeconomic level (Duangthip, Gao, Lo, & Chu, 2017; P. Moynihan et al., 2019; Phantumvanit et al., 2018). In the last decades ECC has shown an overall marked increase in several countries and research clearly indicates it is a highly prevalent and widespread condition, though available evidence has shown significant disparities in its prevalence rates, varying from 1% to 90% (K. J. Chen et al., 2019; Edem, 2018; Phantumvanit et al., 2018). Even though evidence has shown ECC is disproportionately present in lower socioeconomic groups, the disease is not by all means restricted to such backgrounds (Anil & Anand, 2017; Becker et al., 2002; Meyer & Enax, 2018; P. Moynihan et al., 2019; Phantumvanit et al., 2018). Epidemiological data has indicated ECC prevalence ranges from 1% to 12% in most developed countries (Çolak, 2013; Congiu et al., 2014; Edem, 2018; Kaya et al., 2018), whereas in developing nations prevalence is reported as between 28% and 90% (Edem, 2018; Phantumvanit et al., 2018). Recent data from Australia, however, has revealed a worrying prevalence rate of more than 50%, despite being considered a developed nation (Chrisopoulos & Harford, 2016).

Some of the highest ECC prevalence rates, ranging up to 90%, have been reported in some African, Middle and South Eastern Asian countries (Anil & Anand, 2017; Çolak, 2013; Duangthip et al., 2017; Edem, 2018; Ganesh et al., 2019; Phantumvanit et al., 2018; X. Wang, Wei, Li, & Mei, 2017; X. Zhang et al., 2016), like Cambodia and Indonesia, where 90% of three to five-year-old children present a dmft score higher than six (P. Moynihan et al., 2019). In North America, published studies concerning preschool children from the United States have indicated a 40% prevalence (Bugis, 2012), whilst investigations conducted in South America have indicated 41.6% (Amaral, Batista, Meirelles, Cypriano, & Sousa, 2014) to 67.8% prevalence rates (Lourenço, Saintrain, & Vieira, 2013) in Brazil, for instance.

In Europe, considerable variation respecting ECC presence has also been found. Some developed countries have reported figures below 36%, such as 2.1 to 11.4% in Sweden (A. I. Ismail & Sohn, 1999; Strömberg, Holmn, Magnusson, & Twetman, 2012), 13% in Switzerland (Phantumvanit et al., 2018), 26.2% in Germany (Grund, Goddon, Schüler, Lehmann, & Heinrich-Weltzien, 2015), and 36% in Norway (Phantumvanit et al., 2018). Conversely, in other European nations as the United Kingdom, Finland, Portugal, Spain and Italy this figure rises up to higher values ranging from 55% to 63% (Albert, Leyda, & Ribelles, 2016; Borralho, 2014; K. J. Chen et al., 2019; Sónia Mendes & Bernardo, 2015; Phantumvanit et al., 2018).

Overall, ECC's reported prevalence around the globe varies widely between nations, but also between investigations conducted in the same country or within distinct regions from the same country. Such variance may partially be ascribed to differences with regard to culture, ethnicity, socioeconomic status, dietary and oral hygiene patterns and further unknown factors fluctuating from country to country or from area to area (Anil & Anand, 2017). However, it should be noted that these estimates have also been largely limited by factors concerning disparities in methodological research features (American Academy of Pediatric Dentistry (AAPD), 2017c; Phantumvanit et al., 2018). Not only investigations have addressed ECC's impact on study samples comprising preschool children of different ages and/

or from high-risk populations (Dye et al., 2007), but have also used discrepant diagnostic thresholds (Phantumvanit et al., 2018). To date, most of surveys conducted to measure ECC prevalence have used presence of cavitation as the minimal caries diagnosis level, instead of considering noncavitated lesions, a criterion consistent with the currently accepted ECC definition (American Academy of Pediatric Dentistry (AAPD), 2017c; Phantumvanit et al., 2018).

The same drawbacks are verified when interpreting epidemiological data on Portuguese ECC-afflicted children. The majority of studies involving larger and probabilistic samples comprised children in the reference age groups recommended by the WHO, that is, six- and twelve-year-old children. Research among Portuguese preschool children are, therefore, scarce and has mainly used cavitated lesions in dentin as diagnostic criteria, *i.e.*, following the WHO 1997 criteria (World Health Organization (WHO), 1997, 2013), leading to underestimation of the disease impact. Although in the last two decades a general improvement regarding oral health status of six-year old children has been verified, as the rate of children free from dental caries has increased from 17.0% to 51%, since 1984 to 2005, respectively (C. M. Almeida, 1997; C. M. Almeida, Emílio, Möller, & Marthaler, 1990a, 1990b, 1991; C. M. Almeida, Petersen, André, & Toscano, 2003; Direção-Geral da Saúde (DGS), 2000, 2008), national epidemiological data from 2013 revealed almost half of the six-year population (45.2%) still suffered from dental caries (Calado, 2017; Direção-Geral da Saúde (DGS), 2015). This latter study evaluated the presence of dental caries following the International Caries Detection and Assessment System II (ICDAS-II) diagnostic criteria (A. Ismail et al., 2007), which considers noncavitated caries lesions in enamel as the minimal diagnosis level. Accordingly, authors stated the caries prevalence rate of 45.2% they had found, relative to a cut-off diagnostic point of cavitated lesions, corresponded, in fact, to a caries prevalence of 68.1% if ICDAS-II criteria were considered (Calado, 2017; Direção-Geral da Saúde (DGS), 2015). In Portugal, studies conducted in subjects younger than six years old have reported an ECC rate varying from 15% to 48.1% according to WHO criteria (C. M. Alves, 2009; S. Mendes, Rodrigues, Abukumail, Guerreiro, & Bernardo, 2009; Vasconcelos, Melo, & Gavinha, 2004). More recently, a study performed in preschools using ICDAS-II diagnostic criteria found an ECC prevalence of 56.4% (or 26%, according to WHO criteria) and a dmft score of 2.5, having also highlighted that most of ECC lesions found in the study sample were untreated (Borralho, 2014; Sónia Mendes & Bernardo, 2015).

ECC's impact on life quality

Due to ECC's rapid and extensive nature, when allowed to progress the disease yields serious impact on patients, comprising broad orofacial, medical and social detrimental consequences during a critical period of their physical and biopsychosocial development (Çolak, 2013; Fontana, 2015; Meyer & Enax, 2018). Indeed, even though ECC affects the primary dentition, numerous long-term consequences have been reported, many of which extending beyond the oral cavity, and thereby emphasizing the marked disease's public health relevance (Davidson et al., 2016). Overall available studies have highlighted the significant impact ECC exerts on children's and their families' oral health-related quality of life (OHRQoL), a subjective multidimensional construct which reflects the impact of oral conditions on the individual's physical functioning and psychosocial well-being, and demonstrated ECC is the oral condition that most negatively affects children's OHRQoL (Abanto et al., 2011; Easton, Landgraf, Casamassimo, Wilson, & Ganzberg, 2008; Kramer et al., 2013; Norman Tinanoff & Reisine, 2009; H. M. Wong, McGrath, King, & Lo, 2011).

Maintaining an intact primary arch and good oral health status during early childhood is widely reckoned as of extreme importance for children's well-being and development of the stomatognathic system

(Davidson et al., 2016) and, accordingly, premature tooth loss often caused by ECC can produce severe sequelae (Anil & Anand, 2017; Bissar, Schiller, Wolff, Niekusch, & Schulte, 2014; Çolak, 2013). In the short- to medium-term, such sequelae include compromised function in eating and speech production (Kagihara et al., 2009), as well as self-esteem issues (Gussy et al., 2006; Kagihara et al., 2009; Yang Li et al., 2011).In fact, disfigurement caused by decayed or missing teeth often affects children psychosocially, as it negatively influences their self-image, socializing abilities and peer interaction (Gussy et al., 2006; Jain, Patil, Shivakumar, & Srinivasan, 2018; Yang Li et al., 2011). Regarding eating constraints, concerns have been raised about an association between untreated ECC and a higher risk for delayed or reduced growth (American Academy of Pediatric Dentistry (AAPD), 2017c; Kagihara et al., 2009; Kawashita et al., 2011). Although available evidence is not consensual, multiple studies have reported ECC-afflicted children who present cavitated lesions in dentin have lower body weight and height in comparison to ECC-free children (Bissar et al., 2014; L.-W. Li, Wong, Peng, & McGrath, 2015; Shen, Bernabé, & Sabbah, 2019).These debilitating outcomes relate to a reduced ability to sleep and malnourishment risk attributed to chewing difficulties, disinclination to eat and/or avoidance of certain types of foods mainly caused by tooth loss and/or inflammation and pain of dental origin (Bissar et al., 2014; Jain et al., 2018; Kawashita et al., 2011).

In addition, premature tooth loss, either simply as a result of dentition destruction caused by ECC progress or as an outcome of its treatment, can affect children's facial growth and development and may interfere with permanent teeth alignment, thus increasing the risk for orthognatic problems later in life (Achmad et al., 2019; Kagihara et al., 2009; Paglia et al., 2016). Also, among the long-term complications, longitudinal studies have shown children affected by ECC not only present a great probability of undergoing disease recurrence after treatment, but also of experiencing subsequent dental caries in mixed phase and permanent dentition (American Academy of Pediatric Dentistry (AAPD), 2017c; Berkowitz, Amante, Kopycka-Kedzierawski, Billings, & Feng, 2011; Kawashita et al., 2011; Leong, Gussy, Barrow, de Silva-Sanigorski, & Waters, 2013; Y. Li & Wang, 2002).

Another common consequence of ECC is dental pain, which is mostly associated to untreated teeth and can harmfully impact on children's overall regular activities, from sleeping, eating or playing, to concentrating and learning (Gussy et al., 2006; Kagihara et al., 2009; Leong et al., 2013; Paglia et al., 2016; Rai & Tiwari, 2018; Norman Tinanoff & Reisine, 2009). Diminished ability to learn, scholar performance and cognitive development have been reported in ECC-afflicted children, since pain and discomfort interfere adversely with nutrition, concentration capacity and school participation (American Academy of Pediatric Dentistry (AAPD), 2017c; Gussy et al., 2006; Paglia et al., 2016; Turton, Durward, Manton, Bach, & Yos, 2016). Besides from presenting impaired speech (Bissar et al., 2014; Kagihara et al., 2009; Mothupi, Nqcobo, & Yengopal, 2016) and poorer concentration capacity while in the classrooms, affected children also experience increased days with restricted activity (Phantumvanit et al., 2018) and higher absenteeism rates from school due to ECC-related symptoms or to attend dental appointments (American Academy of Pediatric Dentistry (AAPD), 2017d; Turton et al., 2016). In fact, epidemiological data has revealed children five to seven years of age in the United States of America are estimated to miss more than seven million school hours annually as a consequence of caries lesions which began during the preschool period (Norman Tinanoff & Reisine, 2009).

Children with ECC also exhibit higher risk of emergency room visits and hospitalizations, owing to pain and infection of dental origin (American Academy of Pediatric Dentistry (AAPD), 2017c; Anil & Anand, 2017; Gussy et al., 2006; Leong et al., 2013; Paglia et al., 2016). Such acute or chronic oral infectious processes can cause more widespread issues, considering that patients present an increased risk of developing potentially life-threatening infections and bacteremia (S. S. Gao, Duangthip, Lo, & Chu, 2018;

Kagihara et al., 2009; P. Moynihan et al., 2019; Paglia et al., 2016). Even though direct and indirect costs of ECC have not been systematically documented, the economic impact of the disease is considered a significant burden for both families and society (American Academy of Pediatric Dentistry (AAPD), 2017c; S. S. Gao et al., 2018; Kagihara et al., 2009; Rai & Tiwari, 2018; Twetman & Dhar, 2015). The increased ECC costs reflect not only expenses related to dental treatment, but also work absenteeism and possible loss of income of parents or legal guardians (G. L. Ribeiro et al., 2015). ECC's cost of care also poses a large impact on health systems, considering a substantial amount of hospital resources is spent in many countries treating ECC lesions and its sequelae (S. S. Gao et al., 2018; Paglia et al., 2016; Rai & Tiwari, 2018; Norman Tinanoff & Reisine, 2009). Lastly, despite not thoroughly reported to our knowledge, estimates of ECC's human costs have included reports of several deaths related to sepsis, as well as a number of other complications occurring after dental treatments performed under general anesthesia (Casamassimo, Thikkurissy, Edelstein, & Maiorini, 2009; Kagihara et al., 2009; Norman Tinanoff & Reisine, 2009).

1.1.3. ECC management: challenges in standard care and disease prediction

Regardless of being a preventable disease, ECC's aggressive pattern can rapidly cause dental destruction in very young children (Gussy et al., 2006; Kagihara et al., 2009; Vadiakas, 2008). Failure to prevent ECC and/or to diagnose its initial lesions often leads to several adverse effects and, furthermore, delays in providing proper treatment usually allow patients' oral condition to worsen, consequently increasing treatment's complexity and cost (Meyer & Enax, 2018). The nature of the dental treatment instituted in ECC patients depends on several aspects relative to the severity of the disease, child's age, development level, comprehension skills and ability to cooperate, as well as the patient's and family motivation towards dental treatment (American Academy of Pediatric Dentistry (AAPD), 2017c; Slayton, 2015; Taneja & Singh, 2019). While disease management should include simple interventions as dental prophylaxis, fluoride application and oral hygiene and diet assessment and counselling, the majority of ECC-afflicted children usually necessitate multiple complex treatments like dental restorations, pulp treatments and more complex rehabilitation (American Academy of Pediatric Dentistry (AAPD), 2017c; Ganesh et al., 2019; Taneja & Singh, 2019). In general, as patients frequently need either full mouth or considerable extensive rehabilitation, the mentioned procedures typically imply performing numerous prolonged chairside appointments, obviously requiring some degree of cooperation from the patient (Slayton, 2015). Therefore, in very young and/or uncooperative children, ECC therapy is often only viable under deep sedation or general anaesthesia (Anil & Anand, 2017; Davidson et al., 2016; Ganesh et al., 2019; Kraljevic, Filippi, & Filippi, 2017). Accordingly, not only ECC current standard care can be expensive and involve possible health risks, but it also poses considerable personal and professional implications on the child's family (American Academy of Pediatric Dentistry (AAPD), 2017c; S. S. Gao et al., 2018; Rai & Tiwari, 2018; Norman Tinanoff & Reisine, 2009). In addition, since the disease cannot be successfully addressed with rehabilitative treatment alone (Twetman & Dhar, 2015; Wulaerhan et al., 2014) because affected children present an increased risk for future caries lesions, either soon thereafter or later in adolescence or adulthood (American Academy of Pediatric Dentistry (AAPD), 2017c; Becker et al., 2002; Kawashita et al., 2011; Kraljevic et al., 2017), its consequences are not limited to the childhood period. With regard to immediate ECC recurrence, relapse rates of approximately 40% within the first year after dental rehabilitative surgery under general anesthesia have been described (Berkowitz et al., 2011; Petti, 2010; Twetman & Dhar, 2015) and thus disease control cannot, therefore, solely rely on restorative and surgical procedures (Kawashita et al., 2011).

In recent years ECC management has shifted towards a more preventive and preservative approach, rather than the longstanding surgical or restorative perspective (American Academy of Pediatric Dentistry (AAPD), 2016; Kawashita et al., 2011). Establishment and modification of the individual risk factors inherent to ECC onset and development, as well as providing demineralization control strategies and instructing caregivers on oral matters are practices of the upmost importance regarding disease management (Kawashita et al., 2011; P. Moynihan et al., 2019). To promote ECC's prevention, multiple preventive concepts should be explored in order to favor protective factors and minimize caries-promoting aspects, mostly strategies regarding minimization of early Mutans Streptococci acquisition, implementation of proper dietary and oral hygiene practices and opportune access to oral health care (American Academy of Pediatric Dentistry (AAPD), 2017c, 2017a; Kagihara et al., 2009).

Currently, despite available risk assessment programs assess numerous factors including salivary flow rate, pH and buffering capacity, amounts of cariogenic pathogens and dietary and oral hygiene parameters, such screening models present limited accuracy and validity (Fontana, 2015; Pappa, Kousvelari, & Vastardis, 2018; Vadiakas, 2008). Unfortunately, to date, the single most consistent risk predictor of ECC is still the previous occurrence of dental caries (Anil & Anand, 2017; Gussy et al., 2006; Hart et al., 2011; Vadiakas, 2008). The use of this clinical criterion as a predictor of future ECC does not enable more susceptible children to be identified before the establishment of the disease, thus not fulfilling the main goal of primary prevention (Anil & Anand, 2017; Gussy et al., 2006; Hart et al., 2011; Vadiakas, 2008). The lack of effective predictors of ECC has clearly limited management strategies and the development of a reliable and precise risk assessment test would assist identification of highly susceptible individuals, with obvious preventive and therapeutic implications (Hajishengallis, Parsaei, Klein, & Koo, 2017; Vadiakas, 2008).

1.2. Saliva: biological aspects and potential for disease diagnosis

1.2.1. Salivary characteristics and principles of secretion mechanisms

Human saliva is a mucoserous exocrine secretion resulting from a complex mixture of fluids from varied sources (Humphrey & Williamson, 2001; Kaufman & Lamster, 2002; Nunes, Mussavira, & Bindhu, 2015). In fact, this mixed fluid found in the oral cavity, known as whole saliva, is composed by the secretions of three pairs of major salivary glands and of numerous minor salivary glands, combined with various amounts of gingival crevicular fluid, desquamated epithelial cells and food debris, serum and blood derivatives, suspended microorganisms and their metabolites, as well as gastric reflux liquid and mucosal transudations from the nasal cavity and pharynx (P. D. Almeida, Grégio, Machado, Lima, & Azevedo, 2008; Chiappin, Antonelli, Gatti, & De Palo, 2007; Humphrey & Williamson, 2001; Mahvash Navazesh & Kumar, 2008; Pappa et al., 2018; Pedersen et al., 2018; Roi et al., 2019).

Saliva is a clear and very dilute biofluid that, despite being constituted by approximately 99% water, also contains an important and wide variety of functional organic and inorganic components (Chojnowska et al., 2018; Humphrey & Williamson, 2001; Kubala et al., 2018; Pappa et al., 2018; Roi et al., 2019; Streckfus, 2015). Salivary inorganic compounds include many electrolytes, mainly cations of sodium, potassium, calcium and magnesium and anions of phosphate and carbonate (Chiappin et al., 2007; Chojnowska et al., 2018; Greabu et al., 2009; Humphrey & Williamson, 2001; Roi et al., 2019), whilst its fraction of organic substances involves protein and non-protein components (Chiappin et al., 2007; Roi et al., 2019). A large number of protein compounds are supplied by this biofluid, including amino acids, peptides (as defensins, histatins, statherins and cathelicidins), glycoproteins (as mucins, proline-rich proteins (PRPs), immunoglobulins (Igs), agglutinin and lactoferrin), enzymes (as amylase, lipases, proteases, peroxidase and lysozyme) and lipoproteins, among others (Chiappin et al., 2007; Cuevas-Córdoba & Santiago-García, 2014; Feron, 2019; Gröschl, 2017; Hemadi, Huang, Zhou, & Zou, 2017; Yeh et al., 2010). Small amounts of non-protein components as nitrogenous products (*e.g.* ammonia and urea), carbohydrates (*e.g.* glucose), lipids (such as cholesterol and free fatty acids) and hormones are also present in saliva (Chiappin et al., 2007; Chojnowska et al., 2018; Feron, 2019; Humphrey & Williamson, 2001; Matczuk, Żendzian-Piotrowska, Maciejczyk, & Kurek, 2017; Nunes & Macedo, 2013; Roi et al., 2019).

The major salivary glands comprise bilateral pairs of the parotids, located next to the ears and extending to the lower border of the mandible, and the submandibular and sublingual glands, placed on the floor of the oral cavity (Humphrey & Williamson, 2001; Pedersen et al., 2018; Streckfus, 2015). About 600 to 1000 minor glands, ranging from 1 to 5 mm, can be found in distinct locations of the submucosa throughout the mouth, from the labial, buccal and palatal, to the lingual, retromolar and regions (Chojnowska et al., 2018; Humphrey & Williamson, 2001; Pedersen et al., 2018; Streckfus, 2015).

The designations *major* and *minor* denote to the anatomic size of the glands; however, and even though the large glands are responsible for the majority of the whole-saliva volume (90%) and electrolyte content (Kubala et al., 2018; Mahvash Navazesh & Kumar, 2008; Pedersen et al., 2018), the contribution of minor glands for total secretion is of the upmost relevance, due to the protective and lubricating nature of its mucous secretions (Humphrey & Williamson, 2001; Mahvash Navazesh & Kumar, 2008).

The composition of secretions differs according to the type of salivary gland it was produced by (P. D. Almeida et al., 2008; Neyraud, Tremblay-Franco, Gregoire, Berdeaux, & Canlet, 2013). Acinar cells, the secretory end pieces of the glands in which saliva is primarily produced, determine whether secretions are serous, mucous or of mixed nature (Aps & Martens, 2005; Gröschl, 2017; Streckfus, 2015). While parotids are categorized as purely serous glands, presenting less viscous secretions that lack mucins and present more ions and enzymes (P. D. Almeida et al., 2008; Chiappin et al., 2007), submandibular and sublingual are mixed glands, secreting mixed serous and mucous fluids (Chiappin et al., 2007; Chojnowska et al., 2018; Humphrey & Williamson, 2001; Roi et al., 2019). Mucous secretions exhibit higher amounts of mucins and little or no enzymatic properties and tend to be viscoelastic, a relevant characteristic for lubrication and retention of saliva in oral structures (P. D. Almeida et al., 2008; Pedersen et al., 2018; Proctor, 2016). As to minor glands, those located on the palate and base of tongue are purely mucous and labial, buccal and molar salivary glands are mixed (Chiappin et al., 2007; Chojnowska et al., 2018; Proctor, 2016).

Salivary secretion is regulated by neural reflexes arising from the autonomic nervous system (by both sympathetic and parasympathetic nerves) and is also under hormonal control (due to gastrointestinal hormones) (Humphrey & Williamson, 2001; Pappa et al., 2018; Pedersen et al., 2018). Accordingly, the salivary output will vary significantly in terms of flow, total volume and composition depending on the differential activation of glands by distinct sources and types of stimuli (Neyraud et al., 2013; Pappa et al., 2018; Pedersen et al., 2018). In resting circumstances and absence of any significant triggers, a continuous basal flow of unstimulated saliva (USS) is produced under dominance of sympathetic innervation (C. Alves, Brandão, Andion, & Menezes, 2010; Chiappin et al., 2007; Mahvash Navazesh & Kumar, 2008; Takeda et al., 2009). This salivary flow is relatively low and nearly 65% of it is produced by the submandibular glands, 20 to 25% by the parotids, 4 to 8% from sublingual and less than 10% from the minor salivary glands (Gröschl, 2017; Humphrey & Williamson, 2001; Kubala et al., 2018; Proctor, 2016; Roi et al., 2019). Conversely, in the presence of a trigger, such as gustatory, olfactory or mechanical (through the act of chewing) stimuli, signals are transmitted to the salivary nuclei in the brainstem, leading to glandular stimulation and altering salivary secretion (Chiappin et al., 2007; Humphrey & Williamson, 2001; Proctor, 2016; Streckfus, 2015). As a response to stimulation, parasympathetic pathway drastically alters the percentage of contribution from each gland: parotids generate most of the total secretions, submandibular glands produce about 35% and sublingual and minor glands contribute only 7 to 8% each (Chiappin et al., 2007; Humphrey & Williamson, 2001; Neyraud et al., 2013; Nunes & Macedo, 2013; Streckfus, 2015). The produced saliva, known as stimulated saliva (SS), is consequently waterier, more serous and exhibits a markedly increased flow rate (P. D. Almeida et al., 2008; Chiappin et al., 2007; Humphrey & Williamson, 2001; Streckfus, 2015).

1.2.2. The role of saliva in the oral environment and in dental caries

Saliva fulfills a variety of roles imperative for maintaining oral homeostasis (Baum, Yates, Srivastava, Wong, & Melvin, 2011; Castagnola et al., 2011; Kubala et al., 2018; Lenander-Lumikari & Loimaranta, 2000; Pedersen et al., 2018). By moistening, lubricating and protecting dental structures and the oropharyngeal mucosa, saliva enables multiple oral functions as speech articulation, masticating and swallowing, and also exerts some digestive action and participates in the perception of taste and thermal stimuli (Chojnowska et al., 2018; Kubala et al., 2018; Lenander-Lumikari & Loimaranta, 2000; Pedersen et al., 2018). Moreover, saliva's protective nature against biological, mechanical, thermal and chemical injuries to oral tissues constitutes one of its main functions (Chiappin et al., 2007; Chojnowska et al., 2018; Humphrey & Williamson, 2001; Kubala et al., 2018). All this large array of salivary actions is related to its fluid characteristics and specific biologically relevant components (Humphrey & Williamson, 2001; Ruhl, 2012). With regard to the relevance and complexity of its composition, it is important to highlight that the majority of its compounds are multifunctional, redundant and amphifunctional, meaning that they perform more than one function, play similar functions to different extents and act both for and against the host, respectively (A. V. N. Amerongen et al., 2004; Humphrey & Williamson, 2001; Ruhl, 2012; Tabak, 2006).

The complex role of saliva in protecting against dental caries has been widely studied and documented (Dodds, Johnson, & Yeh, 2005; Idrees, Nassani, & Kujan, 2018). It is known saliva considerably influences all processes involved in caries dynamics, as it affects all three major components in caries' etiology: dental surfaces, substrate and dental plaque (Dodds et al., 2005). Summarily, the part that saliva plays in caries dynamics is dependent upon its flow rate and clearance, pH and buffer capacity, impact on calcium phosphate homeostasis and effects on bacterial metabolism (Dodds et al., 2005; Puy, 2006).

Oral clearance, the capacity of diluting and eliminating from the oral cavity food debris (and, to a lesser extent, free microorganisms), is one of saliva's most basic and important functions and it is intrinsically related to salivary flow rates (C.-G. Crossner, Hase, & Birkhed, 1991; Dawes et al., 2015; Humphrey & Williamson, 2001; Puy, 2006). The average total daily flow of whole saliva in adults ranges from 500 to 1500 mL, under physiological conditions (P. D. Almeida et al., 2008; Gröschl, 2017; Mahvash Navazesh & Kumar, 2008; Pappa et al., 2018). In healthy adults, any value higher than 0.1 mL per minute for USS flow rates is considered acceptable, whereas for SS flow rate, the minimum normal volume per minute is about 0.2 mL (P. D. Almeida et al., 2008; Humphrey & Williamson, 2001; Nunes et al., 2015). Nonetheless, research has shown there is great intra and interindividual variability concerning salivary flow rates, especially in children, in which the stimulated saliva flow rate is reported to range between 0.1 to 6 mL per minute (P. D. Almeida et al., 2008; Humphrey & Williamson, 2001; Sánchez-Pérez et al., 2009). Salivary secretion markedly increases during mastication and also displays circadian rhythm: it reaches its maximum peak at around 12 a.m. and during sleep hours the reported salivary flow is nearly zero (Aps & Martens, 2005; Humphrey & Williamson, 2001; Proctor, 2016; Puy, 2006). Further aspects affecting salivary production quantitatively (as well as qualitatively) include physiological and pathological conditions, *e.g.* the type and intensity of stimulation, individual level of hydration, stress, body posture, physical exercise, intake of medication, circannual variations over seasons and many diseases (P. D. Almeida et al., 2008; Aps & Martens, 2005; Chiappin et al., 2007; Dodds et al., 2005; Humphrey & Williamson, 2001; Nunes et al., 2015). The dilution and removal of substrate from the oral cavity after food ingestion is also highly dependent on the residual volume, the amount of saliva remaining in the mouth after swallowing (C.-G. Crossner et al., 1991; Dawes et al., 2015; Humphrey & Williamson, 2001). In terms of oral health, clearance is considered a crucial capacity, as it diminishes the retention of fermentable carbohydrates, fundamental for the acid production in dental plaque (C.-G. Crossner

et al., 1991; Humphrey & Williamson, 2001). It is known that the salivary flow is not even throughout the oral cavity and regional variations occur (Humphrey & Williamson, 2001; Puy, 2006). Structures in mandibular lingual regions, for instance, are subjected to high volume salivary flows, thereby also experiencing improved clearance rate of bacterial acids (Humphrey & Williamson, 2001). This fact partially justifies why primary mandibular incisors are usually less susceptible to ECC, as previously stated (Edem, 2018; Humphrey & Williamson, 2001; Kawashita et al., 2011).
The salivary buffer capacity is vital for the maintenance of saturation with regard to HAP in saliva, as well as of a balanced oral microbiota, and it is closely related to salivary flow (Dawes et al., 2015; Humphrey & Williamson, 2001; Pedersen et al., 2018). Saliva is a slightly acidic biological fluid, its normal pH is about 6 to 7, and it may range from 5.3 in low salivary flow to 8 when in peak flow (Greabu et al., 2009; Humphrey & Williamson, 2001; Spielmann & Wong, 2011). Its buffer capacity exerts a crucial role in the regulation of pH levels in both saliva and dental plaque and, therefore, in the maintenance of oral homeostasis, by neutralizing acids ingested or produced by microorganisms in the oral cavity (Pappa et al., 2018; Pedersen et al., 2018).

Saliva's ability to maintain a proper acid-base balance relies on the bicarbonate, the phosphate and the protein systems and urea (Kubala et al., 2018; Pedersen et al., 2018). The carbonic acid and bicarbonate system is the major determinant of saliva's buffering capacity and can increase salivary pH through the conversion of the carbon dioxide dissolved in saliva into a gaseous state (Aps & Martens, 2005; Dawes et al., 2015; Nirmala, Sulthana, Jagadeeswari, & Girija, 2013; Pappa et al., 2018; Petersen, 2003; Vuletic, Peros, Spalj, Rogic, & Alajbeg, 2014). This mechanism occurs mostly in high flow rates during stimulation, owing to higher concentrations of bicarbonate than in resting circumstances (Aps & Martens, 2005; Nirmala et al., 2013; Pappa et al., 2018; Pedersen et al., 2018; Vuletic et al., 2014), whereas the phosphate system contributes to the buffer capacity especially during low salivary flow rates (Kubala et al., 2018; Pappa et al., 2018; Vuletic et al., 2014). The mechanism dependent on proteins only contributes to salivary buffer capacity at very low flow rates and at low pH values (below 5) and it is mostly attributed to low-molecular weight histidine-rich peptides (Humphrey & Williamson, 2001; Pedersen et al., 2018; Vuletic et al., 2014). Lastly, urea also contributes to pH regulation by releasing ammonia as a result of being metabolized by dental plaque (Dawes et al., 2015; Hicks, Garcia-Godoy, & Flaitz, 2004; Humphrey & Williamson, 2001).

Saliva is also accountable for maintaining calcium phosphate homeostasis, thus directly interfering with demineralization and remineralization processes (Dawes et al., 2015; Hicks et al., 2004). The fact that saliva is supersaturated with calcium and phosphate with respect to HAP promotes the resistance of dental surfaces to cariogenic processes, as it reduces the probability of demineralization occurrence and favors the remineralization of susceptible enamel surfaces (Dawes et al., 2015; Hicks et al., 2004; Puy, 2006). A number of salivary proteins, namely statherins, some PRPs, cystatins and histatins, bind to HAP and aid maintaining such stability of calcium and phosphate saturation in saliva (Dawes et al., 2015; Dodds et al., 2005; Hicks et al., 2004; Lenander-Lumikari & Loimaranta, 2000; Puy, 2006; Schenkels, Veerman, & Nieuw Amerongen, 1995). Fluoride in saliva, on the other hand, also assists in inhibiting dissolution of enamel, since it maintains saliva supersaturated with respect to any fluorapatite formed in the tooth surface (Dawes et al., 2015; Humphrey & Williamson, 2001).

Saliva also provides specific immune response through Igs (IgA, IgM and IgG) (Humphrey & Williamson, 2001; Kubala et al., 2018; Pedersen et al., 2018). Salivary Igs, in particular IgA, which has long been recognized as the first line of host defense, present antimicrobial action, inhibit microbial adhesion and promote phagocytosis (Dawes et al., 2015; Pappa et al., 2018; Pedersen et al., 2018). In parallel, many non-specific defensive factors can also be distinguished (Dawes et al., 2015; Gröschl, 2017; Hicks et al., 2004; Kubala et al., 2018). Several components, the majority of which enzymes, peptides, proteins and

glycoproteins, are accountable for saliva's anti-bacterial, anti-fungal and anti-viral mechanisms, including mucins, histatins, cystatins, statherins, PRPs, peroxidases, amylase, lysozyme, lactoferrin and defensins (A. V. N. Amerongen et al., 2004; Dawes et al., 2015; Humphrey & Williamson, 2001; Pedersen et al., 2018). Particularly, mucins, PRPs, cystatins, statherins, lysozyme and lactoferrin, among others, participate in the formation of the acquired enamel pellicle, a thin protein biofilm covering enamel's surfaces and protecting it from acid challenges, and modulate microbial colonization by interacting with dental surfaces and microorganisms (Dawes et al., 2015; Humphrey & Williamson, 2001; Kubala et al., 2018; Lenander-Lumikari & Loimaranta, 2000; Pedersen et al., 2018). Lysozyme, for instance, is able to hydrolyze the bacterial cellular membranes and lactoferrin binds to iron, a vital nutrient to microbes (A. V. N. Amerongen et al., 2004; Dawes et al., 2015; Humphrey & Williamson, 2001; Pappa et al., 2018; Pedersen et al., 2018). Salivary peroxidases catalyze the oxidation of salivary thiocyanate into hypothiocyanate, which is highly toxic to bacterial systems, and cystatins act as protease inhibitors, thus preventing microorganisms to metabolize salivary proteins (A. V. N. Amerongen et al., 2004; Dawes et al., 2015; Farnaud, Kosti, Getting, & Renshaw, 2010; Humphrey & Williamson, 2001). Salivary agglutinins, statherins and mucins also promote bacterial aggregation, hence facilitating its clearance from the oral cavity (A. Amerongen, 2002; Dodds et al., 2005; Lenander-Lumikari & Loimaranta, 2000).

1.2.3. Diagnostic potential of saliva: salivary sample advantages and current applications

The use of saliva as a diagnostic fluid has gained attention over the past years in several medical fields and has been successfully employed in the screening of many systemic disorders (Chojnowska et al., 2018; Malamud, 2011; Martí-Álamo, Mancheño-Franch, Marzal-Gamarra, & L., 2012; Roi et al., 2019; Spielmann & Wong, 2011; Streckfus, 2015; Wallner-Liebmann et al., 2016). As previously described, since saliva contains a number of substances as hormones, nucleic acids, proteins, enzymes, cytokines, interleukins and antimicrobial constituents, many of which plasma-derived (either actively or passively transferred) and therefore also found in systemic circulation, this biofluid is considered a *mirror* of the oral and general health status (Chiappin et al., 2007; Chojnowska et al., 2018; Lee, Garon, & Wong, 2009; Miller et al., 2010; Spielmann & Wong, 2011; Streckfus, 2015). Many of these salivary compounds can be related to oral and systemic diseases, thus presenting the potential to act as biomarkers for multiple disorders and, in addition, saliva offers distinct advantages over other bodily fluids (Chiappin et al., 2007; Greabu et al., 2009; Roi et al., 2019). Saliva has been frequently referred to as the ideal non-invasive diagnostic material for many purposes, as it is an easily accessible fluid whose collection is painless, convenient, inexpensive and safe, with minimal risk of infections for both patients and health professionals (Amado, Ferreira, & Vitorino, 2013; Chojnowska et al., 2018; Nunes & Macedo, 2013; Ruhl, 2012; Streckfus, 2015). This diagnostic fluid allows obtaining multiple samples within a short period of time through easy procedures, as often no specialized equipment is necessary for its completion, and requires less sample manipulation after collection (Greabu et al., 2009; Martí-Álamo et al., 2012; Miller et al., 2010; Roi et al., 2019). Salivary sampling also tends to be stress-free and is usually better tolerated than blood collection, the current most popular and widely accepted fluid used in clinical diagnostics (Chojnowska et al., 2018; Cuevas-Córdoba & Santiago-García, 2014; Greabu et al., 2009).

To use saliva as a specimen numerous methods for saliva sampling can be performed, namely harvesting whole saliva, individual gland saliva, with or without stimulation (Cuevas-Córdoba & Santiago-García, 2014; Nirmala et al., 2013; Nunes & Macedo, 2013; Topkas, Keith, Dimeski, Cooper-White, & Punyadeera, 2012). USS whole saliva can be relatively easily collected by passive drool (PD), the most recommended

method for open-field studies or routine sampling (Gröschl, 2017; Mahvash Navazesh & Kumar, 2008; Nunes et al., 2015). In the PD method the subject allows saliva to pool in his mouth, with his head tilted forward and without performing any oral movements, and then gently drools saliva directly into a test container (Chiappin et al., 2007; Kaufman & Lamster, 2002; M. Navazesh, Mulligan, Kipnis, Denny, & Denny, 1992; Mahvash Navazesh & Kumar, 2008). SS, on the other hand, can be collected by mastication action (of paraffin wax or a neutral gum base) or by gustatory stimulation (through application of an acid, as citric or malic acid, in the subject's tongue or use of candy drops, for instance) (C. Alves et al., 2010; Chiappin et al., 2007; Donzella, Talge, Smith, & Gunnar, 2009; Kaufman & Lamster, 2002; Mahvash Navazesh & Kumar, 2008; Nunes et al., 2015).

Some commercial devices designed to facilitate saliva collection are currently available (Khurshid et al., 2016; Nunes et al., 2015; Pfaffe, Cooper-White, Beyerlein, Kostner, & Punyadeera, 2011; Topkas et al., 2012). Besides from suction devices (micropipettes, syringes or saliva aspirators), most strategies involve using an absorbent base for saliva absorption, either under stimulated or unstimulated circumstances, and after collection devices must be subjected to centrifugation and/or compression, in order to obtain the salivary sample (Chiappin et al., 2007; Kubala et al., 2018; Michishige et al., 2006; Nunes et al., 2015; Priya & Prathibha, 2017; Voegtline & Granger, 2014). Available absorbing devices include a variety of materials, from synthetic or cotton swabs to filter papers and hydrocellulose microsponges and some companies have even designed specialized versions of devices for children and babies (C. Alves et al., 2010; Chiappin et al., 2007; Cuevas-Córdoba & Santiago-García, 2014; Donzella et al., 2009; Granger et al., 2007; Gröschl, 2017). These devices present several advantages, as they enable saliva sampling in populations in which collecting saliva would be challenging or would not yield sufficient sample volume, as newborns, children with poor cooperation skills and elderly participants (Granger et al., 2007; O'Farrelly & Hennessy, 2013; Topkas et al., 2012). The major drawbacks of most absorbing devices involve not allowing direct and accurate measurement of the collected volume and potential inference with the detection or quantification of specific analytes (Chiappin et al., 2007; Granger et al., 2007; Nunes et al., 2015; Topkas et al., 2012).

Secretions from individual salivary glands can, however, be more suitable samples for the diagnosis of gland-specific pathology or for certain research purposes (some otorhinolaryngologic investigations, for instance), rather than whole saliva (which contains secretions from all glands and components of non-glandular origin as well) (Chiappin et al., 2007; Humphrey & Williamson, 2001; Nirmala et al., 2013; Nunes et al., 2015; Roi et al., 2019). Gland-specific collection can be performed through glandular ducts cannulation or specific collecting devices to the glandular ducts emergence area (Chojnowska et al., 2018; Gröschl, 2017; Mahvash Navazesh & Kumar, 2008; Nunes et al., 2015). Nonetheless, such procedures are extremely intricate, slow, invasive and require skilled operators (Chojnowska et al., 2018; Gröschl, 2017; Nunes et al., 2015).

Overall, the choice of sample type, collection system or device should be carefully pondered, taking in consideration multiple issues including the sampling purposes, the analytes to be investigated, the volume of saliva needed to complete analysis, and also the characteristics of the individuals to be sampled (Chiappin et al., 2007; Hofman, 2001; Nunes et al., 2015).

1.3. Saliva metabolomics in oral health research

1.3.1. Metabolomics: concepts and strategies

Over the last decade major advances in global understanding of biological systems have been made, mainly due to the increasing popularity and significance in biomedical sciences of the study of genes and their expression (genomics and transcriptomics, respectively), of protein expression (proteomics) and of metabolic compounds (metabolomics) (Kussmann, Raymond, & Affolter, 2006; Lindon, Nicholson, & Holmes, 2007; Lindon, Nicholson, Holmes, & Everett, 2000). In fact, the integration of these '*omic*' (a neologism widely adopted in research fields) technologies has not only provided valuable insights into several physiological and pathological mechanisms, but also allowed significant breakthroughs in the search for accurate diagnostic and prognostic biomarkers, as well as predictors for treatment response (Alonso, Marsal, & Julià, 2015; Nicholson, Holmes, et al., 2012; Rattner & Bathe, 2017; A. Zhang, Sun, & Wang, 2012b).

'*Omic*' approaches adopt a holistic snapshot of the molecules that constitute a cell, tissue or organism(Lindon et al., 2000) (Lindon et al., 2000). While genomics comprises the systematic study of an organism's set of genes, transcriptomics assesses changes in genome expression and transcription into proteins, hence giving rise to proteomics as the large-scale study of proteins within a living cell, system or organism (Kussmann et al., 2006; Villas-Bôas, Roessner, Hansen, Smedsgaard, & Nielsen, 2007). The complex interaction between genes and environmental factors reflects on several biochemical processes, thus inducing functional alterations on the proteome and metabolome, a term that refers to the vast group of metabolites (*i.e.* low molecular weight compounds, M_w < 1500 Dalton) in a biological system (S. Barnes et al., 2016; Beale et al., 2016; A.-H. M. Emwas, Salek, Griffin, & Merzaban, 2013; Shah, 2018; Smolinska, Blanchet, Buydens, & Wijmenga, 2012). Metabolites, as the intermediates or final downstream products of genome expression, protein synthesis and cell's response to environmental and external perturbations, are considered more reliable indicators of homeostasis and non-homeostasis status than those occurring at upstream levels; in fact, impact on the metabolic profile can be significant even when alterations in proteins and transcripts are not detectable (A.-H. M. Emwas et al., 2013; Romano et al., 2018; Wallner-Liebmann et al., 2016; A. Zhang, Sun, Wang, Han, & Wang, 2012). In this context, metabolomics, commonly defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (A.-H. M. Emwas et al., 2013; A. Zhang, Sun, & Wang, 2012a), is a promising and fast growing '*omics*' platform (Dona et al., 2016; Lenz & Wilson, 2007; Lindon, Holmes, Bollard, Stanley, & Nicholson, 2004; Lindon et al., 2000). Since metabolomics enables the assessment of the overall levels of metabolites, that is, the parallel measurement of both endogenous and exogenous metabolites of biological samples (A. Zhang, Sun, Wang, et al., 2012; A. Zhang, Sun, & Wang, 2012b), it provides a global overview of the metabolic dynamics that reflect the biochemical activity underlying physiological and pathological states (Alonso et al., 2015; A.-H. M. Emwas et al., 2013; Lenz & Wilson, 2007; Lindon et al., 2000). Metabolomics' significant contribution towards defining individual and disease-specific metabolic fingerprints and understanding biochemical pathways involved in many disorders (Kolokolova, Savel'ev, & Sergeev, 2008; Lindon et al., 2000; A. Zhang, Sun, Wang, et al., 2012; A. Zhang, Sun, & Wang, 2012b), should help to anticipate diagnosis, to define predictive and progression biomarkers of several diseases, as well as to monitor therapeutics impact (A.-H. M. Emwas et al., 2013; Gebregiworgis & Powers, 2012; Nicholson, Connelly, Lindon, & Holmes, 2002; Nicholson, Wilson, & Lindon, 2011; Nicholson, Holmes, et al., 2012).

The human metabolome comprises a broad group of small biomolecules, including lipids, amino acids, peptides, nuclei acids, organic acids, vitamins, thiols and carbohydrates (Grimaldi et al., 2018; Shah, 2018; A. Zhang, Sun, Wang, et al., 2012), that exhibit a diverse range of concentration levels and physicochemical characteristics (Beale et al., 2016). Metabolomics has proven to be capable of providing comprehensive data on the metabolome of several types of human samples, namely biofluids such as whole blood, plasma and serum, urine, saliva, breath, bile, cerebrospinal seminal and amniotic fluid and also tissue extracts and cell cultures (Beale et al., 2016; Gil & Duarte, 2018; Kolokolova et al., 2008; Lindon et al., 2004; A. Zhang, Sun, Wang, et al., 2012; S. Zhang, Gowda, Ye, & Raftery, 2010). To date, a total of 18,659 metabolites have been detected in blood (http://www.hmdb.ca/statistics, accessed 16 Jun. 2019), the most studied biofluid in metabolomics (Luque de Castro & Priego-Capote, 2018), and recent data indicates that just over 1200 compounds have been identified in the human salivary metabolome (http://www.salivametabolome.ca, accessed 16 Jun. 2019).

Metabolomics has demonstrated great potential in several areas of the biomedical field and the sum of publications in the context of disease research in peer-reviewed journals has steadily increased each year (Alonso et al., 2015; A.-H. M. Emwas et al., 2013; A. Zhang, Sun, Wang, et al., 2012). Several studies involving animal models of disease or human samples have explored metabolomics' assets to improve disease detection and characterize underlying pathological mechanisms, mainly through the comparison of the metabolic fingerprints of disease samples with ones from healthy controls (A.-H. M. Emwas et al., 2013). One of the first applications of metabolomics in medical areas involved establishing metabolic profiles of tumors through analysis of tumoral cell lines, tissues or biofluids (A.-H. M. Emwas et al., 2013) and, to date, these approaches have unveiled metabolic signatures of numerous cancers, such as human brain (R. Pandey, Caflisch, Lodi, Brenner, & Tiziani, 2017), breast (Silva, Perestrelo, Silva, Tomás, & Câmara, 2019), lung (Duarte, Rocha, & Gil, 2013; Tang et al., 2019), pancreatic (Long et al., 2018), renal (DiNatale, Sanchez, Hakimi, & Reznik, 2019; Gil, Pinho, Monteiro, & Duarte, 2015; Monteiro et al., 2016) and ovarian cancers (Burton & Ma, 2019). Metabolomics has also been employed in the research of a wide range of different medical conditions, including neurological disorders as Alzheimer's disease (Hurtado, Kohler, & de Lange, 2018) and psychosis (C. Li et al., 2018), metabolic diseases like diabetes mellitus (Arneth, Arneth, & Shams, 2019) and gestational diabetes (Pinto et al., 2016) and in many fetal (Pinto et al., 2015) and newborn disorders (Gil & Duarte, 2018). Nonetheless, in addition to the potential for discovery of disease diagnostic and progression biomarkers, metabolomics has allowed great advances in the fields of toxicology (Nicholson et al., 2002; Steuer, Brockbals, & Kraemer, 2019) and pharmacology, namely regarding drug development and safety assessment (A.-H. M. Emwas et al., 2013; Guleria, Kumar, Kumar, Raj, & Kumar, 2018; A. Zhang, Sun, Wang, et al., 2012), and also in therapy monitoring in many medical domains (Araújo et al., 2019; Rattner & Bathe, 2017). Currently, and following the emerging personalized medicine trends, pharmacometabolomic studies aiming to predict the effect of drug exposure prior to its administration have also been performed (Nicholson, Everett, & Lindon, 2012; Nicholson et al., 2011).

Generally, in metabolomics research, biofluids, tissues or cells are analyzed in order to unveil differences in either the nature or levels of metabolites characterizing phenotypes and/or biochemical reactions to perturbations. However, these analyses can be performed under targeted or untargeted approaches (S. Barnes et al., 2016). While targeted metabolomic studies are hypothesis-driven experiments which aim to accurately measure limited and predefined sets of metabolites, untargeted studies focus on analyzing the global metabolomic profile within a biological sample (Alonso et al., 2015; S. Barnes et al., 2016; A.-H. M. Emwas et al., 2013). The latter approach, also known as "top-down strategy", does not require a prior specific hypothesis on a particular group of metabolites, generating therefore a large and complex amount of data (Alonso et al., 2015; S. Barnes et al., 2016). Nonetheless, both approaches constitute challenging tasks, thus relying in information-rich analytical technologies (Nicholson et al., 2002; S. Zhang et al., 2010).

1.3.2. Analytical techniques: Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry

Although a variety of analytical technologies can be employed in metabolomics to identify and quantify known and unknown metabolites, the two most commonly used techniques are Nuclear Magnetic Resonance Spectroscopy (NMR) and Mass Spectrometry (MS) (Aimetti, Cacciatore, Graziano, & Tenori, 2012; Alonso et al., 2015; Lindon et al., 2004; A. Zhang, Sun, Wang, et al., 2012; S. Zhang et al., 2010). Though both techniques are suitable for studying human biofluids, considering they enable the collective measurement of a large amount of data on the metabolome of complex mixtures in one single run, each technique presents its associated advantages and drawbacks (A. Zhang, Sun, Wang, et al., 2012).

1.3.2.1. Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is an established and acclaimed technique for determining molecular structure and probing the metabolic composition of biological samples, based on the energy absorption and re-emission of some atomic nuclei (with nuclear spin) due to the variations of an external magnetic field (Alonso et al., 2015; Lenz & Wilson, 2007; Mikkonen et al., 2018). In NMR spectroscopy studies of biofluids hydrogen is the most commonly targeted nucleus (^{1}H-NMR), essentially due to its natural abundance in organic molecules from biological samples (about 99.98%), but also as hydrogen provides the highest relative sensitivity of all naturally occurring spin-active nuclei (Alonso et al., 2015; Kussmann et al., 2006; Lenz & Wilson, 2007). Other less frequently targeted nucleus include carbon (^{13}C-NMR), phosphorous (^{31}P-NMR) and fluorine (^{19}F-NMR) (Alonso et al., 2015; Kussmann et al., 2006; Lindon et al., 2007).

^{1}H-NMR experiments are based on the principle that the effects of the applied external magnetic field on the behavior of nuclei will vary according to chemical environment experienced by each nuclei within a molecule (Abraham, Fisher, & Loftus, 1987; Lindon et al., 2007). Accordingly, each hydrogen nuclei present in a molecule will resonate at a particular frequency and produce a distinctive set of signals, highly characteristic of the specific compound, in the resulting NMR spectrum (Lindon et al., 2007). Furthermore, the area beneath a signal in the spectrum is proportional to the relative number of nuclei originating it (*i.e.* molecule concentration), allowing for quantitative analysis under specific conditions (Mikkonen et al., 2018). After an NMR experiment, the resulting spectral data will, therefore, not only provide detailed information on the molecular structure of each compound, enabling its identification, but, in principle, also allow the indirect measurement of its quantity within a biological sample (Alonso et al., 2015).

The spectral data obtained after an NMR spectroscopy can be referenced to a single frequency axis (one-dimensional, 1D), in which the peaks of each molecule are placed within its resonant frequencies, or two frequency axes (two-dimensional, 2D) (Alonso et al., 2015). To date, 1D ^{1}H-NMR experiments have been largely used in metabolomics, owing to the relatively fast acquisition times, making them particularly suitable for high-throughput studies with large sample arrays (Alonso et al., 2015; Gebregiworgis & Powers, 2012). Despite these advantages, the small spectral width of 1D ^{1}H-NMR spectroscopy often leads to crowding of most metabolites' resonances within a narrow chemical shift, causing extensive overlapping (Alonso et al., 2015; A.-H. M. Emwas et al., 2013; Gebregiworgis & Powers, 2012). Such spectral overlap can make it difficult to differentiate between multiple peaks, hindering metabolite assignment and spectral interpretation, especially in complex and heterogeneous

samples like human biofluids. The second dimension in 2D-NMR spectra enables the separation of the otherwise overlapping spectral peaks, dispersing the peaks into two dimensions and significantly increasing the resolution (Alonso et al., 2015; Lindon et al., 2007). Accordingly, 2D-NMR spectroscopy enable researchers overcoming the abovementioned overlapping issue and are usually performed to characterize compounds that were not unambiguously identified through the 1D approach (A.-H. M. Emwas et al., 2013). Their use is, nonetheless, restricted to selected samples used for peak assignment and not for routine metabolomics analysis, due to the fact that they require significantly longer acquisition times, up to hours per experiment (Gebregiworgis & Powers, 2012).

The fundamental concepts underlying NMR spectroscopy and, in particular, the general principles inherent to 1D and 2D experiments, will be addressed in detail in section 4.1 of chapter 4 (*'Basic principles of NMR spectroscopy'*).

1.3.2.2. Mass Spectrometry

In MS analysis spectral data is acquired in the form of a mass-to-charge ratio (*m/z*) against the relative abundance of the measured compounds (Alonso et al., 2015; Girolamo, Lante, Muraca, & Putignani, 2013). In general, these experiments comprise three distinct steps: firstly, the molecules within the biological sample are subjected to the action of an ionizing energy and converted into gas-phase ions (Villas-Bôas, Mas, Åkesson, Smedsgaard, & Nielsen, 2005). The resulting ionic compounds will then be further separated based on their *m/z* values via magnetic or electric fields through a component (mass analyzer) (Girolamo et al., 2013). Finally, the separated ionic species are converted into electrical signals, detected and their abundance is assessed, hence creating a mass spectrum, which will be characteristic of each molecule in the analyzed sample (Girolamo et al., 2013).
Currently, a previous additional separation step is usually required to identify metabolites efficiently in MS-metabolomics (A.-H. M. Emwas et al., 2013; Lindon et al., 2004), due to the limited molecular-weight dispersion of metabolites and depending on the type of metabolites of interest (Gebregiworgis & Powers, 2012). In metabolomic approaches this pre-separation step is usually performed by gas chromatography (GC-MS) to separate volatile compounds or liquid chromatography (LC-MS) for studying polar, less-polar and neutral metabolites, though capillary electrophoresis (CE-MS) can also be carried out to separate highly polar and ionic metabolites (Girolamo et al., 2013; Lindon et al., 2004; Nicholson, Everett, et al., 2012; Villas-Bôas et al., 2005). Both GC and LC separation techniques are based on the interaction of different metabolites present in the sample with the adsorbent materials within the chromatographic column of the separation device coupled to the mass spectrometer (Alonso et al., 2015; Girolamo et al., 2013). Summarily, metabolites with different chemical properties require different intervals of time to move through the chromatographic column (*i.e.* retention time). The retention time values, along with the *m/z* values generated through MS, originate the two axes present in a LC-MS or in a GC-MS spectra (Alonso et al., 2015).

Since MS experiments often require a separation step which depends on the nature of the metabolites on interest, this technique is considered more advantageous for targeted researches, for instance, the detailed study of lipids present in a tissue, biofluids or cell culture, although it can also be employed for global profiling studies (Nicholson, Everett, et al., 2012). Differently, NMR spectroscopy constitutes a non-selective technique, providing multiparametric data on the sample metabolome, without necessarily anticipating the nature of the molecules to be identified (Lenz & Wilson, 2007; C. J. L. Silwood, Lynch, Claxson, & Grootveld, 2002). Additionally, in NMR spectroscopy several chemi-

cally different components can be simultaneously detected in a single run and no further techniques have to be associated in order to reliably identify metabolites, forasmuch as an NMR spectrum entails comprehensive data on the structure of a wide range of compounds (Kolokolova et al., 2008). Sample handling can, therefore, be more complex and time-consuming in MS approaches, in which samples may have to be aliquoted and prepared in order to be subjected to different conditions for different classes of metabolites detection, contrarily to the straightforward sample preparation procedures in NMR experiments (Figueira et al., 2017; Gebregiworgis & Powers, 2012; Lenz & Wilson, 2007; Lindon et al., 2004; S. Zhang et al., 2010). NMR is also considered a non-destructive technique, *i.e.* samples may be recovered for subsequent analysis by other methods to identify specific metabolites, while MS involves a destructive process (A.-H. M. Emwas et al., 2013; Kolokolova et al., 2008; Lenz & Wilson, 2007; Lindon et al., 2004). Though both methods require small sample sizes, MS spectrometry necessitates amounts on the low µL range, whilst in an NMR experiment about 200 to 500 µL of sample are typically used (Gebregiworgis & Powers, 2012; Lindon et al., 2004). Moreover, the metabolic profile of the sample can be acquired through NMR spectroscopy within a few minutes (approximately 10-30 minutes for 1D ^{1}H-NMR), while longer acquisition periods of time might be required in some MS experiments (Alonso et al., 2015; Lenz & Wilson, 2007; Lindon et al., 2004; C. J. L. Silwood et al., 2002; S. Zhang et al., 2010). MS-based studies are also more expensive in a per-sample cost basis and in relation to maintenance expenses (A.-H. M. Emwas et al., 2013; Lenz & Wilson, 2007).

Low sensitivity represents a relevant inherent drawback of NMR spectroscopy comparing to the exquisite sensitivity of MS spectrometry (Gebregiworgis & Powers, 2012; Kolokolova et al., 2008; S. Zhang et al., 2010). In fact, while the latter provides lower detection limits, detecting analytes present at less than picomolar quantities, in comparison to the submillimolar levels detected by NMR (Gil & Duarte, 2018; Lindon et al., 2004; Nicholson, Everett, et al., 2012). However, MS tends to be less reproducible and more susceptible to variability (Nicholson, Everett, et al., 2012) and NMR is considered a robust technique (Alonso et al., 2015; Lenz & Wilson, 2007; Nicholson, Everett, et al., 2012; S. Zhang et al., 2010). Lastly, it is reckoned that one disadvantage of NMR-based studies is that very skilled technicians are needed to perform and interpret experiments.

In sum, though NMR and MS techniques can identify and quantify data on a broad range of molecules with good analytical precision and accuracy, the two present associated benefits and disadvantages and, accordingly, their complementary nature has been increasingly demonstrated in several metabolomic-based researches which combined both approaches to gain a broader perspective on the metabolome (Gebregiworgis & Powers, 2012; Nicholson, Everett, et al., 2012; A. Zhang, Sun, Wang, et al., 2012).

1.3.3. Statistical tools in metabolomics

Attending to the complex nature of NMR spectra, the relevance of all of the subsequent procedures that allow extracting valuable and "*human readable*" information from raw metabolomic data is indisputable. A general scheme of a typical methodological pipeline of an NMR-based untargeted metabolomics study is presented in Figure 1.2. Briefly, after sample collection and preparation each sample is placed within a tube, introduced in the NMR spectrometer and its NMR spectrum is acquired. After several spectra are acquired and accumulated, spectral data is processed through numerous methods in order to generate sample metabolic features (Nicholson et al., 2002). The data resulting from these processes is arranged

and reduced to a feature quantification matrix (FQM), a data table which comprises the quantification of the metabolic features of all the analyzed samples (Alonso et al., 2015; Nicholson et al., 2002). This matrix of data is subsequently subjected to univariate and multivariate statistical analysis to reduce the dimensionality of complex spectroscopic data and to unveil how distinct biochemical patterns that relate with the samples phenotypic data (Alonso et al., 2015; Lindon et al., 2004). The main principles of the procedures underlying spectral pre-processing, processing and data analysis will be presented below.

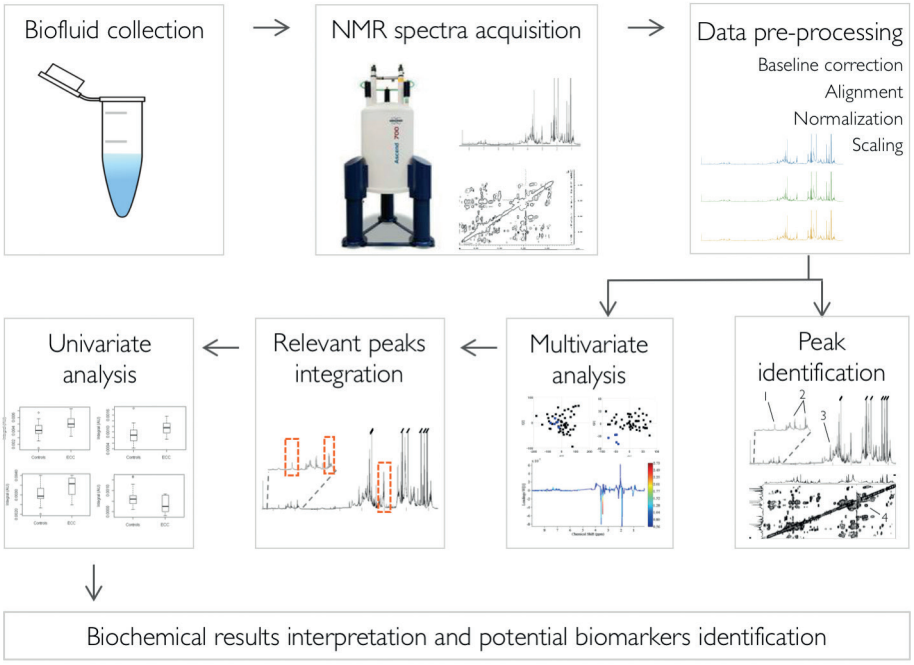

Figure 1.2. Illustrative scheme of a metabolomics study flowchart using NMR spectroscopy.

Data preprocessing

NMR-based studies often deal with numerous biological samples from multiple subjects (animals or tissues) with inherent variability and, correspondingly, it would be unreasonable to expect uniformity in NMR data. Inevitably, the obtained data often presents variations in signals positions and intensity due to differences in the chemical environment of the sample matrix, like ionic strength, pH or protein content, as well as phase and baseline distortions caused by unwanted macro-molecule signals (S. Zhang et al., 2010). Non-biological factors can also lead to undesired variability in NMR data, mainly due to experimental artifacts relative to suppression of the water region, shimming (that is, the processes performed during each NMR experiment to correct inhomogeneities in the applied magnetic field) and acquisition parameters defined for each sample (A.-H. M. Emwas et al., 2013; Gebregiworgis & Powers, 2012). Data preprocessing consists on a series of computational steps, between raw spectra and data analysis, to improve signal quality and ensure dataset robustness, through correction or minimization of such systematic or random biases, so that only biologically relevant differences are present in the data (Alonso et al., 2015; Beale et al., 2016).

Initial preprocessing steps involve spectra phase correction to produce pure absorption line shapes and baseline correction, during which each spectrum's baseline is adjusted in order to remove low frequency

artifacts and differences between samples produced by experimental variations (Alonso et al., 2015; Gebregiworgis & Powers, 2012; Lindon et al., 2007). Distortions in spectra baseline can potentially affect quantification of the metabolites and also the statistical analysis. Although both processes, phasing and baseline correction, can be carried out automatically, such processes present limited reliability and, therefore, performing manual correction and visual inspection of processed spectra for artifacts is of the upmost importance (Lindon et al., 2007). The subsequent step, alignment, is one of the most critical processing steps and aims to reduce the abovementioned shifts in peak positioning among different spectra, observed in the parts per million (ppm) axis, while maintaining spectral resolution (Alonso et al., 2015; Lindon et al., 2007; S. Zhang et al., 2010). Spectral alignment is firstly performed globally to full resolution spectra using a signal reference peak, and then a fine peak-based alignement is carried out. Fine alignment corrects individual peaks across samples and can be completed either manually or through algorithms such as the recursive segment-wise peak alignment (Veselkov et al., 2009; S. Zhang et al., 2010). Essentially, this algorithm realignes peak positions in a ^{1}H-NMR spectrum with respect to peak positions of a reference spectrum (Veselkov et al., 2009). After alignment spectral dataset are converted to FQM, a data representation in which rows correspond to the analyzed samples and columns correspond to each variable, *e.g.* chemical shift.

Subsequently, a normalization step is generally performed to overcome variations between concentration, dilution or total amount of samples and allow accurate comparison and quantification of the metabolomic features within datasets (Alonso et al., 2015; Lindon et al., 2007). Normalization is applied to each row of the matrix, dividing each row by a constant, which can be, for instance, the total integrated intensity across the whole NMR profile (method known as total area or total intensity) (Lindon et al., 2007). The last pre-processing step is data scaling, a method necessary to prevent that most abundant metabolites do not dominate projections, hindering the detection of potentially valuable variations in metabolites in lower concentrations (S. Zhang et al., 2010). Multiple scaling methods are available, of which Unit Variance (UV), Pareto and logarithmic transformation are among the most commonly used (S. Zhang et al., 2010). UV scaling consists in mean centering, that is, subtracting the mean value of the sample, followed by the division of each variable by its standard deviation (Veselkov et al., 2011). Differently, in Pareto scaling each variable is divided by the square root of the standard deviation of that variable and in logarithmic transformation a logarithm is applied to each variable (Veselkov et al., 2011).

Multivariate analysis tools

Once metabolomic features are adequately quantified, several statistical methods can be applied to chemical data in order to reduce complexity from the NMR dataset, detect meaningful patterns and generate or test scientific hypothesis (Lindon et al., 2004; Nicholson, Everett, et al., 2012; A. Zhang, Sun, & Wang, 2012b). Multivariate statistical analysis (MVA) methods are considered pattern-recognition methods that take in account all the metabolomic features simultaneously and that can be categorized into two distinct groups: unsupervised and supervised methods (Alonso et al., 2015; Lindon et al., 2004, 2007).

In unsupervised analysis methods the similarity patterns within the data are detected without *a priori* knowledge of the type or class of the study samples (e.g control group *vs.* disease group) and, therefore, these techniques are based strictly on data intrinsic variations (Nicholson, Holmes, et al., 2012; Veselkov et al., 2009). Principal Component Analysis (PCA), the most commonly used technique in MVA, provides an unbiased overview of the dataset and is frequently used as an exploratory tool, enabling clustering trends and outlier detection (Dona et al., 2016; A.-H. M. Emwas et al., 2013). PCA is

considered a projection-based method, meaning it projects and reduces multivariable NMR data into a simple visual format, since metabolic features are transformed into and expressed as a set of linearly uncorrelated variables, the principal components (PCs) (Lindon et al., 2004, 2000). After NMR integrals are converted, clustering tendencies in data can be visualized in scores plots and explained by loading plots. In PCA scores plots each point represents an NMR spectrum and the coordinate axis corresponds to the PCs (PC1, PC2, etc.) (Gebregiworgis & Powers, 2012). The first PC contains the largest part of the variance of the data, whereas the subsequent PCs contain correspondingly smaller amount of variance (Lindon et al., 2000). As the scores plot represents the observations, with the position of each single observation being given by the relative similarity between samples, similar observations appear closely positioned in the plot. PCA also enables the detection of the dominant variant in the dataset, which may occasionally be related to a secondary factor, like gender or diet, for instance, and not associated to the biological effect in study (A.-H. M. Emwas et al., 2013). Consequently, PCA can suggest a particular dataset may need to be stratified, *i.e.*, divided into more matched subgroups, in order to allow establishing more reliable conclusions.

Supervised methods involve techniques that, with previous knowledge of the class information, identify data patterns correlated with the phenotypic variable of interest and optimize the discrimination between two or more sample classes for a particular factor (Dona et al., 2016; Lindon et al., 2007). These methods are critical in metabolomic-based researches since they enable sample classification and are useful to build prediction models (A.-H. M. Emwas et al., 2013). To generate prediction models a "training set" of the data is used to build a mathematical model that is able to predict correctly the group each sample belongs to (Lindon et al., 2000).

Partial Least Squares-Discriminant Analysis (PLS-DA), a widely used supervised method, is also a projection-based technique which aims at maximizing clustering behavior, again expressed in scores and loadings plots, this time as a function of latent variable components (LVs, with a similar meaning to PCs in PCA) (Lindon et al., 2007; Nicholson, Everett, et al., 2012). In PLS-DA data is approximated in a low-dimensional space by decomposing variation into LVs, the linear combinations of the original variables (Lindon et al., 2007). Between groups, the highest source of variation detected between classes is contained in the first LV, even though it might not necessarily be the highest reason of variation within the dataset. Contrarily to PCA, PLS-DA does not maximize the explained dataset variance, but the covariance between the factor of interest and metabolomic data (Dona et al., 2016; Lindon et al., 2000). Consequently, metabolites contributing to group separation are usually ascribed by plotting the loadings plots, along with the variable importance of the projection (VIP), a measure of how much each variable (or NMR peak, in the present context) contributes to group separation in the scores plot. Accordingly, in the model, metabolites presenting higher VIP values are considered the most relevant analytes to the discrimination.

Variable selection methods

Datasets arising from NMR-based studies are complex and contain vast amount of information, comprising also some irrelevant or redundant variables considering the classification purpose. Nonetheless, cautious simplification of original datasets can be achieved using variable selection (VS) methods, approaches involving removal of irrelevant, unreliable or noisy variables (Smolinska et al., 2012). Consequently, with minor loss of information, model complexity is reduced, spectral regions with stronger correlation to class definition are selected and the model's performance can potentially be improved

(Smolinska et al., 2012). Though numerous approaches have been proposed to perform VS, one of the most popular methods is based on a MVA parameter, the VIP criteria (Gromski et al., 2014; Smolinska et al., 2012). The VIP value refers to the relevance of variables in the model considering, not only their correlation to the response, but also with respect to the projection. Summarily, whenever a VIP value from a variable is inferior to 1, the given variable is not relevant for the projection, so it can be removed from the model. After such procedure, a smaller set of predictive variables correlated to the particular response will be selected, enhancing the accuracy of estimation.

Validation of MVA models

In metabolomic studies the typically large amount of variables in comparison to the sample number may potentially conduce to an over fitting of MVA models, that is, an overestimation of success (Alonso et al., 2015; Smolinska et al., 2012). This possibility justifies performing rigorous validation approaches, another fundamental step in metabolomics, in order to evaluate the predictive ability of a model (Smolinska et al., 2012; Westerhuis et al., 2008). Indeed, properly validated statistical models tend to provide more reliable findings, particularly if the obtained results (for instance, a set of metabolites found to contribute to the progression of a particular disease) are to be clinically applied in the future. Numerous tools are currently available to validate a model obtained after MVA, including permutation tests and cross-validation methods (Rubingh et al., 2006). In permutation tests, labels from the two classes tested in the model (*i.e.* controls and disease) are subjected to multiple random rearrangements and models are recomputed (Westerhuis et al., 2008). Such rearrangements aim to assess whether the particular classification in the two groups is significantly better than other random classification in two arbitrary groups (Westerhuis et al., 2008). Monte Carlo Cross validation (MCCV) is a validation approach that splits data into training and prediction sets, and the former set is used to predict class membership (Xia, Broadhurst, Wilson, & Wishart, 2013; Q.-S. Xu, Liang, & Du, 2004). In MCCV, 500 to 1000 iterations are performed and, for each particular iteration, confusion matrices and Q^2 distribution of the true and permuted models are calculated, enabling the production of a receiver operating characteristics graph (ROC), which informs on the performance and the prediction ability of the classification model (Q.-S. Xu et al., 2004). The calculated confusion matrix comprises four distinct outcomes: true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) (Westerhuis et al., 2008). These outcomes will constitute the basis for multiple metrics, such as true positive rate (TPR) or sensitivity, false positive rate (FPR) or 1-specificity and classification rate (CR). While sensitivity is defined as the number of true positives as percentage of all positives, 1-specificity is considered the amount of false positives as a percentage of all negatives (Westerhuis et al., 2008). The CR refers to the ratio between the sum of true positives and negatives and the total number of classifications, that is, the percentage of samples correctly classified in the model. The graph presenting Q^2 distribution displays the calculated Q^2 values for permuted and true models in all of the iterations carried out in MCCV. It should be noted that predictive power Q^2 is a default parameter used to evaluate PLS-DA performance, particularly the level of discrimination obtained by the PLS-DA model. Ideally, the Q^2 value should be as closer to 1 as possible, since such value suggests predicted classes match original classes. Therefore, the Q^2 distribution graph obtained through MCCV constitutes a valuable tool to assess a model's predictive power, bearing in mind that in a model with good predictive ability, minimal overlapping of Q^2 values of true and permuted models should occur (Xia et al., 2013; Q.-S. Xu et al., 2004). Finally, the ROC plot is built from confusion matrices and presents sensitivity on the *y* axis and 1-specificity on the *x* axis of the plot (Westerhuis et al., 2008). In a ROC plot of an ideal model true classes should appear, not only distinctively separated from the permuted classes, but also present high sensitivity and specificity values (as close to 1 as possible).

Data correlation tools: statistical total correlation spectroscopy

Statistical total correlation spectroscopy (STOCSY) is a method of correlation analysis increasingly applied in the metabolomics context, which has improved the obtainment of data from complex biological biofluids (A.-H. M. Emwas et al., 2013; S. Zhang et al., 2010). Based on the computation of correlation statistics between the peak intensities in a set of NMR spectra, STOCSY generates a one- or two-dimensional map, presented in the form of an NMR spectrum, and displaying correlation among spectra intensities (Cloarec et al., 2005). Through this correlation between signals from molecules that vary in concentration between samples, STOCSY allows the identification of peaks from the same molecule, in addition to detecting different molecules that interact in the same metabolic pathways (Cloarec et al., 2005; Coen, Holmes, Lindon, & Nicholson, 2008). In the pseudo spectrum built through STOCSY, the correlation of each resonance (relatively to the selected signal on which STOCSY was performed) is revealed by a color scale ranging from low correlation to high correlation (typically 0 and 1, respectively) (Dona et al., 2016).

Currently, this method can be highly useful in peak assignment and measurement of metabolite correlations in both 1D and 2D spectra, either homo or heteronuclear data. Nevertheless, in complex samples analysis, in which this technique is often necessary, overlapping of signals (the spectral issue that may primarily hinder peak assignment) also interferes with STOCSY capacity to accurately detect correlations.

Univariate statistical methods

As previously addressed, metabolomic data arising from biological samples reveals great complexity, presenting correlations among features from the same metabolites and between metabolites from the same pathway, in addition to the effect of potential confounding variables (*e.g.*, gender and diet). In this context, metabolomic data analysis relies mostly in MVA, in consideration that univariate statistical methods compare particular variations, not accounting for the rest of the metabolic profile, that is, the presence of interactions between the multiple remaining features in study (Alonso et al., 2015). Accordingly, a given metabolite can be considered relevant through MVA and, simultaneously, not individually statistically significant if the remaining metabolic profile is not being considered (as in univariate analysis) (Saccenti, Hoefsloot, Smilde, Westerhuis, & Hendriks, 2014). On the contrary, individual analytes may seem to vary significantly between groups when analyzed by univariate methods and no relevant changes are detected using MVA procedures. Still, univariate statistical methods are frequently used mainly to complement information provided by MVA, supporting its results, as well as to confirm identification of metabolic biomarkers statistically significant according to a critical threshold (Vinaixa et al., 2012).

Univariate statistical methods, being based on hypothesis testing, define a *null hypothesis* (H_0), which postulates the absence of difference between the mean (or median) areas of the metabolites detected in the studied groups, and pre-determine the threshold of probability for H_0 rejection (*p*-value). After applying the statistical test(s), a *p*-value that reflects the probability of wrongly rejecting the H_0 is obtained. H_0 is rejected if the *p*-value is below the pre-defined threshold of probability (, commonly set at 5%) (Vinaixa et al., 2012). This means that, if p-value < 0.05, there is sufficient evidence to reject H_0 in 95% of cases (for a significance level of 95%). Though several univariate tests can be applied in the metabolomics context, the selection of the method is dependent upon the statistical characteristics of the feature, namely the type of groups and whether their respective data follows a Gaussian or normal distribution (Ghasemi & Zahediasl, 2012; Vinaixa et al., 2012). Assumption of normality distribution should be assessed through the Kolmogorov-Smirnov normality test or Bartlett's homogeneity of variances test

(Ghasemi & Zahediasl, 2012; Vinaixa et al., 2012). Normally distributed data is usually analyzed through parametric tests including Student t-test (or just t-test) to compare two groups or ANOVA (analysis of variance) to assess differences between more than two groups (Alonso et al., 2015; Vinaixa et al., 2012). Inversely, when data normality cannot be assumed, non-parametric tests are preferable. In such cases, tests like Wilcoxon signed rank test, Wilcoxon rank sum test, Mann-Whitney test or Kruskal-Wallis one-way analysis of variance can be applied (Alonso et al., 2015; Vinaixa et al., 2012).

Univariate analysis methods can also be performed in metabolomics with respect to correction methods applied to multiple testing, in order to reduce the occurrence of type I errors, finding a statistically significant result by chance (that is, a FP). One of the most conservative multiple test correction approach is the Bonferroni correction (Alonso et al., 2015). The Bonferroni correction adjusts *p*-values through minimization of the number of false positives regarding the total number of tested hypothesis, at the expense of increasing the amount of FN (Alonso et al., 2015; Armstrong, 2014). In this approach, the significance level for one hypothesis, that is, the α value, is divided by the number of hypothesis being tested simultaneously (Alonso et al., 2015).

1.3.4. State of art of saliva metabolomics

Following on blood/urine metabolomics advances in relation to disease, salivary metabolomics has revealed great potential regarding diagnostic utility and monitoring disease progression and therapeutic impact (Bonne & Wong, 2012; Shah, 2018; Yilmaz et al., 2017; A. Zhang, Sun, Wang, et al., 2012; A. Zhang, Sun, & Wang, 2012a; Y. Zhang et al., 2014). The broad set of saliva's advantages as a diagnostic fluid, particularly its' informative nature and its' quick, non-invasive and simple sample collection, along with metabolomics' capability of providing comprehensive and reliable insights into the complex composition of human biosamples have ensured the emergence of this research field (Cuevas-Córdoba & Santiago-García, 2014; Koneru & Tanikonda, 2014; Mikkonen et al., 2016; Shah, 2018; D. T. W. Wong, 2012; A. Zhang, Sun, Wang, et al., 2012; Y. Zhang et al., 2014). In fact, the potentialities of saliva metabolomics in accurately portraying, discriminating and predicting disease profiles, led to an increased popularity among research groups (Beale et al., 2016; Dame et al., 2015; A. Zhang, Sun, Wang, et al., 2012) and, in the last years, the number of published scientific researches involving the salivary metabolome has grown, though in an inferior proportion than for plasma and urine metabolomics (Gardner, Parkes, Carpenter, & So, 2018). The dynamic and translational nature of metabolomic-based salivary researches has fostered the integration of research results into clinical applications and, presently, multiple biomarkers capable of discriminating between healthy and diseased subjects have already been identified (Shah, 2018; A. Zhang, Sun, & Wang, 2012a). The application of metabolomic approaches to non-invasively diagnose systemic pathologies is being explored in several areas ranging from sarcoidosis (Duchemann et al., 2016), to autoimmune diseases like Sjögren's syndrome (Kageyama et al., 2015; Mikkonen et al., 2012) or neurodegenerative disorders such as dementia (Figueira et al., 2016) and Alzheimer's disease (Gleerup, Hasselbalch, & Simonsen, 2019; Huan et al., 2018; Liang et al., 2015; Yilmaz et al., 2017). Metabolic profiling of saliva has also allowed to discriminate between moderate asthma and healthy control samples with good diagnostic performance (Malkar, Wilson, Harrrison, Shaw, & Creaser, 2016), demonstrating an appealing potential to study respiratory diseases (Snowden, Dahlén, & Wheelock, 2012; Turi, Romick-Rosendale, Ryckman, & Hartert, 2018). Similarly, this platform has shown promising results in type II diabetes mellitus (DM II) research, as profiles associated to the disease have been identified in DMII patients' saliva (Virginia M. Barnes et al., 2014;

Yousri et al., 2015). Moreover, in this context, metabolomic studies have established reliable markers of acute, short-term and long-term glycemic control (Mook-Kanamori et al., 2014; Yousri et al., 2015) and, presently, an assay kit which had already been validated to detect the condition in blood is being evaluated through metabolomic approaches to assess its applicability in saliva samples (Halama et al., 2016).

As revealed by evidence from research in other biomedical fields, alterations in cellular energy metabolism inherent to multiple types of cancers are evident in the metabolic profiles of tumors and patient's biofluids, such as blood, urine and saliva (Armitage & Barbas, 2014; X. Chen & Yu, 2019; Spratlin, Serkova, & Eckhardt, 2009). Accordingly, researchers have begun to explore saliva metabolomics with regard to oncologic pathologies such as gastric (Y. Chen et al., 2018), breast (Sugimoto, Wong, Hirayama, Soga, & Tomita, 2010) and pancreatic cancers (Sturque, Berquet, Loison-Robert, Ahossi, & Zwetyenga, 2019; Sugimoto et al., 2010). In 2010, a study using capillary electrophoresis and time-of-flight mass spectrometry (CE-TOF-MS) carried out a comprehensive profiling of metabolites in saliva samples from individuals presenting periodontal disease or breast, pancreatic or oral cancers and found 57 metabolites could accurately predict the probability of suffering from each one of the disorders, demonstrating once again the existence of cancer-specific signatures comprised in salivary metabolome (Sugimoto et al., 2010).

Since then, several studies have addressed the potential of salivary metabolite profiles for early and non-invasive oral cancer screening, in particular to detect oral squamous cell carcinoma (OSCC), one of the most frequent oral neoplasms and whose lesions can go dangerously unnoticed in early stages (X. Chen & Yu, 2019; Ishikawa et al., 2016; Mikkonen et al., 2018; Ohshima, Sugahara, Kasahara, & Katakura, 2017; Vitorino et al., 2010). Wei *et al.* demonstrated a salivary metabolic profile capable of distinguishing patients with OSCC from controls and from those with precursor oral lesions (such as lichen planus and leukoplakia) with good-to-excellent sensitivity, specificity and accuracy using a MS approach (Wei et al., 2011). Recently, one study combining NMR and MS-based approaches, evaluated saliva from OSCC patients with and without metastasis (Lohavanichbutr et al., 2018). Though no significant differences were registered in metabolite levels in the compared groups, further studies are warranted to confirm such results, since metabolomic profiling of saliva is also expected to provide a potentially useful complementary tool to physical exams and radiologic imaging regarding metastasis detection in subjects presenting OSCC (Lohavanichbutr et al., 2018). Still in respect to neoplastic pathologies of the oral cavity, metabolomic analysis of saliva through NMR spectroscopy has also been recently applied in studying parotid tumors (Grimaldi et al., 2018). Although this preliminary data suggesting the existence of a distinctive metabolomic profile (mainly characterized by an altered profile of amino acids) associated to these tumors still requires thorough confirmation, such findings would allow major improvements regarding disease early screening, a particularly relevant breakthrough considering salivary gland cancers usually enclose poor prognosis and survival rate (Grimaldi et al., 2018).

1.3.4.1. Salivary metabolomics in the context of oral diseases research in adults

In the last years salivary metabolomics has been increasingly explored concerning oral pathologies, specially periodontal diseases in adults (X. Chen & Yu, 2019; Mikkonen et al., 2016). Periodontal diseases cover a group of inflammatory diseases of the periodontium caused by multifactorial interactions between the host and pathogenic bacteria and characterized by loss of connective tissue around teeth, whose diagnosis relies on clinical and radiological examination (Liebsch et al., 2019; Mikkonen et al., 2016). Considering the altered cellular metabolism of the affected host and microbes releases multiple intermediary end products into oral fluids, and in the expectation that saliva metabolic profiling would provide a valuable overview on the pathogenesis of these diseases, multiple investigations have compared

and distinguished salivary profiles of healthy and periodontal conditions by either MS (V M Barnes et al., 2011; Virginia M. Barnes et al., 2014; Liebsch et al., 2019) and NMR-based approaches (Aimetti et al., 2012; García-Villaescusa et al., 2018; Romano et al., 2018; Singh, Saxena, Saimbi, Arif, & Roy, 2017).

To date, researches have revealed significant alterations fatty acid metabolism (V M Barnes et al., 2011; Virginia M. Barnes et al., 2014), indicative of the increased inflammatory condition and oxidative stress typically associated to periodontal chronic bacterial infection state. Several changes in other classes of metabolites have also been detected, namely regarding dipeptides, amino acids, carbohydrates, lipids and nucleotides (V M Barnes et al., 2011; Virginia M. Barnes et al., 2014; Liebsch et al., 2019), probably as a result of the high degree of macromolecular degradation of proteins, triacylglycerol, glycerolphospholipids, polysaccharides, and polynucleotides in affected subjects (V M Barnes et al., 2011; Liebsch et al., 2019). The observed changes not only reflect the interactions between host and bacteria in disease (mainly related to levels of bacterially modified amino acids and creatine) but, more importantly, the creation of a more favorable environment for oral flora through the increased availability of metabolites including fatty acids, dipeptides and monosaccharides for bacterial energy production (attributed to an increased activity of lipase, protease and glycosidase enzymes) (V M Barnes et al., 2011; Mikkonen et al., 2016). Overall, studies have generally agreed that metabolites associated with periodontal status mostly concern a reflection of bacterial species (*e.g.* lactate, pyruvate and formate) (Rzeznik et al., 2017), tissue degradation (*e.g.* proline, phenylalanine and tyrosine) (Virginia M. Barnes et al., 2010) and host defense mechanisms (*e.g.* valine, isoleucine) (S. Zhang, Zeng, Ren, Mao, & Qiao, 2017). Recently, a comprehensive MS study involving a larger sample (n=909) in comparison to previous studies identified a large panel of metabolites related to periodontal disorders (Liebsch et al., 2019). Nevertheless, considerable differences in metabolites variations were found when considering participant's age and, across all age groups, phenylacetate was the only metabolite consistently associated to periodontal condition. Accordingly, and although many previous researches had suggested the identification of a single biomarker associated to periodontitis (rather than a combination of metabolites) would be unlikely, authors considered phenylacetate might constitute a promising biomarker to screen the disease and future well-controlled experimental studies with large but tightly defined cohorts are needed to validate such finding.

Similarly, other aspects of periodontal diseases such as therapy response monitoring have been explored (Huang et al., 2014; Kuboniwa et al., 2016; Romano et al., 2019). One recent exploratory study used NMR spectroscopy to unveil whether the salivary metabolome of periodontal patients is altered by non-surgical periodontal therapy to patterns more similar to the ones compatible with periodontal health (Romano et al., 2019). The individual treatment-related impact detected in the study cohort allowed a discrimination with 92.5% accuracy between groups, ascribable to the quantitative and qualitative changes in inflammation processes and oral microflora after treatment (Romano et al., 2019). However, the distinct salivary changes detected in patients after treatment were not strong enough to shift the salivary metabolomic signature of periodontitis towards a fingerprint associated with healthy status, a finding which authors attributed to the small sample size of the study. Preliminary data in this field is, nonetheless, promising and efforts to explore and validate biomarkers capable of evaluating periodontal health/disease in a point-of-care environment are currently being made.

1.3.4.1.1. Saliva metabolomics and dental caries

Except for a limited number of studies, saliva metabolome has been largely overlooked as an interesting tool to address dental caries dynamics, in comparison to research regarding periodontal disease. It is well known that the composition of saliva influences, not only the risk of developing dental caries,

but also its progression rate, thus saliva's metabolomics capacity of mirroring the dynamic of biological pathways would represent a valuable tool in caries research. Despite its recognized assets, NMR-based techonologies have been scarcely used in this context.

In 1999 one study investigated samples of active primary root carious lesions and USS of adults through ^{1}H-NMR spectroscopy for the first time (C. J. Silwood et al., 1999). The study reported that the USS NMR spectra contained several proeminent and sharp peaks ascribable to the presence of many biomolecules, including some organic acid anions (acetate, propionate, lactate, formate, *n*-valerate and *iso*-butirate). Similarly, organic acids were found in carious dentin, though samples presented higher levels of acetate and formate in comparison to lactate, propionate, pyruvate, butyrate and succinate concentrations (C. J. Silwood et al., 1999). At the time, findings provided valuable insight on the relevance of organic acids in the development and progression of dental caries and revealed great potential of saliva metabolomics in oral health research. Nonetheless, since then, metabolomic approaches have been predominantly used to explore oral biofilms in adults through MS, mainly in an attempt to compile basic knowledge on the metabolic pathways of oral bacteria of dental plaque, in particular the carbohydrate metabolism by caries-associated bacteria like *Streptococcus* and *Actinomyces*, and clarify its relationship with oral health and disease (Nascimento, Zaura, Mira, Takahashi, & Ten Cate, 2017; N. Takahashi, 2015; N. Takahashi, Washio, & Mayanagi, 2010; Nobuhiro Takahashi, Washio, & Mayanagi, 2012).

Understanding bacterial metabolic pathways through these studies has definitely expanded knowledge on dental caries processes in the last years (Nascimento et al., 2017). Carbohydrates from diet, as well as from salivary glycoproteins (previously degraded by bacterial or human glycosidases into sugar molecules and proteins), are metabolized by supragingival saccharolytic bacteria (e.*g. Streptococcus, Actinomyces*, and *Lactobacillus)* to organic acids, decreasing oral pH (Nascimento et al., 2017; N. Takahashi, 2015; N. Takahashi & Yamada, 1999; Washio et al., 2016). Such metabolization into organic acids, obviously implicated in caries demineralization process, occurs via the Embden-Meyerhof-Parnas pathway (EMP pathway; glycolysis), the pentose-phosphate pathway and the tricarboxylic acid cycle (TCA cycle) pH (N. Takahashi, 2015; N. Takahashi & Yamada, 1999; Nobuhiro Takahashi et al., 2012), which are near to be fully disclosed by metabolomics recent advances (Nascimento et al., 2017). On the other hand, proteins, peptides and amino acids continuously provided by salivary flow gingival crevicular fluid into the oral cavity can be used as substrate for bacterial growth, being further metabolized through various metabolic pathways, mainly into short-chain fatty acids, carbon oxide, amines and ammonia (Nascimento et al., 2017; N. Takahashi, 2015; Washio et al., 2016). These metabolites have been related to oral malodor and tissue inflammation, but also to acid neutralization within the biofilm, thus possibly counteracting tooth demineralization (Nascimento et al., 2017; N. Takahashi, 2015; Washio et al., 2016).

Bacterial metabolic pathways responsible for alkalinization were further investigated in a MS study of supragingival plaque, which explored the contribution of amino acid degradation pathways to pH homeostasis (Washio et al., 2016). The study revelead these pathways include deamination, decarboxylation and transamination reactions that are likely interconnected to each other, as well as coupled to carbohydrate pathways in a manner yet to be fully clarified (Washio et al., 2016). Additionally, the research demonstrated that glutamine and glutamate, amino acids commonly present in dental plaque, might be the main source of ammonia production, along with arginine (resultant of arginine deiminase pathway), contributing to pH homeostasis and balancing the acid-induce demineralization in plaque (Washio et al., 2016).

1.3.4.2. Potentialities of salivary metabolomics in pediatrics

The large majority of assays exploring salivary diagnostics through metabolomic approaches, either to explore systemic or oral pathologies dynamics', have been performed in adult cohorts (Hassaneen & Maron, 2017). However, it is recognized that infants and children would benefit the most from such non-invasive health monitoring and diagnostic tools. Biomarkers detected in serum have been considered the gold standard in diagnostic testing for numerous disorders in general; nonetheless, saliva collection appears to constitute a quicker, inexpensive and relatively benign alternative to blood sampling for health professionals working with children (Hassaneen & Maron, 2017; Javaid, Ahmed, Durand, & Tran, 2016; Sugimoto et al., 2013). Salivary assay development for pediatric populations may also be particularly advantageous to higher risk patients, since saliva sampling procedures involve minimal trauma and absence of anemia risk, contrary to the anemia risk often associated to serial phlebotomy in such patients (Hassaneen & Maron, 2017). Although in a considerable inferior number comparing to researches in adult cohorts, metabolomic analysis of saliva has recently started to be applied in some pediatric medical fields (P. A. Almeida et al., 2017; Morzel et al., 2017; Oliveira et al., 2016).

After urine and plasma metabolic profiling of type I diabetes mellitus (DMI) in children cohorts demonstrated the existence of different panels of metabolites associated to disease (Culeddu et al., 2012; Oresic et al., 2008), research on the salivary metabolome of diabetic children raised interest. To our knowledge, two NMR spectroscopy studies have comparatively analyzed the salivary metabolome of healthy and DMI children (Costa, 2013; Oliveira et al., 2016). One of the studies corroborated previous findings on children's DMI urine samples (Culeddu et al., 2012), as it revealed distinct salivary profiles between controls and DMI subjects, predominantly owing to variations in acetate, *N*-acetyl-sugars, lactate and sugar levels (Oliveira et al., 2016), whereas in the other research the DMI cohort exhibited higher salivary levels of butyrate and decreased concentrations of alanine, ethanol and malate, in comparison to controls (Costa, 2013). Interestingly, both salivary metabolomic studies collected and included as study variables a set of oral findings detected in participants (such as measurement of salivary flow rate and buffer capacity, parental report of having experienced xerostomia and the presence of soft tissue or dental caries lesions, for instance). Such methodological options were based on the fact that DMI patients often present oral mucosa alterations and experience lower salivary flow rates, local conditions which could potentially affect the salivary metabolome and/or mask the impact of the systemic disease on it (Costa, 2013; Oliveira et al., 2016). Although currently there is no consensus concerning a higher susceptibility to oral diseases such as dental caries in DM children, authors considered the commonly alterations in salivary glands they frequently present could possibly lead to altered salivary buffered capacity or composition, with potential effects on dental caries lesions' onset or progression. Whilst in one study oral conditions presented no impact on the metabolomic profiles (Oliveira et al., 2016), associations with some oral parameters were found in the other (Costa, 2013); nonetheless, comparison of their findings should be cautiously performed, as results refer to cohorts from different backgrounds, age intervals (20 months-6 years and 5-15 years, respectively) and sizes (n=68 and n=205, respectively).

Recently, another NMR-spectroscopy research evaluated the effects of hemodialysis treatment on the saliva of children and adolescents with end-stage renal disease (P. A. Almeida et al., 2017). The study findings disclosed not only the existence of a salivary fingerprint associated to chronic kidney disease, but also demonstrated that even though hemodialysis altered that particular fingerprint, the treatment did not completely reestablish patients' salivary profile to normal conditions (that is, to a profile similar to the observed in controls) (P. A. Almeida et al., 2017).

In addition to medical diagnosis, saliva metabolic profiling has also raised interest in the sensory physiology and dietary behavior domains. Saliva metabolome is believed to provide valuable information in this field, since this fluid plays an important role on food taste acceptance and texture perception and some salivary compounds have been linked to preference for fat, salty or sweet aliments (Morzel et al., 2017). One preliminary study explored the associations between food consumption patterns and saliva metabolome, using both MS and NMR methods, in children presenting eating difficulties and receiving more that 50% of their total energy intake through artificial nutrition (namely enteral or parental route) (Morzel et al., 2017). All in all, the study confirmed salivary composition differed according to diet qualitative features and paved the way towards further researches exploring the potentialities of saliva metabolomics in this field.

1.3.4.2.1. Metabolomics of children's saliva and dental caries research

To date, the literature on the effect of dental caries on the salivary metabolome of children is still scarce. Similarly to the approaches previously described for the study of dental plaque in adult populations, one pilot study has investigated the impact of dental caries on children's dental biofilm through GC/MS analysis (Zandona et al., 2015). The research included a small cohort composed by caries-afflicted (CA) and caries-free (CF) controls (n=11 and n=4, respectively, from 10 to 15 years old) and demonstrated that biofilm from caries-active locations exhibit a distinct metabolic profile characterized by high levels of branded alcohols and esters, compared to caries-free sites (Zandona et al., 2015). Though preliminary, these findings indicated that some metabolites present in the oral biofilm may be capable of providing a characteristic metabolomic signature for caries activity in children.

In respect to saliva, one untargeted LC-MS study has assessed the impact of dentition stage and caries on children salivary metabolomic profiles (Foxman et al., 2014). In addition, authors also gauged the impact of sibship (meaning children descending from same parents, or siblings) on the metabolome, as it could possibly reflect the effect of shared genes, environment and/or behaviors. In this report, a strong effect of sibship on the metabolite profiles was identified and associations of metabolite profile with dentition and dental caries were also found nested in the familial effects.

Other MS studies of saliva have indeed investigated ECC-afflicted children but focusing on salivary proteome rather than the metabolome (Ao et al., 2017; Hemadi et al., 2017; Si, Ao, Wang, Chen, & Zheng, 2015; X. Sun et al., 2016; Tian et al., 2017). In fact, in the last decade several studies have extensively explored the salivary protein profile features associated to the development of dental caries and/or ECC, in particular (Fidalgo et al., 2014; X. Gao, Jiang, Koh, & Hsu, 2016; Guo & Shi, 2013; Hemadi et al., 2017; Lorenzo-Pouso et al., 2018; C. Martins, Buczynski, Maia, Siqueira, & Castro, 2013; Piekoszewska-Ziętek, Turska-Szybka, & Olczak-Kowalczyk, 2019; Vitorino et al., 2005). Such interest has risen along with the development of increasingly more advanced protein profiling technologies, as from the known pivotal role of salivary proteome in oral homeostasis, since oral proteins not only provide several protective functions but, at the same time, can contribute to inflammation and infection processes (Ao et al., 2017; Lorenzo-Pouso et al., 2018). The majority of the performed studies consisted on cross-sectional researches in which the salivary proteome of ECC-afflicted or S-ECC children was compared to the profile of already treated subjects or healthy controls (Hemadi et al., 2017; Piekoszewska-Ziętek et al., 2019; Si et al., 2015). More recent studies addressing the salivary proteome in ECC dynamics have, nonetheless, involved longitudinal designs to assess changes in proteomic profiles that, over follow up periods, either correlated to disease development (Ao et al., 2017), were altered after treatment (X. Sun et al., 2016) or were associated to ECC recurrence after treatment (Tian et al., 2017).

Available evidence indicates higher levels of specific proteins, such as PRPs (Bhalla, Tandon, & Satyamoorthy, 2010; Hemadi et al., 2017; T. R. Ribeiro et al., 2013), or peptides as histatins (Ao et al., 2017; Hemadi et al., 2017; X. Sun et al., 2016) might be associated to an increased risk of ECC. The relationship between ECC and levels of salivary Igs, particularly IgA and IgG, still comprises controversy, as studies have reported distinct results (Hemadi et al., 2017; Piekoszewska-Ziętek et al., 2019). However, most of the studies have suggested higher levels of IgA (Fidalgo et al., 2014) and IgG (Bagherian & Asadikaram, 2012; Farias & Bezerra, 2003) in children with ECC, thus indicating the potential of such antibodies as biomarkers for ECC, in the future. To date, due to conflicting data or insufficient evidence, the role of other proteins and peptides, such as defensins (Malcolm et al., 2014; T. R. Ribeiro et al., 2013), mucins (Angwaravong, Pitiphat, Bolscher, & Chaiyarit, 2015; Bhalla et al., 2010), lactoferrin (Hao & Lin, 2009; Moslemi et al., 2015) and lysozyme (Lertsirivorakul, Petsongkram, Chaiyarit, Klaynongsruang, & Pitiphat, 2015; Moslemi et al., 2015) regarding ECC is still unclear. Moreover, reports on total salivary protein concentration refer its relationship with ECC still needs to be fully elucidated (X. Gao et al., 2016; C. Martins et al., 2013) and ascribe the lack of association probably to the function redundancies of protein compounds found in saliva (Hemadi et al., 2017).

Saliva metabolic composition remains, therefore, largely under-explored concerning dental caries research in children. To our knowledge, only two NMR studies of saliva have addressed CA children, compared to CF controls (Fidalgo, Freitas-Fernandes, Almeida, Valente, & Souza, 2015; Fidalgo et al., 2013). In 2013, one report characterized children salivary metabolome as a function of presence of dental caries (n=15 CA, mean age=7.23±2.01; n=18 age-matched controls) and dentition stage (Fidalgo et al., 2013). USS of children bearing caries lesions exhibited higher levels of lactate, fatty acid, acetate and *n*-butyrate and a reduction in levels of phenylalanine, propionate and saccharides. Moreover, the obtained metabolomic data enabled the classification of children with and without caries, since almost all of the subjects were correctly classified through cross validation procedures, except for two participants who produced false positive results. As increased levels of lipids had already been previously reported in parotid saliva from CA adults (Tomita, Miyake, & Yamanaka, 2008), authors suggested the variation in lipid levels and fatty acid composition was probably associated to the development of dental caries, considering lipids presence on the salivary pellicle of tooth surfaces may inhibit bacterial acid diffusion, hence enhancing dental caries lesions progression. The higher concentrations of the organic acids lactate, acetate and n-butyrate, known products of bacterial metabolism, were ascribed to a likely higher oral colonization of cariogenic microorganisms in CA children. When these organic acids are produced by cariogenic bacteria salivary and plaque pH are reduced and the porosity of the dental plaque matrix is increased. In fact, similar findings had been reported in two previously mentioned studies involving MS analysis of adults' supragingival dental plaque (N. Takahashi et al., 2010) and NMR spectroscopy of biopsies samples from active caries lesions (C. J. Silwood et al., 1999). Conversely, authors speculated the decreased levels of saccharides detected in the CA group resulted from the use of these metabolites as a substrate in bacterial energetic metabolism.

Aiming to assess whether the salivary metabolome suffered alterations during development, this untargeted NMR approach also gauged the effect of dentition stage within the CF cohort (n=15 primary dentition, mean age=4.27±1.27; n=18 mixed phase, mean age=7.94±2.09 and n=17 permanent dentition, mean age 10.88±1.05). Overall, a very similar metabolomic profile was detected among CF children. In this subcohort only acetate, saccharides and propionate were identified at larger concentrations in the saliva of children with permanent dentition, as compared to the children with primary dentition. Authors hypothesized the slight differences detected among dentition stages may relate to both physiological (mostly hormonal and related to salivary gland maturation) and social behavioral

changes during the pre-pubertal period. Similarly, it is possible that the eruption of permanent teeth and increased contact surface areas and sites for bacterial adhesion also contribute to higher concentrations of the mentioned microbial metabolites in the permanent dentition subjects' saliva (Fidalgo et al., 2013).

As the first NMR-based study exploring children saliva, this research provided a significant overview on caries dynamics, along with a contribute for describing both the metabolite profile patterns found in healthy children's saliva and the possible age-dependent changes on metabolome. Despite that, caution should be taken when interpreting the study findings, particularly since it involved a relatively small sample size when analyzing disease (n=15 CA *vs.* n=18 CF children) and health conditions (n=15 primary dentition, n=18 mixed phase, and n=17 permanent dentition subjects). In addition, when dental caries and controls groups were compared, the mean age of the CA subcohort was 7.23±2.01 years (with age-matched controls). The inclusion of younger CA children would have been of particular interest, considering a younger age group would have comprised children still in primary instead of mixed phase dentition, allowing for the potential obtainment of more valuable results concerning future clinical applications of metabolomic data in disease prevention and management (Fidalgo et al., 2013).

A second report by the same research group conducted the first longitudinal NMR-based study targeted at clarifying if the salivary metabolome associated to ECC in children was altered after treatment (Fidalgo et al., 2015). Thus, authors explored the metabolome of USS from ECC-afflicted children, sampled before and 7 days after treatment, as well as at a 1-month, 2-month and 3-month time point follow-ups, in comparison to controls (n=20 ECC-afflicted, mean age=2.8±0.83; n=10 controls, mean age=3.0±1.2) (Fidalgo et al., 2015). Distinct salivary profiles were found after dental treatment, particularly a reduction in the levels of acetate, propionate, fatty acid, butyrate and saccharides, though clearly more evident in the samples collected at 2 and 3 months after treatment than at the 7 day and 1-month follow up. However, even after 3 months, ECC treatment had conduced to a profile closer, but not similar, to the one found in controls, leading authors to hypothesize that the 3-month follow up period used in the study was not sufficient to completely recover oral homeostasis (Fidalgo et al., 2015).

As in findings obtained in the previous study, propionate, acetate and butyrate were among the metabolites that contributed the most to differences between groups, leading authors to confirm these organic acids' association with disease activity. The higher levels of saccharides' in saliva before treatment was, in this study, attributed to the fact that dental surfaces with caries lesions present irregularities, thus boosting dental plaque retention and delaying oral clearance of several metabolites arising from food ingestion, leading to a longer maintenance of saccharides in caries lesions with consequence increase in the production of organic acids. Albeit this study involved a similar sample size comparing to the previously referred research, the cohort comprised children in much younger ages (mean age=2.8±0.83 in ECC-afflicted and mean age=3.0±1.2 in controls), therefore targeting the specific impact of ECC on saliva (Fidalgo et al., 2015). Subsequent researches on larger (but appropriately matched) cohorts are presently required in order to provide stronger evidence supporting these preliminary findings. Further evidence may enlighten the present knowledge on ECC complex pathogenesis, possibly validating salivary metabolomic biomarkers and thus allowing the development of more effective evidence-based early diagnosis and/or prevention strategies.

1.3.4.3. Salivary metabolome under physiological conditions

Currently, the limited knowledge on the salivary metabolic profile of healthy children is still a major limitation hampering the development and clinical application of metabolomic researches in several medical fields (Hassaneen & Maron, 2017). It is known the human metabolome (and particularly the salivary composition) can be potentially influenced by a variety of endogenous features including age, gender and body mass index, as well as by exogenous factors (fasting/diet and level of hydration, medication intake, physical activity, pathological insults and other environmental factors, for instance) (Dame et al., 2015; Pedersen et al., 2018; Pfaffe et al., 2011; Rist et al., 2017; Wallner-Liebmann et al., 2016). As performed to explore the role of any other human biofluid within a specific biological system affected by disease, the starting point to unveil the role of saliva's metabolome in the oral cavity in pathological conditions is to first establish its composition in healthy subjects under homeostasis conditions. A thorough characterization of the salivary metabolome of healthy children would therefore constitute a key foundation for further studies assessing specific disease fingerprints, potentially useful in clinical diagnostics, as to better interpret and compare findings of previous studies. In fact, children's salivary metabolome has not been thoroughly catalogued nor described in terms of physiological conditions, such as gender or age (and dentition stage) yet.

Inter- and intra-individual variability

Similarly to the research efforts made to unveil and establish the human salivary proteome (Cabras et al., 2014), in the last years several metabolomic studies have addressed saliva's inter-individual compositional variability in healthy adults, through MS (Quintana et al., 2009) and NMR (Bertram, Eggers, & Eller, 2009; Lemanska, Grootveld, Silwood, & Brereton, 2012; C. J. L. Silwood et al., 2002; Wallner-Liebmann et al., 2016; M. C. Walsh, Brennan, Malthouse, Roche, & Gibney, 2006) techniques. Although multiple studies have assessed inter-individual differences in saliva metabolome, strong evidence on this matter was achieved in 2016, when one NMR study analyzed salivary samples from healthy adults collected for ten consecutive days, of which in the first seven days participants performed their average daily routine and in the last three days they were subjected to a standardized diet, plus a physical exercise program at the tenth day (Wallner-Liebmann et al., 2016). Obtained data indeed confirmed the clear existence of a strong individual metabolic phenotype in saliva, slightly weaker than the metabolic signature found in urine, however less influenced by dietary variations (Wallner-Liebmann et al., 2016).

The significance of salivary intra-individual variability has also been explored in studies using MS (Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012; Sugimoto et al., 2013) and NMR (Bertram et al., 2009; Dame et al., 2015; C. J. L. Silwood et al., 2002; M. C. Walsh et al., 2006) approaches. It is widely known that the human circadian rhythm modulates many human behavioral and physiological features, particularly at metabolic levels (Dame et al., 2015). Salivary glands function also displays circadian variation, as pointed out by multiple prior reports stating discrepancies in saliva composition and secretory rates, which present its maximum levels in the mid-afternoon or during stimulation periods (Li-Hui et al., 2016), and its minimum flow during sleep and night time (Pedersen et al., 2018; Wu et al., 2008). Moreover, seasons of the year also yield circadian-related changes in salivary characteristics, usually towards higher secretion rates in winter compared to summer (Cuevas-Córdoba & Santiago-García, 2014). Presently, metabolomic evidence has shown, not only that profiles of adults' diurnal and nocturnal salivary samples can be distinguished with great predictability (Bertram et al., 2009), but also significant differences can be found in diurnal samples collected just within a few hours apart (Dame et al., 2015). This reported

variability should be taken in account when analyzing saliva for research purposes, mainly through performing samples collection at the same time-span, to minimize the effect of circadian rhythm and avoid undesired bias in metabolomic data (Li-Hui et al., 2016; Pedersen et al., 2018).

Influence of gender and age

The impact of gender differences in saliva has been explored mostly in adults from a variety of perspectives comprising studies characterizing saliva's flow (Idrees et al., 2018; Inoue et al., 2006; Li-Hui et al., 2016; R. S. Percival, Challacombe, & Marsh, 1994; Pfaffe et al., 2011) and several particular biochemical analytes or properties (P. D. Almeida et al., 2008; Dame et al., 2015; Hemadi et al., 2017; Li-Hui et al., 2016; Prodan et al., 2015; S. Sun et al., 2014; Zaura et al., 2017). Authors have referred that some variations are expected to occur in saliva owing to the common variances among genders in body mass index and salivary gland size, as well as due to differences in estrogen-related metabolism and in gene expression profiles (Li-Hui et al., 2016). In children, the assumption that boys present higher salivary flow rates in comparison to girls has been stated; nonetheless, to date, the available literature regarding this issue is not unequivocal (Björnstad & Crossner, 2007; Bretz et al., 2001; C. G. Crossner, 1984; Rotteveel, Jongerius, Limbeek, & Hoogen, 2004; Torres et al., 2006; Tukia-Kulmala & Tenovuo, 1993; Tulunoglu, Demirtas, & Tulunoglu, 2006).

Gender specific differences in children's salivary biochemical compounds have also been explored but not systematically characterized or fully clarified yet (Davidopoulou, Diza, Menexes, & Kalfas, 2012; Dezan, Nicolau, Souza, & Walter, 2002; Granger, Schwartz, Booth, & Arentz, 1999; Jafarzadeh, Sadeghi, Karam, & Vazirinejad, 2010; Netherton, 2004; Tulunoglu et al., 2006; Watanabe, Tanaka, Shigemi, Hayashida, & Maki, 2009). In the metabolomics context, gender has been shown to impact pronouncedly on the NMR profiles of adult's urine (Kochhar et al., 2006; Psihogios, Gazi, Elisaf, Seferiadis, & Bairaktari, 2008; Rist et al., 2017) and blood (Kochhar et al., 2006; Rist et al., 2017; Vignoli, Tenori, Luchinat, & Saccenti, 2018). Differences between adult males and females have also been detected in saliva's metabolome and proteome through MS techniques (Fleissig et al., 2010; Sugimoto et al., 2013, 2010; S. Sun et al., 2014; X. Xiao et al., 2017). The existing ^{1}H-NMR reports concerning this matter are limited and not consensual, as they have registered both no significant changes (Bertram et al., 2009) and possible changes in the salivary metabolome as a result of gender-related characteristics (Takeda et al., 2009; Wallner-Liebmann et al., 2016). In Takeda et al. investigation, organic acids, glycine and methanol, the metabolites which significantly varied between genders, presented higher levels in males (Takeda et al., 2009). Accordingly, gender-related differences are expected to impact on salivary metabolite profiles of children as well.

In addition, the impact of age on saliva characteristics has been investigated by a number of studies in children and adults, as during childhood salivary glands undergo growth and maturation processes and later are prone to structural and degenerative changes (stromal alterations and loss of secretory acini, for instance), experiencing anatomical and physiological alterations with individual's advancing age that can lead to gland functional alterations (Affoo, Foley, Garrick, Siqueira, & Martin, 2015; Pedersen et al., 2018; Vissink, Spijkervet, & Van Nieuw Amerongen, 1996).

Until recently the evidence on specific age-related variations in saliva flow rates of healthy adults remained somewhat unclear, since many studies had reported both stable (Bakke et al., 2004; Ben-Aryeh et al., 1986; Salvolini et al., 2000; Ship & Fischer, 1997) or decreasing salivary flow rates with age

(Moritsuka et al., 2006; M. Navazesh et al., 1992; R. S. Percival et al., 1994; Smith et al., 2013; X. P. Wang et al., 2012). In 2015 one meta-analysis concluded that USS and SS whole, as well as submandibular/sublingual gland flow rates were significantly decreased in older subjects comparing to younger adults (independently of medication usage), while parotid and minor salivary glands flow were not affected by age (Affoo et al., 2015). Furthermore, significant differences were not found for SS flow rates after excluding the subcohort under medication intake from the statistical analysis (Affoo et al., 2015). In spite of age-related loss of secretory tissue being known to occur in all salivary glands, such results suggest the extent of functional impairment with age may vary in a gland-specific manner, as the salivary flow of parotid glands (the major contributors to SS secretion) seems to remain stable during ageing in health non-medicated adults (Affoo et al., 2015; Pedersen et al., 2018). Concerning children and adolescents, while some publications have pointed out an increase of salivary flow during childhood (C. G. Crossner, 1984; Hemadi et al., 2017; Wu et al., 2008), several reports failed to detect significant age-specific differences (Björnstad & Crossner, 2007; Bretz et al., 2001; Dezan et al., 2002; Rotteveel et al., 2004; Torres et al., 2006).

With respect to the biochemical composition of saliva, age-dependent variations are believed to occur from early childhood to adolescence and throughout adulthood, as already established in relation to several electrolytes and particular compounds (Dame et al., 2015; Fidalgo et al., 2013; Hemadi et al., 2017; P. Pandey, Saxena, Chaudhary, Reddy, & Rao, 2015), such as calcium and magnesium (Pappa et al., 2018), hormones (*e.g.* salivary cortisol) (Pappa et al., 2018), proteins and peptides (Hassaneen & Maron, 2017; Malcolm et al., 2014; Manconi et al., 2013; Messana et al., 2015; S. Sun et al., 2014) and enzymes (*e.g.* α-amylase) (Pappa et al., 2018). Large efforts have been made to characterize age-related characteristics of the human urine and blood metabolomes (Kochhar et al., 2006; Psihogios et al., 2008; Rist et al., 2017; Vignoli et al., 2018); still the influence of age in the salivary metabolomic profile has yet been systematically addressed. To our best knowledge, regarding NMR spectroscopy-based salivary studies in children, this variable has only been preliminary evaluated in Fidalgo et al. report, as described above, and whose findings apparently support the hypothesis of growth-related changes (Fidalgo et al., 2013).

Influence of sampling conditions

Additionally, in studies focusing on saliva composition, further biological and technical aspects concerning saliva sampling can influence the obtained results. Regarding sample collection settings, factors such as saliva collection method, the type of stimulus used, time of day and duration of collection, participants' body posture and level of stress should be closely standardized and/or monitored (Dame et al., 2015; Hanrahan, McCarthy, Kleiber, Lutgendorf, & Tsalikian, 2006; Mahvash Navazesh & Kumar, 2008; Neyraud et al., 2013; Pedersen et al., 2018). It is also recommended collections should follow a period of fasting of at least one hour (Gardner et al., 2018; Ishikawa et al., 2017) and delayed oral hygiene, to prevent contamination from diet and oral hygiene products' sources and to suppress salivary glandular stimulation (Gardner et al., 2018; Ishikawa et al., 2017; Pedersen et al., 2018; Priya & Prathibha, 2017; Wallner-Liebmann et al., 2016; M. C. Walsh et al., 2006; Zaura et al., 2017). Alongside, sample handling, storage and processing conditions can potentially affect saliva's metabolome, thus introducing undesired variability in datasets and also making the comparison of separate studies' results more difficult (Dame et al., 2015; Pedersen et al., 2018; Prodan et al., 2015).

Stimulation, regardless of the stimulus nature, is known to alter saliva's pH, secretion rate and chemical composition (Chiappin et al., 2007; Mahvash Navazesh & Kumar, 2008; Neyraud et al., 2013; Okuma et

al., 2017; Pfaffe et al., 2011), as upon stimulation conditions the salivary glands contribute to whole saliva in different extents than in resting circumstances (Affoo et al., 2015; Humphrey & Williamson, 2001; Li-Hui et al., 2016). The effect of stimulation on the '*omic*' signature of saliva has mostly been reported in terms of proteome (Neyraud, Sayd, Morzel, & Dransfield, 2006; Pfaffe et al., 2011; Quintana et al., 2009; Schipper et al., 2007; X. Xiao et al., 2017). Available literature on stimulation-induced changes on the metabolome is, to date, scarce and, to our knowledge, has only been studied in adults, via MS (Okuma et al., 2017) and NMR approaches (Figueira et al., 2017; Gardner, Parkes, So, & Carpenter, 2019; Neyraud et al., 2013; Takeda et al., 2009).

Although some conflicting results have been found in NMR spectroscopy studies, all of them reported substantial differences in USS and SS metabolite composition (Figueira et al., 2017; Neyraud et al., 2013; Takeda et al., 2009) suggesting that, even though further research is required to fully clarify such aspect, the impact of saliva stimulation on metabolome should not be disregarded. Comparison of the limited data available is hindered due to divergences in the stimulants used in reports: while two NMR studies used saliva samples obtained through gum chewing (Figueira et al., 2017; Neyraud et al., 2013), the protocol performed in the other report involved application of citric acid solution directly to the participants' tongue (Takeda et al., 2009). In general, caution is recommended when planning a research in which SS composition is to be analyzed, as each stimulation method presents limitations that if not taken in account can interfere with the results of the study. Paraffin, a popular material used to obtain SS through chewing movements, is well-accepted in children cohorts, for instance, and constitutes a flavor and odorless material, thus not inducing any other stimulation type than the one produced by mastication (Okuma et al., 2017). However, samples collected through this method may possibly include a higher bacterial content, removed from dental plaque during chewing movements (Motisuki, Lima, Spolidorio, & Santos-Pinto, 2005). The use of acid stimulation has also raised concerns, as authors have suggested it may alter the sample pH and composition (Beale et al., 2016; Granger et al., 2007; Hanrahan et al., 2006; Neyraud et al., 2013; Nunes & Macedo, 2013; Nunes et al., 2015; Voegtline & Granger, 2014) and has also been reported to interfere with the measurement of some analytes (Chiappin et al., 2007).

The most recent NMR study characterizing the metabolome of different types of saliva covered a comparison between USS and parotid gland saliva collected by placing a sterilized Lashley cup over the parotid duct opening, the latter sampled through both resting and stimulation with tartaric acid conditions (Gardner et al., 2019). Metabolome analysis of saliva specifically secreted by parotid glands using NMR spectroscopy had been performed in a previous research, in which parotid saliva was collected from only one individual and compared to a USS sample of the same subject (Figueira et al., 2017). Gardner *et al.* study involved a larger sample constituted by healthy adults (n=11) and, in addition, plasma samples were further analyzed with NMR and USS samples were cultured on sucrose and peptide-enriched media. Researchers intended to gauge the extent to which metabolites in whole saliva mirrored the host or microbial metabolic activity, given that USS is a complex mixture containing fluid secreted by glands, bacterial and host-derived cells, while parotid saliva, if aseptically collected directly from the glands, is composed exclusively by glandular secretions, thus reflecting the host metabolic activity (Gardner et al., 2019). Interestingly, in this study, stimulation with tartaric acid almost did not affect the NMR profile of parotid saliva, excepting for urea, which was higher in unstimulated parotid samples.

To the best of our knowledge, this was the first time such comparison was performed through NMR, as previous studies explored the effect of stimulation on whole saliva (Figueira et al., 2017; Takeda et al., 2009) or specifically on parotid secretion in comparison to USS (again, referring to whole saliva) (Figueira et al., 2017). Considering that in this study urea levels were lower in USS comparing to parotid

saliva and that an inverse correlation with the bacterial load was also detected, authors stated these findings could be due to a utilization of host-derived metabolites by oral microbiota, possibly as a bacterial survival mechanism (since urea is implicated in increasing oral pH via conversion to ammonia). In general, USS inter-individual variation was found to be much greater than the one found in plasma or parotid saliva and USS samples presented considerably high short-chain fatty acids levels, namely acetate, propionate, butyrate and formate. These analytes were, however, detected in residual concentrations in parotid saliva and plasma of the same individuals, highly suggesting such compounds are almost entirely generated from bacterial fermentation occurring in the oral cavity, rather than constituting endogenous host metabolites. Contrastingly, no differences between the levels of lactate, an organic acid formerly suggested to arise in saliva from saccharolytic bacteria origin (Takeda et al., 2009), were found varying between groups. This finding led researchers to believe lactate could also be, in fact, a host-derived metabolite appearing in saliva from circulation via salivary glands and that its concentration in saliva reflected baseline levels from plasma, considering participants had fasted before sampling (thus excluding the hypothesis of lactate production due to recent ingestion of food). In sum, despite the study limitations, overall results showed saliva metabolome can reflect more the oral microbiota activity than the underlying host metabolism. If confirmed, such findings will possibly influence the methodology of further researches in this matter, as well as the affect the interpretation of investigations regarding the impact of physiological and pathological modifiers in saliva metabolome (Gardner et al., 2019).

Most studies performed in children populations in non-metabolomic contexts have involved SS, which is easily and rapidly collected and does not require specialized skills (Chiappin et al., 2007). However, USS composition is believed that better represent baseline oral environment (Chiappin et al., 2007; Granger et al., 2007). Unexpectedly, USS collection can often be considerably a challenging and time-consuming procedure, especially in young children, occasionally hindering the obtainment of sufficient volume (Chiappin et al., 2007; O'Farrelly & Hennessy, 2013). To address this issue, saliva absorbing devices commercially available have been reported to provide benefits concerning children collaboration and the amount of collected saliva (Chiappin et al., 2007; Granger et al., 2007; O'Farrelly & Hennessy, 2013; Topkas et al., 2012). Nonetheless, their potential interference with sample composition has been noted (Chiappin et al., 2007; Granger et al., 2007; O'Farrelly & Hennessy, 2013; Sugimoto et al., 2013; Topkas et al., 2012). One MS study comparing saliva collected through PD and a cotton-based absorbing device in an adult cohort has pointed out significant differences should be expected in the salivary metabolome when using absorbing methods (Sugimoto et al., 2013). As far as we know, the use of absorbing devices in saliva collection for NMR-based metabolomic analysis has not been studied on adults or children, thus it is currently unknown whether they interfere with sample metabolic composition.

Lastly, in addition to the imperative need of studies accurately examining the impact of physiological and collection sampling parameters in the metabolome of saliva, cautious optimization and standardization of experimental conditions are also required in future researches (Dame et al., 2015; A.-H. M. Emwas et al., 2013; Gardner et al., 2018). Lack of methodological standardization has been pointed out as one of the main causes for inconsistent or not comparable results in salivary metabolomic researches (Gardner et al., 2018). Presently, in contrast to existing NMR-based studies of urine or plasma, investigation fields in which validated guidelines on sample collection, handling and analysis have been published, available NMR studies of saliva present highly varied protocols (Chetwynd, Dunn, & Rodriguez-Blanco, 2017; Gardner et al., 2018).

Until recently, no study had assessed or standardized technical aspects such as saliva sampling, storage, centrifugation or processing conditions for NMR spectroscopy, parameters that can possibly affect saliva's metabolome and thus introduce unwanted variations in data and lead to misleading conclusions

(X. Chen & Yu, 2019; A.-H. M. Emwas et al., 2013; Pedersen et al., 2018). In 2018, aiming to assess multiple fundamental aspects of saliva preparation for NMR, one study examined the impact of centrifugation (particularly centrifugation force and timing, that is, if centrifugation should be performed of after initial freezing), freeze-thaw cycles and different quantification methods on the NMR salivary profile of healthy subjects (Gardner et al., 2018). Results indicated ^{1}H-NMR salivary metabolome is, in fact, resilient to several changes resulting from freezing, even when it is performed after centrifugation. This constituted a relevant analytical issue, as freezing whole blood prior to conversion to plasma or serum can produce leakage of intracellular metabolites into the blood, for instance (Gardner et al., 2018).The need for centrifuging saliva to remove cellular content from samples was indeed confirmed through this study, though similar effects were noticed the multiple between centrifugation forces applied (from 750 to 15000g).

Lastly, authors highlighted the need to keep samples at 4ºC from the moment of collection until storage or processing phases, to use metabolic inhibitors such as sodium azide (NaN_3), as well as to use Na^+/3-trimethylsilyl-propionate (TSP) solution with phosphate buffer as an internal standard (Gardner et al., 2018). Nonetheless, several questions regarding protocol considerations remain unanswered, in particular the effects of prolonged storage at -80ºC before processing or the impact of keeping samples at room temperature while awaiting analysis in the NMR spectrometer.The establishment of evidence-based protocol recommendations that clarified all of the above-mentioned aspects would most definitely promote the accomplishment of sample collection/processing under well-controlled conditions and encourage the development of future NMR studies of saliva.

1.4. Dissertation topic choice

The present project intends to shed light on ECC, a disorder which constitutes a public health problem of global proportions with devastating effects among toddlers and young children and whose prevention and management remains a challenge to the medical community (Phantumvanit et al., 2018; Slayton, 2015). To date, the lack of accurate and consistent risk predictors has hampered effective identification of children at high-risk for ECC before the onset of the disease (Edem, 2018; Zemaitiene et al., 2017). Improvements concerning disease timely diagnosis and/or management would not only decrease the significant social and economic consequences of ECC, but would also improve children's immediate and long-term OHRQoL.

In the last years, saliva has been increasingly viewed as one of the most appealing and promising screening tools due to its' potential to monitor physiological and pathological states of an individual, hence enabling early diagnosis and assessment of disease severity (Idrees et al., 2018; Streckfus, 2015). Its non-invasive and convenient collection, along with the multiple protective roles it is known to perform in the oral cavity, in particular regarding dental caries (Chiappin et al., 2007; Greabu et al., 2009; Roi et al., 2019), make saliva the ideal biofluid to explore ECC dynamics. Through saliva metabolomics, valuable insights on several pathological processes have been attained, providing major breakthroughs in many biomedical fields (A.-H. M. Emwas et al., 2013; A. Zhang, Sun, & Wang, 2012a; Y. Zhang et al., 2014) and, accordingly, we believe the application of this powerful approach to ECC research has great potential.

Saliva metabolomics remains, however, largely unexplored in caries research, especially in pediatric populations, and the scarce studies that have been conducted have either involved small sample sizes or cohorts of children in mixed phase dentition evaluated in a single time period (Fidalgo et al., 2015, 2013). The present observational investigation is therefore based on the potentialities of saliva metabolome regarding ECC. A 1H-NMR based strategy was performed to analyze the salivary metabolic profiles of a large-scale five-year-old cohort, aiming to identify metabolomic profiles characteristic of ECC's presence or distinct clinical stages. The size of this cohort, along with the age of its children, constituted major strengths of our research approach in comparison to previous studies on this matter.

Furthermore, the absence of bibliographic references providing validated protocols in regard to issues as sample collection, storage and analytical techniques, as well as data on the potential impact of subjects' physiological features in the salivary metabolome represented a challenge to our team. The intention to address these relevant research gaps and to predict feasibility and adverse events of our full-scale research project led us to design a small scale pilot study targeted at establishing a salivary samples collection protocol suitable for 1H-NMR-based studies and performing a preliminary assessment of the effects of gender, dentition stage and presence of dental caries on the salivary metabolome of our cohort.

1.5. Aims of this thesis

Aiming at identifying the impact of ECC on the salivary metabolome of children through 1H-NMR spectroscopy, the present work pursued the following objectives:

1. To determine ECC prevalence, severity and significant associations with sociobehavioral factors in a large preschoolers cohort (main study);
2. To perform a 1H-NMR-based metabolomics cross-sectional pilot study in a small children cohort to:

    2.1. present a comprehensive and detailed description of children's salivary metabolome using NMR spectroscopy, for the first time to our knowledge;
    2.2. establish a suitable protocol for salivary sample collection with minimal interference with the metabolome by assessing the impact of stimulation and of saliva absorbing devices in the metabolome;
    2.3. gauge the effects of physiologic features and dental caries in the salivary metabolome;

3. To perform a main observational longitudinal study using NMR metabolomics to:

    3.1. determine the impact of ECC on salivary metabolome (diagnostic markers)
    3.2. preliminary search for potential ECC predictive markers.

1.6. Outline of this thesis

For the purpose of addressing all the research aims, the present dissertation covers one pilot study and one main large-scale investigation, being structured into seven chapters as follows.

In **chapter 1** the scope of this research is introduced by providing a comprehensive overview of ECC's distinctive clinical pattern and etiology, with particular emphasis on its impact as a public health problem and current challenges on disease management and prediction. Saliva's protective role and potential for disease diagnosis are also reviewed, along with background on the metabolomics methodology applied. A state of the art of saliva metabolomics in oral research and relevant knowledge gaps in this field are then presented. Chapter 1 also includes the justification of the choice of this dissertation topic and the goals of the investigation hereby described.

Chapter 2 describes the experimental details involved in the work performed, including issues on study population selection, metadata and sample collection, data acquisition and data analysis procedures as well.

Chapter 3 includes the analysis of clinical data and metadata arising from the two evaluation periods performed in the main observational study (baseline and six months), which allowed the determination of ECC's prevalence and severity, the investigation of meaningful associations between metadata variables and ECC, as well as definition of subgroups within the cohort.

A comprehensive characterization of children salivary metabolome viewed by 1D and 2D NMR experiments is depicted in **chapter 4**, after a brief account on the fundamental principles underlying the identification of metabolites through these strategies.

In **chapter 5** the effects of stimulation conditions and the use of a saliva-absorbing device on the salivary metabolome of a pilot cohort of children were assessed, in order to establish a suitable salivary collection protocol for subsequent ^{1}H-NMR-based research in pediatric populations.

After optimization of the saliva collection protocol, the relative impacts of gender, dentition stage and presence of dental caries on the USS metabolome of the pilot cohort were gauged by NMR metabolomics, as displayed in **chapter 6**, and a putative saliva metabolomic signature of dental caries in children composed by 21 metabolites was identified.

Chapter 7 outlines the USS NMR metabolomics study of the large five-year-old cohort of the main observational research, performed to unveil the impact of ECC in the salivary metabolome. Although the main investigation involved a longitudinal data collection, in this chapter only the baseline collection metabolomic results are presented, as the six-month evaluation ^{1}H-NMR data is still subject of our ongoing work. A striking novel result involving an alanine variation in ECC was found, the direction of which appeared to be related to gender. Qualitative trends including other metabolites were also found. The reasons underlying such different results from those of the pilot study are also discussed in chapter 7.

Finally, general conclusions and future perspectives in the context of the present work are drawn, highlighting the contribution of the investigation to ^{1}H-NMR-based salivary research and to knowledge on ECC dynamics.

Chapter 2. Experimental procedures

Chapter 2. Experimental procedures

The present chapter describes the study population selection processes, salivary samples and metadata collection procedures, as well as the experimental and data analysis strategies used to perform all the experimental work presented throughout the thesis.

The cross-sectional pilot study, firstly developed mainly to optimize the protocol used in saliva collection and to evaluate the impact of potential confounding factors on saliva metabolome analysis, involved a small cohort of children attending a pediatric dentistry clinic in September 2015 in Coimbra, Portugal. The following main research, conducted in 2016, consisted on an observational longitudinal study exploring the saliva metabolome of five-year old children attending preschools in the same district, followed through two periods: an initial baseline evaluation and after six months. While participants from the pilot study were only subjected to one evaluation in which saliva collections and one oral examination were performed, children from the large cohort participated on saliva collection and oral examination procedures on each of the two evaluation periods.

This work was conducted with the collaboration of the Institute of Pediatric and Preventive Dentistry from the Faculty of Medicine of the University of Coimbra (FMUC) and the Centre for Research in Ceramics and Composite Materials of the University of Aveiro (CICECO/UA). The Laboratory of Biostatistics and Medical Informatics from FMUC also supported this research.

Ethical approval for this study was obtained from the FMUC ethics committee (reference CE-012/2015, in 23rd February 2015; Annex I) and from the Regional Health Administration of the Centre Region (ARSC) (reference 65/2014, in 22nd January 2015; Annex II). The Baixo Mondego Health Centers Group (ACeS Baixo Mondego) also approved the prosecution of this research project (reference 11072, in 27th May 2015; Annex III) within the scope of the National School Health Programme (PNSE), already implemented in the district's preschools. Thoroughly respecting the Declaration of Helsinki principles (amended in 2013) for human research, a written informed consent of each child's legally authorized representative was obtained (Annex IV) and, additionally, child assent was verbally obtained for all subjects after providing simple and brief explanations on the study's purposes and procedures (Annex V).

2.1. Definition of study population

2.1.1. Pilot study (small cohort)

The pilot study involved a convenience sample, which aimed to represent a population of systemically healthy Portuguese children and was composed of four to sixteen-years old healthy children (n=38) who attended a pediatric dentistry clinic in Coimbra, Portugal, for regular dental care, whose legal tutors signed a written consent (Annex IV). Children receiving antibiotics, having used antiseptic mouthwash/toothpaste in the preceding two weeks, with signs of periodontal disease or wearing orthodontic devices were excluded. Further exclusion criteria comprised children under medication that could affect saliva secretion and children who were crying or restless during oral examination or saliva collection.

This small cohort comprised SS and USS groups, each including the same subjects, except for eight additional elements in the former group (Figure 2.1). As described in Table 2.1, average age, gender ratio, dentition stage proportion (primary/mixed/permanent) and CA) / CF ratio were similar in USS and SS groups, which were therefore considered matched for those factors. A small group of four-year old children (*n*=5) donated saliva by both PD and SalivaBio's Children's Swab® (SCS) methods (Figure 2.1).

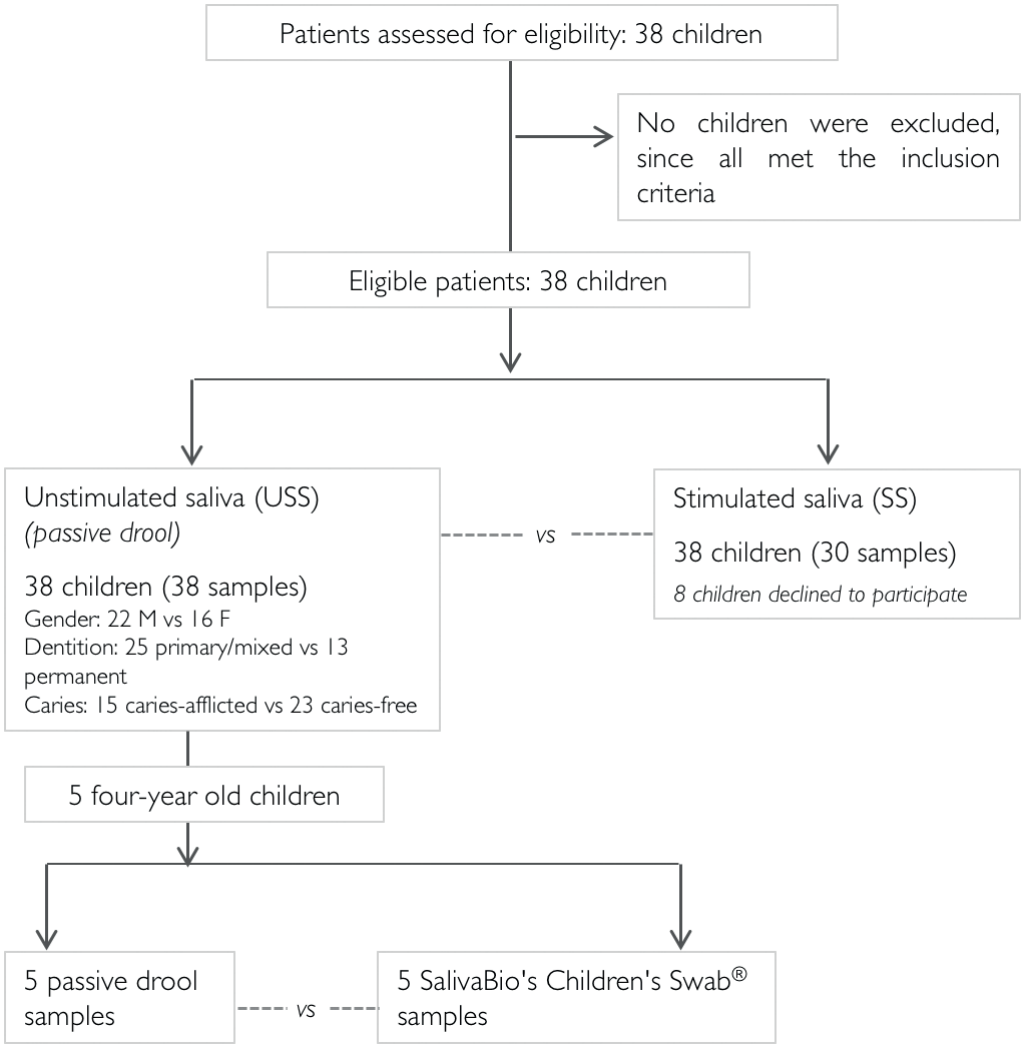

Figure 2.1. STROBE flow-chart of participants in the pilot study.

Sample group	*n*	*Age (years)*	*Gender (M/F)*	Dentition stage: *Primary (%) Mixed phase (%) Permanent (%)*	Dental caries status	
					CA subjects	CF subjects
SS [a)]	30	10 (5-16)	17/13	4 (13%) 14 (47%) 12 (40%)	13	17
USS [a), b)]	38	9 (4-16)	22/16	9 (24%) 16 (42%) 13 (34%)	15	23
PD and SCS [c)]	5	4	3/2	5 (100%) 0 (0%) 0 (0%)	1	4

Table 2.1. Characterization of the study population, comprising number of subjects (*n*), average age in years (ranges in brackets), male (M) / female (F), dentition stage and dental caries status, per subgroup. [a)] single saliva collection per patient; [b)] USS samples collected by PD were used to study the effects of gender, dentition stage and caries; [c)] the same subjects provided samples for both PD and SCS groups.

For the study of gender, dentition stage and caries on salivary metabolome, USS subsets were defined: 1) gender: 22 males (primary/mixed/permanent staging: 6/8/8); CA/CF=10/12) and 16 females (staging: 3/8/5; CA/CF=5/11); 2) staging: primary and mixed stages were grouped due to clinical similarity, resulting in 25 subjects at primary/mixed stages (M/F=14/11; CA/CF=10/15) and 13 subjects at permanent stage (M/F=8/5; CA/CF=5/8); and 3) caries status: CA *n*=15 (staging: 2/8/5; M/F=10/5) and CF *n*=23 (staging: 7/8/8; M/F=12/11). Although not perfectly matched, these groups will help gauge the relative magnitude of each factor (gender, staging, caries), compared to the remainder. In particular, CA *vs.* CF comparison will unveil if caries play a stronger role on saliva metabolome than gender and dentition stage; the alternative use of matched groups might unveil new metabolic information, which should however already include any effects observed in the present study. Our preliminary power calculations indicated about 20-30 samples per group; however, the sample sizes used were those realistically available and roughly approximate to calculated size, namely subcohorts of 13-23 individuals (Table 2.1). These group sizes were comparable to previous reports (Fidalgo et al., 2015, 2013).

2.1.2. Observational study (large cohort)

The main study sample involved a convenience sample, which included five-year old children attending preschools in the area of Coimbra during 2016-2017 academic year. Similarly to other ECC observational studies, sample size determination could have been calculated through Krejcie & Morgan formula, which considers the available disease prevalence data (Krejcie & Morgan, 1970). However, due to the particularities of statistical methodologies applied in metabolomic analysis, to the fact that there are currently no standard methods for sample size estimation in metabolomics and considering previous studies sample size, 200 children was settled as minimum sample size. To account for potential dropouts, 20% more than the calculated minimum sample size was considered, accounting for a total of 240 participants.

After obtaining approval from all research ethics boards, contact was established with several preschools, with the collaboration of two district school nurses who had already implemented PNSE projects' in the abovementioned education institutions. The nurses' accessibility and proximity to these preschools definitely promoted the obtainment of consent and cooperation from the preschool principals, enabling the establishment of an appropriately sized convenience sample within the time constraints of the present research. Accordingly, 17 preschools from the rural and urban area of Coimbra, including public, for-profit and not-for-profit private preschools (namely Private Institutions of Social Solidarity, IPSS) were selected to enrol the study. A detailed characterization of the preschools included in the present study is presented on Table 2.2.

Type of preschools	Number of preschools	**Preschools' location** *(U/R)*	Eligible children		Number of intraoral examinations		Number of saliva collections	
			n	***Gender*** *(M/F)*	Baseline evaluation	Six-month evaluation	Baseline evaluation	Six-month evaluation
Public	11	6/5	81	40/41	78	62	64	54
Private	2	2/0	30	22/8	29	25	27	23
IPSS	4	2/2	35	16/19	35	27	31	23

Table 2.2. Characterization of the main study sample, comprising type, number and location of included preschools (urban (U) / rural (R)), as well as number (*n*) and gender ratio (male (M) / female (F)) of participating children. Number of intraoral examinations and saliva collections performed at each evaluation moment are also presented.

The researcher presented the main purposes and proceedings of the research to each preschool and also highlighted the potential benefits for all participant children and respective legal tutors, since all tutors were informed on the oral health status of their child after each examination (Annex VI) and, additionally, children were given a kit containing one toothbrush, a dentifrice, an educational sticker and a small toy for their participation. A future health benefit to children in general arising from potential improvements in ECC management due to the research was equally emphasized and, in addition, all preschools were given the opportunity to host an oral health promotion session for all school children, promoted by the research team. After describing all the research design, the main researcher provided a written description of the project and each school board was latterly contacted to inquire whether they were interested in participating.

Once the baseline evaluation date was scheduled with each preschool, a study presentation letter (Annex VII) and a duplicate written consent form were sent days before the initial evaluation to the legal tutors of all children born in 2011 (who were five-year old at the time or who would turn five during that year). These documents comprised detailed yet concise explanations written in non-technical language regarding the aforementioned study purposes, proceedings and benefits for participants, aside from referring the absence of expenses or risks in taking part in the study. They also strictly mentioned all participation in research was confidential and voluntary, stated all subjects could refuse to participate or withdraw from the study at any time, without penalty or loss of benefits to which the participant was otherwise entitled and included details on how collected data would be handled and protected. Lastly, the participant information sheet requested a brief set of data concerning the participant's medical status, sociodemographic characteristics and oral health related behaviors (Annex VIII). The main researcher contact information was also available for further information regarding the study methodology. Children legally authorized representatives who consented with enrolment in the study were asked to return to the preschool's teacher(s) or staff the signed original consent form along with the information sheet providing the child's aforesaid data in an enclosed sealed envelope and to retain the duplicate consent form. Aiming to enhance parental adherence to participating in the study, whenever possible, non-respondents legal tutors were contacted by preschool teachers before the scheduled evaluation day.

At the baseline evaluation day at each preschool, eligibility for enrolling the study was defined by children born in 2011 whose legally authorized representatives returned a signed written consent form and provided the requested participant's medical data. Subjects with any relevant systemic illnesses, who were on medication that could affect saliva secretion or who had been on antibiotic therapy or oral antimicrobials within the preceding two weeks were excluded from the study. Further exclusion criteria comprised children who had eaten or performed oral hygiene in the 120 minutes prior to evaluation, children with signs of periodontal disease or wearing orthodontic devices, participants who declined to participate after the main researcher explained the study proceedings and children who were crying or restless during oral examination or saliva collection.

The diagram flow presented in Figure 2.2 illustrates the study sample definition process. A total of 327 children born in 2011 from the selected preschools received the informed consent and were given the opportunity to enrol the study. Of these, 150 returned the original signed form and the requested data on the participant's medical status (83 children from public preschools, 30 from private and 37 from IPSS) but only 146 subjects were eligible to enrol the study (4 children presented relevant systemic diseases).

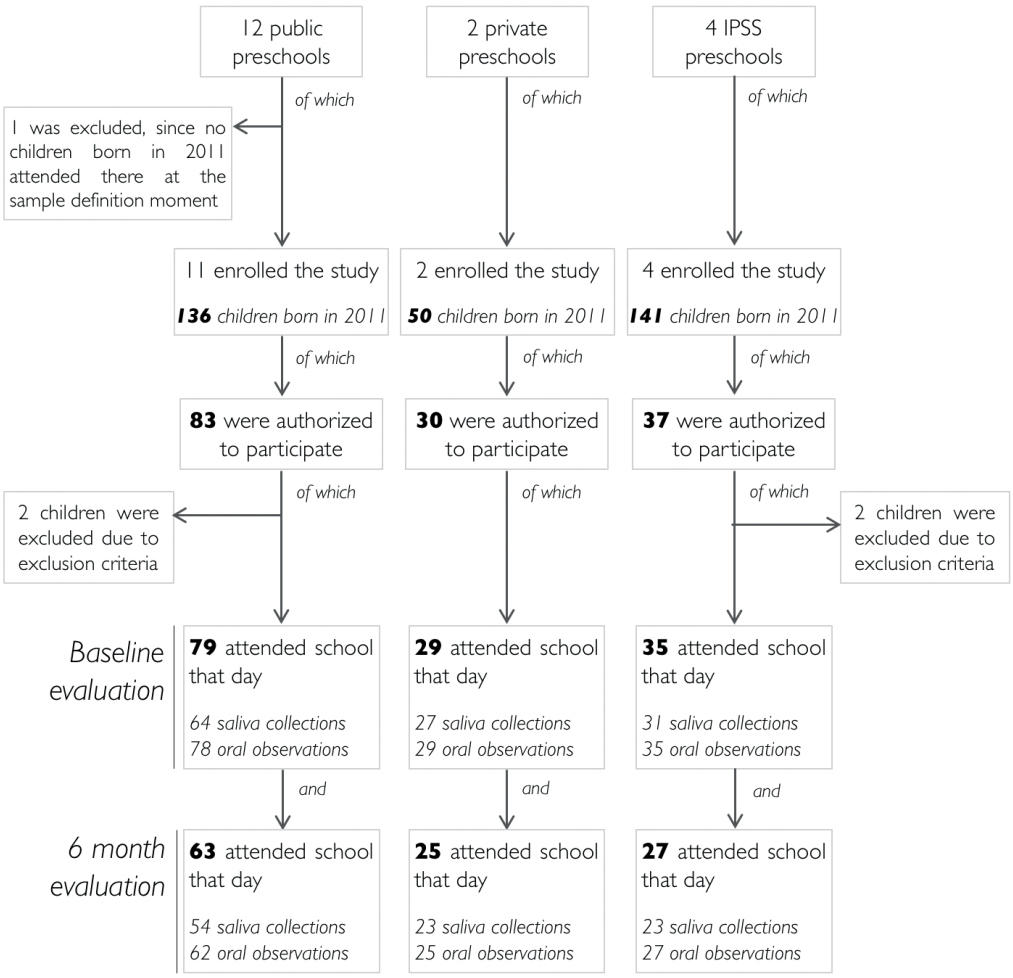

Figure 2.2. Diagram flow of participants and samples in the main study.

Since 3 recruited children did not attend preschool at the baseline evaluation day, the first study moment involved 143 participants, and, of those, 1 child refused to collaborate on oral examination. About 122 saliva samples were successfully collected and analyzed at the first evaluation, since the minimal required saliva volume was not obtained in 4 of the included children, 16 spectra were excluded due to distortion in the water region after processing and 1 subject was crying/restless during saliva collection procedures and did not manage to collaborate.

From all the 150 children whose legal tutors returned the original signed form, only 100 provided the requested data on the participant's sociodemographic characteristics and oral health related behaviors.

At the six-month evaluation day, 31 children who had firstly enrolled in the research did not attend preschool that day. Of the 115 children participating in this evaluation, 114 cooperated on oral examination protocol and 100 saliva samples were collected (1 child did not collaborate on saliva collection and 14 subjects did not expectorate the minimal required saliva volume).

2.2. Collection of saliva and metadata

2.2.1. Saliva collection

Salivary collections were performed in the midmorning period, between 9-11:30 *a.m.*, to minimize circadian rhythm interference (Dawes, 1972) and to ensure subjects had not eaten, drank or used oral hygiene products within the 120 minutes preceding sample collection, as previously had been instructed to legal tutors and to preschools' staff (in the large cohort study, specifically). All salivary samples were collected by the main investigator as described below; though it should be noted while three different saliva collection procedures were conducted in the pilot study (USS collected through PD and through an absorption device and SS), the collections performed in the main study involved only the PD method. USS was collected under resting conditions using the PD method as described by Navazesh & Kumar for 5 minutes or until 1.5 mL were obtained (Mahvash Navazesh & Kumar, 2008). After asking the participants to sit comfortably and undisturbed in a chair, with their heads tilted slightly forward and their eyes opened, subjects were instructed to restrict orofacial movements, allowing saliva to accumulate in the floor of the mouth and then drooling into a sterile codified tube with wide opening and lid.
To collect USS using the absorption device SalivaBio's Children's Swab®, SCS (Salimetrics, State College, PA, USA), the swab was placed in each subject´s oral cavity while the child sat motionless and upright, until saturation of the device was achieved. Saliva was then extracted from the device by centrifugation (15 minutes, 3,000 rpm, Eppendorf 5810R centrifuge with r=180 mm as instructed by the manufacturer) and stored as described below for the samples obtained though the other used methods.
Regarding SS collection, children were asked to chew a paraffin wax pellet (CRT® bacteria, Ivoclar Vivadent AG, Schaan, Liechtenstein) for 3–5 minutes, while expectorating secretions into a laboratory container equivalent to the one used in PD collections (Mahvash Navazesh & Kumar, 2008).
All salivary samples storage and handling were strictly standardized under the following conditions: after rejecting the initial saliva portion, samples were kept at -20 ºC during the remaining procedures, for up to a maximum of three hours, then appropriately placed in ice-chilled boxes and transferred to -80 ºC storage until posterior analysis was performed. A general illustration of salivary samples handling, storage and preparation for NMR analysis is presented in Figure 2.3.

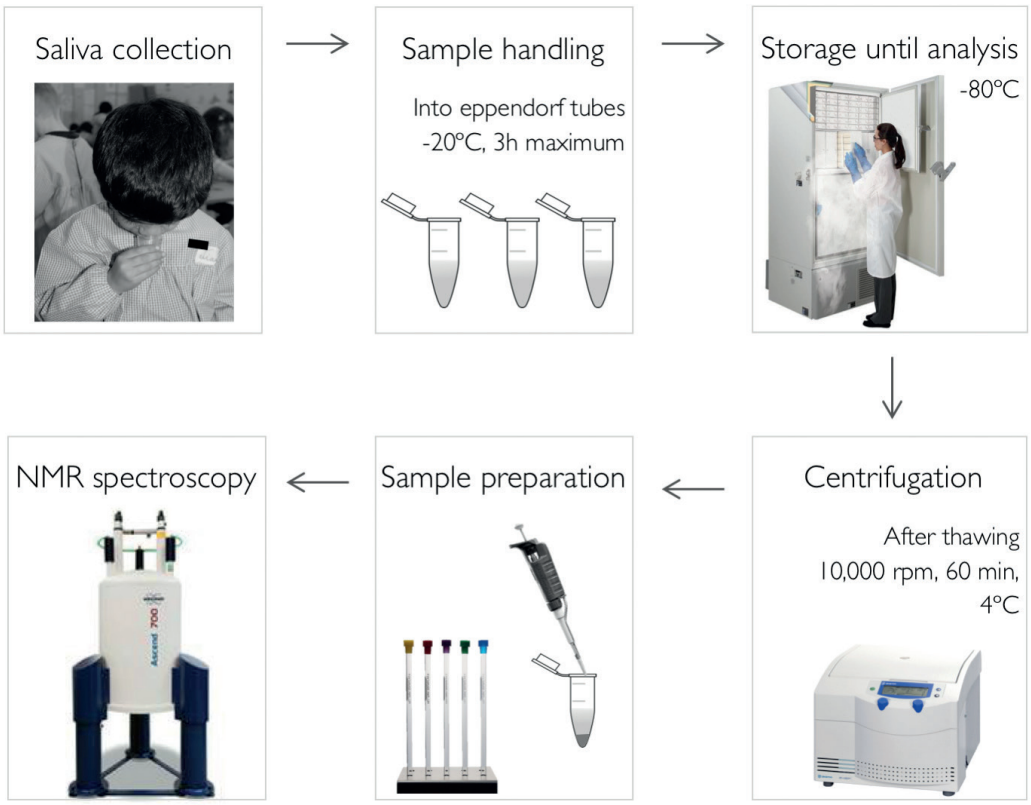

Figure 2.3. Illustrative scheme of sample handling and preparation.

2.2.2. Clinical oral examination

After completion of saliva collections, all clinical examinations were conducted systematically by a single examiner, a previously calibrated and trained dentist, and clinical data elements were registered on an assessment form (Annex IX) by a recording clerk, who was fully knowledgeable concerning the examination details and the overall recording protocol. During observations, the recording clerk sat close to the examiner, to ensure findings were correctly heard and recorded and to allow the examiner to check the finished records for accuracy and completeness. Additionally, copies of the recording instructions and coding criteria were readily available. The examiner daily reviewed and extracted all clinical findings into a Microsoft Excel® spreadsheet.

On average, each child clinical examination took about five to ten minutes and the number of examined children was limited to 15 per session, to avoid fatigue and to ensure the reliability and consistency of the obtained data. Although the examiner was an experienced dentist who had previously been trained in accordance with the recommended methodology for basic oral health surveys, the operator was submitted to a recalibration process before the beginning of the study and intra-examiner reproducibility was tested in the observational study through random complementary reexamination of 10% of the sample. Those examinations occurred within a period not exceeding a month after the initial observation and were accompanied by saliva collection and further analysis contributing, therefore, to the applied laboratory techniques accuracy confirmation.

Although the pilot study was conducted in a pediatric dentistry office setting, all oral examinations in this small cohort were performed without using any dental chair equipment, aiming to reproduce the conditions that expectably would be found in the main study. In fact, preschools involved in the main study seldom presented ideal physical or logistic conditions and the examination areas had to be previously arranged for maximum ease of operation. All subjects were seated in a chair, next to a window, in order to receive maximum illumination from natural light yet avoiding discomfort caused by direct sunlight. A standardized artificial light source was used when necessary, as preconized by the WHO (World Health Organization (WHO), 2013). The examiner positioned himself at the child's eye level, allowing a thorough observation of all oral structures.

Previously to intraoral examination, tooth brushing was performed to each participant by the operator using a conventional toothbrush without dentifrice and, whenever justified, additional existing debris were removed using cotton rolls, gauze or dental floss. In intraoral examinations sterilized material was used, namely a standard mouth mirror (#4 mouth mirror, Hu-Friedy™, Chicago, IL, USA) and a ball tip probe (CP-11.5B screening color-coded probe, Hu-Friedy™, Chicago, IL, USA). All preconized recommendations regarding prevention of contamination and cross-contamination were strictly followed. Disposable masks and gloves, as well as protective glasses were used to reduce the risk of cross-infection. In the observational study, a sufficient number of instruments was accessible at each session, to avoid the need of immediate reutilization. Additionally, an appropriate container for contaminated instruments and a disinfectant solution were also available in each session at preschools. In none of the conducted studies radiographs were taken, as the required equipment is impractical to use in field situations (World Health Organization (WHO), 2013).

Each oral examination was conducted systematically and followed a clock-wise sequence starting from the last tooth of the upper right quadrant. Data relative to dental status was registered following ICDAS-II diagnostic criteria (A. Ismail et al., 2007). Since the primary foundation for applying the ICDAS-II system is the clinical visual assessment of clean and dry teeth, an approved epidemiological modification allowing the use of gauze for dental surfaces drying was applied (N. B. Pitts, 2009). ICDAS-II recommends tooth surfaces should be assessed and scored for both caries and restoration status, leading to a two-digit number, in which the first digit refers to the presence of sealants or any type of restorations (Table 2.3), while the second digit represents the dental caries status (Table 2.4). In the present work, after assessing individually every dental surfaces, data was recorded per tooth, considering each tooth received the "worst" ICDAS code encountered in one of its five surfaces.

Score	Diagnosis	Diagnostic criteria
0	Un-restored or unsealed	There is no evidence of restorations or sealants on the assessed surface.
1	Sealant, partial	A sealant that does not cover all pits and fissures on a tooth surface is detected.
2	Sealant, full	A sealant that covers all pits and fissures on a tooth surface is detected.
3	Tooth colored restoration	A tooth colored (resin or glass-ionomer cement) restoration is present.
4	Amalgam restoration	An amalgam restoration is present.
5	Stainless steel crown	A stainless steel crown is present.
6	Ceramic or gold or porcelain-fused-to-metal crown or veneer	A ceramic, gold or porcelain-fused-to-metal crown or veneer is present.
7	Lost or broken restoration	There is evidence of a lost or fractured restoration.

Table 2.3. Restoration and sealant coding system of ICDAS-II (adapted from Ismail 2007).

Score	Diagnosis	Diagnostic criteria
0	Sound	There is no evidence of caries after prolonged air drying (suggested drying time 5 seconds). Surfaces with developmental defects such as enamel hypoplasias, fluorosis, tooth wear (attrition, abrasion and erosion) and extrinsic or intrinsic stains should be recorded as sound.
1	First visual change in enamel	An initial enamel lesion consistent with a carious opacity or discoloration (white or brown lesion) is detected following prolonged air drying. However, if a discoloration not compatible with sound enamel is visible and limited to the pit and fissure area, whether the surface is seen wet or dry, it should be also recorded as code 1.
2	Distinct visual change in enamel	A distinct lesion, consistent with a white spot lesion and/or a brown carious discoloration is detected in enamel when viewed wet.
3	Localized enamel breakdown	A carious white opacity and/or a brown carious discoloration involving loss of surface integrity without visible dentin is present. If in doubt or to confirm the visual assessment, the WHO probe can be used gently to confirm the presence of a cavity apparently confined to the enamel.
4	Underlying dark shadow from dentin with or without localized enamel breakdown	This lesion appears as a shadow of discolored dentin visible through an enamel surface, which may or may not show signs of localized breakdown. The darkened area is more evident when the surface is wet and it appears as a grey, blue or brown shadow. This code should be attributed to the surface where the lesion was originated and not necessarily to the surface where the shadow is detected.
5	Distinct cavity with visible dentin	An evident cavitation in opaque or discolored enamel exposing the dentin beneath is present.
6	Extensive distinct cavity with visible dentin	There is an obvious and extensive loss of tooth structure, with visible dentin, and the cavity involves at least half of the tooth surface or possibly reaches the dental pulp.

Table 2.4. Dental caries coding system of ICDAS-II (adapted from Ismail 2007).

Four additional ICDAS codes were also considered: 96 – tooth surface that could not be examined; 97 - tooth missing due to caries; 98 - tooth missing because of other reasons; 99 - unerupted teeth. During examination, whenever a deciduous tooth occupied the same space of a permanent tooth, the latter was examined and registered. In this study, code 1, relative to first visual change in enamel, was not considered, attending to the ICDAS-approved modification for epidemiology in which drying teeth with compressed air is not possible. As preconized for epidemiological studies, the letter A was used instead of the ICDAS codes 1 and 2. Furthermore, in the present study, parameters such as the activity of the dental caries lesions or the presence of root caries were not assessed.

The presence of extrinsic dark-pigmentation of the tooth enamel was registered whenever detected and enamel defects, such as hypoplasia, demarcated or diffused opacities, were also recorded and dichotomized as absent or present. The determination of traumatic dental injuries was registered and detected lesions were classified as uncomplicated or complicated. Discoloration stemming from trauma was also investigated.

In the oral examination the presence of additional soft tissue alterations, including fistulas, abscesses, aphthous ulcers, median rhomboid glossitis or geographic tongue, was further assessed and registered.

2.2.3. Collection of medical, sociodemographic and oral health related behavior metadata

The participant information sheet (Annex VIII), sent along with the study presentation letter and filled by the children legal tutors, surveyed several variables comprising some medical, sociodemographic and oral health related behavior metadata. The gathered parameters, presented on Table 2.5, allowed completing the assessment for eligibility for participating in the study, but also classifying children into multiple subgroups which shall be described throughout this thesis. Subject's privacy and data confidentiality were assured since parents were asked to return this document within an enclosed sealed envelope and, additionally, the response option "don't know or prefer not to answer" was available in all of the aforementioned surveyed items.

Relevant metadata	Surveyed items
Child's medical status	Presence of any systemic diseases Presence of growth or developmental problems Current intake of medication Intake of medication in the last 2 weeks Use of antiseptic mouthwash or toothpaste in the last 2 weeks
Sociodemographic parameters	Gender Preschool location Preschool type Number of people in the child's household Existence of siblings Child's birth order Child's place of birth Area of residence Place of birth of the subject's mother Place of birth of the subject's father Parental educational level Parental occupation group
Oral hygiene habits	Age at which the child began tootbrushing habits Toothbrushing frequency Help during toothbrushing Type of dentifrice used Use of dental floss Age at which the child had its first dental visit Frequency of dental visits
Dietary habits	Breastfeeding habits after the first year of life Number of current daily meals Frequency of sweets consumption

Table 2.5. Metadata collected through the participation information sheet, filled by legal tutors.

2.3. NMR metabolomics

2.3.1. Sample preparation for NMR analysis

Saliva samples (previously stored at -80ºC for a maximum of 15 months) were thawed at room temperature and then centrifuged (60 minutes, 10,000 rpm, Sigma 2-16P centrifuge with r=82 mm, 4ºC). In the pilot study, 70 µL of a 10% D_2O (Eurisotop, France) and TSP solution (Eurisotop, France) for chemical shift referencing were added to 630 µL of supernatant (Dame et al., 2015). The mixture was vortexed (30 seconds) and 600 µL were transferred to NMR tubes (5mm diameter).

After conducting the pilot study some minor adjustments to the sample preparation protocol were made in order to optimize this procedure in future studies. Sample preparation protocols reported previously were found to be significantly variable concerning several key parameters, including the use of buffer and NaN_3 or the amount of saliva required for NMR analysis. The use of NaN_3 has been reported to prevent bacterial growth during sample preparation and/or NMR acquisition (Aimetti et al., 2012). Following a thorough literature review, three main adjustments were made to the protocol:

1) a smaller amount of saliva was selected (about 400 µL instead of 630 µL), since not all participants could provide enough saliva to obtain about 630 µL of supernatant after centrifugation;
2) a NaN_3 solution was added to the buffer, to avoid sample degradation;
3) a 100% D_2O solution was used in the buffer preparation, improving water suppression in comparison to the previous protocol.

The overall metabolic profile of spectra, namely peak intensity and width, was not visibly altered by the protocol modifications.

Accordingly, in the large cohort study saliva samples were thawed and centrifuged as described for the pilot study. 300 µL of a 70 mM sodium phosphate buffer (100% D_2O with 0.086% (m/v) TSP solution containing 6.15 mM NaN_3 (Sigma Aldrich, Portugal)) were added to 400 µL of supernatant, after registering the sample initial pH (HI2221-02, Hanna instruments®, Italy). The mixture was vortexed (30 seconds), the final pH was measured and 600 µL were then transferred to NMR tubes. During sample preparation, and whenever possible, the viscosity of each sample was rated in a qualitative scale ranging from 1 (fluidic saliva) to 3 (viscous saliva). Sample viscosity may play a role in spectral quality (high viscosity leading to broader signals), therefore this information, even though of a qualitative nature, was found useful.

2.3.2. Analysis by NMR spectroscopy

Standard 1D ^{1}H-NMR spectra were recorded on a Bruker Avance III spectrometer (500 MHz proton frequency), at 298 K, using a *noesypr1d* pulse sequence (Bruker library) with 100 ms mixing time, 3 µs t1 delay, and water suppression during mixing time and relaxation delay. 256 transients were acquired into 64 k data points, with 10,000 Hz spectral width, 4 seconds relaxation delay, and 3.28 seconds

acquisition time. Each FID (record of signal intensity) was multiplied by a 0.3 Hz exponential function prior to Fourier-transformation to obtain the spectra (recorded as a function of radiofrequency energy, expressed in chemical shift).

2D NMR experiments comprised total correlation spectroscopy (TOCSY) spectra (*'dipsi2phpr'* pulse sequence in the Bruker library), acquired in phase sensitive mode using time proportional phase incrementation (TPPI) and the MLEV17 pulse sequence for spin locking, with acquisition into 256 data points in F1 and 4096 data points in F2, and 8012.82 Hz spectral width (SW) in both dimensions, a 2 seconds relaxation delay and 70 ms mixing time. Heteronuclear single quantum correlation (HSQC) experiments were recorded with inverse detection and 13C decoupling (*'hsqcetgp'* pulse sequence in the Bruker library), a total of 2048 data points in F1, 512 data points in F2, and SW 8012.82Hz (F1) and 20831.98Hz (F2). A relaxation delay of 2 seconds was employed and a refocusing delay of 1.72 ms was used.

Whenever possible, Carr-Purcell-Meiboom-Gill (CPMG) spectra (*'cpmgpr'* from Bruker library) of random samples were also acquired with water presaturation, with 80 loops (n) and a total spin−spin relaxation time (2nτ) of 64 ms (with τ = 400 μs).

2.3.3. Pre-processing and multivariate analysis of NMR data

Spectra were manually phased and baseline-corrected and chemical shifts referenced internally to TSP (δ 0.0). Peak assignments and metabolite identification were performed according to literature (Dame et al., 2015; C. J. L. Silwood et al., 2002; Takeda et al., 2009), 2D NMR experiments on selected samples and spectral databases (Bruker Biorefcode and the human metabolome database) (Wishart et al., 2018). Metabolite identification followed the recommendations in (Salek, Steinbeck, Viant, Goodacre, & Dunn, 2013).

MVA was applied to ^{1}H-NMR saliva spectra, after water region exclusion (4.57 - 5.19 ppm). Spectra were aligned by recursive segment-wise peak alignment (Veselkov et al., 2009) and normalized to total area to account for sample concentration differences. PCA and PLS-DA were performed after UV scaling (SIMCA-P 11.5, Umetrics, Sweden). Loading weights were obtained and colored according to VIP. PLS-DA model validation was carried out by MCCV (seven blocks) with 500 runs, with recovery of Q^2 (predictive power) values and confusion matrices. Classification rates, specificity, and sensitivity were computed and model predictive power further assessed using a ROC map. To improve PLS-DA models, VS was applied as previously described (Diaz et al., 2013). For selected models, relevant peaks were integrated (Amix 3.9.14, BrukerBioSpin, Rheinstetten, Germany), normalized to total area and variations assessed through effect size and *p*-values (Wilcoxon test, $p < 0.05$), the latter corrected for multiple comparisons by the Bonferroni correction (Saccenti et al., 2014). Statistical tests, boxplots, loading plots were performed using R-statistical software and MATLAB (8.3.0, MathWorks).

2.3.4. Variable description and operationalization

Metadata collected through the participant information sheet and from the clinical oral examination in the large cohort study were grouped according to the type of data and its purpose; the operationalization of these variables was conducted as listed below.

2.3.4.1. Participants' identification and medical status data

Each participant was assigned a numerical code, maintained in all the study tasks and documents throughout the duration of the project. The use of study codes instead of the children identifying information ensured confidentiality of subject data was maintained.
The participant information sheet, filled by legal tutors, covered five initial items concerning the child's medical status, namely presence of any systemic diseases, presence of growth or developmental problems, current intake of medication, intake of medication in the last two weeks or use of antiseptic mouthwash or toothpaste in the last two weeks (all of which were considered dichotomous variables: yes or no). These variables enabled completing the assessment of eligibility for participating in the study: children who presented an affirmative answer to any of these items were excluded from the study.

2.3.4.2. Sociodemographic and oral health related behavior data

The following well-known variables, relative to the sociodemographic characteristics (Table 2.6) and oral health related behaviors (Table 2.7) of the sample, enabled the characterization of the study sample and the establishment of several subgroups. The association between these variables and the prevalence and severity of ECC was further investigated.

Variable	Type of variable (answer options)
Participant gender	Dichotomous (feminine or masculine)
Subject's preschool	Multinomial (preschool each child attended)
Type of preschool	Multinomial (public; for-profit; or IPSS private preschools)
Preschool area	Dichotomous (urban or rural)
Number of people in the child's household	Discrete, quantitative
Existence of siblings	Dichotomous (yes or no)
Child's birth order	Ordinal (first; second; third or fourth; or higher)
Area of residence	Dichotomous (urban or rural)
Place of birth of the child's mother or father	Multinomial (Portugal; other European country; or non-european country)
Parental eduactional level	Ordinal (Licenciate, master or doctoral degree; bachelor degree; secondary education; basic education; primary education or illiterate)
Parental occupation	Ordinal (group 1: senior managers/owners in large public/private business organisations, university lecturers, defence forces commissioned officers, qualified professionals or occupations in government administration; group 2: other medium business owners/managers, associate professionals, defence forces officers or basic/secondary education teachers; group 3: tradesmen/women, clerks and skilled office, sargents or related occupations; group 4: small farmer, worker in agriculture or in related fields, office assistants, machine operators, defence forces without qualification not included above; group 5: unpaid worker, labourer, related workers without qualification or occupation not yet referred)

Table 2.6. Well-known sociodemographic variables description and operationalization.

Variable	Type of variable (answer options)
Age at which the child began toothbrushing habits	Ordinal (before the 1st year of life; between the 1st and the 2nd years of life; between the 2nd and the 3rd years of life; after the 3rd year of life; or the child has never started toothbrushing habits)
Toothbrushing frequency	Ordinal (seldom/never; once a day; twice a day; or more than twice a day)
Help during toothbrushing	Dichotomous (yes or no)
Type of dentifrice used	Dichotomous (dentifrice for children or adult dentifrice)
Use of dental floss	Dichotomous (yes or no)
Age at which the child had its first dental visit	Ordinal (before the 1st year of life; between the 1st and the 2nd years of life; between the 2nd and the 3rd years of life; between the 3rd and 4th years of life; after the 4th year of life; or the child has never had a dental visit)
Frequency of dental visits	Multinomial (the child has never had a dental visit; only in case of symptoms; or regularly even without any symptoms)
Breastfeeding habits after the first year of life	Multinomial (the child was never breastfed; no; or yes)
Number of current daily meals	Ordinal (up to 5 meals per day; or more than 5 meals per day)
Frequency of sweets consumption	Ordinal (up to once a week; 2 to 3 times a week; or once a day)

Table 2.7. Oral health related behaviors variables description and operationalization.

2.3.4.3. Clinical data from oral examination

The gathered clinical data allowed the determination of ECC prevalence and severity within the study population and the classification of participants into controls and several ECC-related groups. Criteria for the definition of other groups are described throughout this section.

ECC presence

ECC presence was recorded as a dichotomous variable (yes or no). ECC cases were defined according to the AAPD definition (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c), which states ECC as the presence of 1 or more noncavitated or cavitated caries lesions (ICDAS dental caries code ≠ 0), missing due to caries (ICDAS code 97), or filled tooth surfaces (ICDAS restoration/sealant code ≥ 3) in any primary tooth in a child 71 months of age or younger. Children who presented an ICDAS dental caries code of 0, an ICDAS restoration/sealant code inferior to 3 and no missing teeth due to caries were classified into controls.
The variable ECC presence enabled the determination of the ECC prevalence within the study sample (determined by dividing the number of ECC cases for the total number of observed children).

S-ECC

S-ECC presence was considered a dichotomous variable (yes or no). Similarly to the latter variable, the AAPD definition of S-ECC was applied (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c), that is, children who had 1 or more cavitated (ICDAS dental caries ≥ 5), missing due to caries (ICDAS code 97), or filled ICDAS restoration/sealant code ≥ 3) smooth surfaces in primary maxillary anterior teeth or a decayed, missing, or filled score ($d_{A\text{-}6}$mft) ≥ 6 at the age of 5 was considered affected by S-ECC.
This variable enabled the determination of S-ECC prevalence in the present study, by dividing the number of children affected by S-ECC for the total number of observed subjects.

Presence of dentin caries

The presence of caries involving dentin was registered as a dichotomous variable (yes or no). Caries in dentin were diagnosed if subjects presented at least one ICDAS dental caries code ≥ 4, one tooth missing due to caries (ICDAS code 97) or one filled tooth surfaces (ICDAS restoration/sealant code ≥ 3). Measurement of this variable enabled the comparison of the results found in the present study with studies previously published which have considered the WHO criteria in caries diagnosis (World Health Organization (WHO), 1997, 2013) and, therefore, have neglected the presence of noncavitated lesions. The prevalence of caries in dentin was determined by dividing the number of participants presenting caries in dentin by the total number of children.

ECC severity

ECC severity was gauged considering the dmft score, which corresponds to the sum of decayed, filled and missing teeth due to caries in the child's oral cavity. The dmft score was, therefore, considered a discrete quantitative variable that allowed the quantification of ECC severity in the present sample. This parameter was determined through two different approaches:

– *dmft determination according to ICDAS-II criteria ($d_{A\text{-}6}mft$)*
  Sum of decayed, filled and missing teeth due to caries considering ICDAS dental caries code different than 0.

– *dmft determination according to WHO criteria ($d_{4\text{-}6}mft$)*
  Sum of decayed, filled and missing teeth due to caries considering ICDAS dental caries code equal or higher than 4.

The same variables were also registered considering only permanent teeth (DMFT calculated through ICDAS and WHO criteria).

Other clinical variables gathered through intraoral examinations are presented on Table 2.8.

Variable	Type of variable (answer options)
Dentition stage	Multinomial (primary; mixed phase; or permanent dentition)
Number of permanent teeth	Quantitative discrete (number of permanent teeth present in the child's oral cavity)
Number of decayed primary teeth according to: a) ICDAS-II criteria b) WHO criteria	Quantitative discrete (both variables were relative to the number of primary teeth presenting): a) ICDAS dental caries code ≠ 0 b) ICDAS dental caries code equal or higher than 4.
Number of decayed permanent teeth according to: a) ICDAS-II criteria b) WHO criteria	Quantitative discrete (both variables were relative to the number of permanent teeth presenting): a) ICDAS dental caries code ≠ 0 b) ICDAS dental caries code equal or higher than 4.
Number of filled teeth concerning: a) primary teeth b) permanent teeth	Quantitative discrete (both variables were relative to the number of teeth presenting ICDAS restoration/sealant code equal or higher than 3 concerning): a) primary teeth b) permanent teeth
Number of missing teeth due to caries concerning: a) primary teeth b) permanent teeth	Quantitative discrete (both variables were relative to the number of teeth presenting ICDAS code 97 concerning): a) primary teeth b) permanent teeth
Presence of enamel defects	Dichotomous (yes or no) Parameter registered ***per tooth***. The presence of enamel defects was considered if the child presented at least one affected dental surface.
Presence of traumatic injuries	Dichotomous (yes or no) Parameter registered ***per tooth***. The presence of traumatic injuries was considered if the child presented at least one affected tooth. If present, detected lesions were classified as uncomplicated or complicated.
Presence of soft tissue alterations: Fistulas Abscesses Aphthous ulcers Geographic tongue Median rhomboid glossitis	Dichotomous (yes or no; for each of the variables)

Table 2.8. Clinical variables description and operationalization.

2.3.5. Metadata statistical analysis

Prevalence of ECC, S-ECC and dentin caries was assessed through computation of confidence intervals (CI). The description of categorical variables was performed using proportions of their categories and their corresponding CI were also computed. Descriptive statistical methods were applied to explore the relation between ECC, S-ECC and dentin caries prevalence and sociodemographic and oral health related behavior variables. To described quantitative variables the average and the standard deviation (SD) were employed. The Fisher test was used to assess the association between qualitative variables.

Data analysis and verification of the existence of differences, correlations and associations was performed using R-statistical software (version 3.3.2) and Statistical Package for the Social Sciences (version 24, IBM, SPSS Statistics) and through application of adequate statistical tests at a confidence level of 95%.

Chapter 3. Statistical analysis of metadata from the observational study

Chapter 3. Statistical analysis of metadata from the observational study

This chapter comprises the description of clinical data arising from the oral examination of the five-year old children main study cohort at the baseline evaluation and after six months. At the initial evaluation period the prevalence of ECC according to ICDAS-II diagnostic criteria was 40.10% and the d_{A-6}mft score 1.62 (± 2.52). After six months a 46.50% prevalence of ECC was documented in the cohort and participants presented an average d_{A-6}mft of 1.54 (± 2.54). About 11.30% of the sample experienced S-ECC at baseline evaluation, whereas in the six-month assessment this figure reached 13.20%. Prevalence of dentin caries of 30.30% and 39.50% were found in the initial and at the six-month evaluation, respectively. Decayed teeth was the component contributing the most to the d_{A-6}mft indexes in both evaluations, although a decrease in the percentage of untreated caries was verified from the first to the second evaluation (80.40% to 63.40%, respectively).

Regarding oral health related behavior factors, establishment of toothbrushing habits before the first year of age and twice daily toothbrushing were inversely associated to ECC experience and, furthermore, a statistically significant association was also found between daily ingestion of sweets and the disease. Bearing in mind the potential shortcomings of this longitudinal evaluation, and even though its overall findings may not be fully generalized, the present observations mirror the poor oral health condition of the cohort and emphasize the current need to limit ECC's impact.

3.1. Definition of study population

The main cohort of this observational study was composed by healthy five-year old preschoolers who met the inclusion criteria and whose legally authorized representatives consented the participation on the study. Most preschools involved in the study were public preschools (11, in comparison to 2 private and 4 IPSS preschools), located in urban and rural areas of Coimbra, Portugal. Out of the 146 children eligible to enroll the study, only 143 were present at the baseline evaluation day (initial dropout rate of 2.05%) and, of those, one subject did not cooperate on oral examination procedures, accounting for a total of 142 observations. At the six-month assessment, the dropout rate reached 21.23%, as 31 children eligible to enroll the study did not attend preschool that day. Again, one child did not collaborate in the examination procedures and therefore 114 participants were assessed in the second evaluation period. Detailed data on the characteristics of this large cohort can be found in Chapter 2 ('*Experimental procedures*').

From the 150 consent and participant information forms returned in the beginning of the study, only 100 documents contained the requested data on the participants' oral health related behaviors, yielding a response rate of 66.66%.

3.2. ECC experience and sociodemographic characteristics

In the baseline moment the overall prevalence of ECC was 40.10% (about 57 out of 142 participants), while the prevalence of S-ECC was found to be 11.30% (16 out of 142 subjects) (Table 3.1). About 59.90% of the cohort was ECC-free in the initial evaluation. Table 3.1 shows the prevalence of ECC found in the study cohort in both evaluation periods, as well as disease severity expressed as mean dmft index values.

	Baseline evaluation		Six-month evaluation	
	% (*n*)	[95% CI]%	**% (*n*)**	[95% CI]%
ECC prevalence	40.10% (57/142)	[32.40, 48.40]%	46.50% (53/114)	[37.60, 55.60]%
S-ECC prevalence	11.30% (16/142)	[7.10, 17.50]%	13.20% (15/114)	[8.10, 20.60]%
Dentin caries prevalence	30.30% (43/142)	[23.30, 38.30]%	39.50% (45/114)	[31.00, 48.60]%
ECC severity (d_{A-6}mft)				
Mean (SD)	1.62 (± 2.52)		1.54 (± 2.54)	
min. - max.	0 - 12		0 - 12	
Dental caries severity (d_{4-6}mft)				
Mean (SD)	1.05 (± 2.15)		1.22 (± 2.28)	
min. - max.	0 - 12		0 - 12	

Table 3.1. Prevalence and severity of ECC in the study sample at baseline evaluation and after six months. [95% CI]%, 95% confidence interval; dmft, decayed, missing, or filled teeth score; SD, standard deviation; min., minimum value; max., maximum value.

The mean overall d_{A-6}mft score in baseline assessment was 1.62 ± 2.52 and the values of this index ranged from 0 to 12 in this cohort (Table 3.1). The relative contributions of decayed, missing and filled components for this average d_{A-6}mft score were 80.40%, 0.00% and 19.60%, respectively, as illustrated by Figure 3.1.

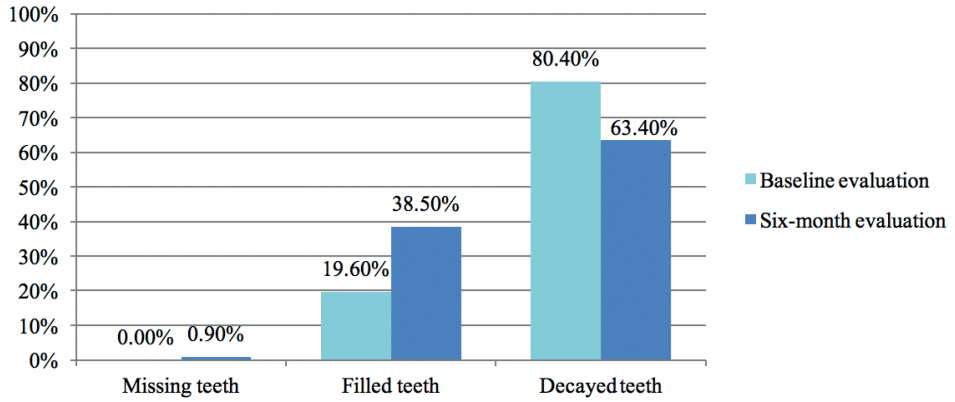

Figure 3.1. Graphic illustration of the distribution of the d_{A-6}mft score components at baseline and at the six-month evaluation.

From the total 142 examined children at the baseline evaluation, 43 presented carious dentin lesions, translating into a prevalence of dentin caries of about 30.30% (Table 3.1). The average d_{4-6}mft score found in the baseline period was 1.05 ± 2.15 and its minimum and maximum values were similar to the figures reported for the d_{A-6}mft index (Table 3.1).

In the six-month assessment, the prevalence of ECC was 46.50%, since 53 of the 114 children participating presented a d_{A-6}mft score > 0 (Table 3.1). Accordingly, 53.50% of the cohort was not afflicted by the disease. With specific regard to S-ECC, a 13.20% prevalence rate was registered and when considering only caries lesions in dentin, a 39.50% prevalence of dentin caries was found (Table 3.1). Even though all disease prevalence rates detected in the second evaluation were higher than those of baseline, such differences were not statistically significant to ECC or S-ECC or dentin caries specifically (Table 3.1).

The average d_{A-6}mft index of the cohort was 1.54 ± 2.54 and the minimum and maximum d_{A-6}mft values corresponded to 0 and 12 (Table 3.1). Assessment of the distribution of the d_{A-6}mft components showed that the decayed component still accounted for the highest input, despite having decreased to 63.40% (Figure 3.1). Proportions of filled and missing teeth components both increased to 38.50% and 0.90%, respectively (Figure 3.1). The mean d_{4-6}mft score in this time period was 1.22 ± 2.28 (Table 3.1).

In this cohort no statistically significant association was disclosed between ECC presence and enamel defects in the initial evaluation (Fisher test, p=0.782) nor at the six-month assessment (Fisher test, p=0.300) (data not shown). Lack of statistically significant association was also found between enamel defects and S-ECC occurrence (Fisher test, p=1.000 for both periods) (data not shown).

Regarding other clinical variables assessed during intraoral examinations, no statistically significant association was found between ECC occurrence and presence of traumatic dental injuries or extrinsic dark-pigmentation of enamel, nor in relation to any of the soft tissue alterations registered (fistulas, abscesses, aphthous ulcers, median rhomboid glossitis and geographic tongue) (data not shown).

The association between ECC occurrence and well-known sociodemographic variables as gender, type of preschool and location of the preschool, was also investigated for both evaluation periods. In the first oral examination, most of the male and female participants were controls and statistically significant differences were found between controls, S-ECC and dentin caries (for males) and between all groups (for females) (Table 3.2.). The same tendency was detected after six months, as the majority of both genders were controls, although statistical significance was only reached between controls and S-ECC (in both genders) (Table 3.3.).

	Controls % (*n*) [95% CI]%	**ECC** % (*n*) [95% CI]%	**S-ECC** % (*n*) [95% CI]%	**Dentin caries** % (*n*) [95% CI]%
Gender				
Males	57.30% (43/75) [46.10, 67.90]%	42.70% (32/75) [32.10, 53.90]%	14.70% (11/75) [8.40, 24.40]%	32.00% (24/75) [22.50, 43.20]%
Females	62.70% (42/67) [50.70, 73.30]%	37.30% (25/67) [26.70, 49.30]%	7.50% (5/67) [3.20, 16.30]%	28.40% (19/67) [19.00, 40.10]%
Type of preschool				
Public	60.30% (47/78) [49.20, 70.40]%	39.70% (31/78) [29.60, 50.80]	14.10% (11/78) [8.10, 23.50]%	32.10% (25/78) [22.70, 43.00]%
IPSS	54.30% (19/35) [38.20, 69.50]%	45.70% (16/35) [30.50, 61.80]%	8.50% (3/35) [3.00, 22.40]%	31.40% (11/35) [18.60, 48.00]%
Private	65.50% (19/29) [47.30, 80.10]%	34.50% (10/29) [20.00, 52.70]%	6.90% (2/29) [1.90, 22.00]%	24.10% (7/29) [12.20, 42.10]%
Preschool location				
Rural	56.50% (26/46) [42.20, 69.80]%	43.50% (20/46) [30.20, 57.80]%	21.70% (10/46) [12.30, 35.60]%	41.30% (19/46) [28.30, 55.70]%
Urban	61.50% (59/96) [51.50, 70.60]%	38.50% (37/96) [29.40, 48.50]%	6.30% (6/96) [2.90, 13.00]%	25.00% (24/96) [17.40, 34.50]%

Table 3.2. Descriptive statistics for sociodemographic parameters and bivariate associations with ECC, S-ECC and dentin caries at baseline evaluation. [95% CI]%, 95% confidence interval; IPSS, Private Institution of Social Solidarity.

	Controls % (*n*) [95% CI]%	**ECC** % (*n*) [95% CI]%	**S-ECC** % (*n*) [95% CI]%	**Dentin caries** % (*n*) [95% CI]%
Gender				
Males	53.30% (32/60) [40.90, 65.40]%	46.70% (28/60) [34.60, 59.10]%	18.30% (11/60) [10.60, 29.90]%	38.30% (23/60) [27.10, 51.00]%
Females	53.70% (29/54) [40.60, 66.30]%	46.30% (25/54) [33.70, 59.40]%	7.40% (4/54) [2.90, 17.60]%	40.70% (22/54) [28.70, 54.00]%
Type of preschool				
Public	54.80% (34/62) [42.50, 66.60]%	45.20% (28/62) [33.40, 57.50]%	12.90% (8/62) [6.70, 23.40]%	38.70% (24/62) [27.60, 51.20]%
IPSS	40.70% (11/27) [24.50, 59.30]%	59.30% (16/27) [40.70, 75.50]%	11.10% (3/27) [3.90, 28.10]%	48.10% (13/27) [30.70, 66.00]%
Private	64.00% (16/25) [44.50, 79.80]%	36.00% (9/25) [20.20, 55.50]%	16.00% (4/25) [6.40, 34.70]%	32.00% (8/25) [17.20, 51.60]%
Preschool location				
Rural	51.40% (19/37) [35.90, 66.60]%	48.60% (18/37) [33.40, 64.10]%	21.60% (8/37) [11.40, 37.20]%	45.90% (17/37) [31.00, 61.60]%
Urban	54.50% (42/77) [43.50, 65.20]%	45.50% (35/77) [34.80, 56.50]%	9.10% (7/77) [4.50, 17.60]%	36.40% (28/77) [26.50, 47.50]%

Table 3.3. Descriptive statistics for sociodemographic parameters and bivariate associations with ECC, S-ECC and dentin caries at the six-month evaluation. [95% CI]%, 95% confidence interval; IPSS, Private Institution of Social Solidarity.

When the association between ECC and the type of preschool was evaluated, a higher percentage of controls was still initially found in each type of educational establishments: public, IPSS and private. In fact, this increased proportion of controls was significantly different than the prevalence rate of S-ECC and of dentin caries in public and private schools and than S-ECC in IPSS preschools in the first oral examination

(Table 3.2). At the six-month evaluation, significantly higher percentages of controls were found in comparison to S-ECC proportions, though this difference was only verified in public and private preschool establishments (Table 3.3). As to preschool location, during baseline assessment controls were more prevalent in both urban and rural preschools; however, although in rural preschools such difference was only statistically significant between controls and S-ECC subgroups, in urban establishments meaningful differences were found between controls and all the remaining groups: ECC, S-ECC and dentin caries (Table 3.2). Lastly, in the second evaluation period statistically significant differences were only identified in urban preschools: controls were once again more prevalent in relation to children afflicted by S-ECC (Table 3.3).

Discussion

The overall high ECC prevalence rates of 40.10% and 46.50% found in baseline and in the six-month evaluation, respectively, along with the average $d_{A\text{-}6}$mft scores, reflect the poor oral health condition of the cohort. In this study, oral examinations were conducted using the ICDAS-II diagnostic criteria (A. Ismail et al., 2007). The adoption of such criteria definitely constituted a major strength of the present research, considering they enable the diagnosis of caries processes in clinical stages of development that precede cavitation, hence promoting a more accurate estimate of ECC prevalence and impact (N. Pitts, Melo, Martignon, Ekstrand, & Ismail, 2011). This methodological option also allowed the definition of ECC cases according to AAPD's definition of the disease, which includes the presence of non-cavitated caries lesions (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c).

Furthermore, findings obtained from ICDAS-II criteria can be compared to those arising from WHO 1997 diagnostic criteria (World Health Organization (WHO), 1997, 2013), a feature particularly relevant during analysis of results, since most of the previous ECC epidemiological studies have used lesions in dentin as diagnostic thresholds. Nonetheless, the selection of which ICDAS-II threshold corresponds to a caries lesion according to the WHO criteria has been subject of controversy (Borralho, 2014; S. Mendes et al., 2009; Sónia Mendes & Bernardo, 2015; Shoaib, Deery, Ricketts, & Nugent, 2009). Following the WHO criteria, presence of caries should be recorded when a lesion presents an unmistakable cavity, undermined (unsupported) enamel or a detectably softened floor or wall (World Health Organization (WHO), 1997, 2013). Attending to the fact that unsupported enamel may be considered clinically equivalent to ICDAS-II dental caries code 4, '*underlying dark shadow from dentin with or without localized enamel breakdown*', we chose to use the latter as threshold to calculate dentin caries prevalence and severity ($d_{4\text{-}6}$mft), as previously performed in a Portuguese research on ECC (Borralho, 2014; Sónia Mendes & Bernardo, 2015).

Bearing in mind the features of each diagnostic system, it can be easily understood that prevalence rates and dmft indexes calculated using ICDAS-II yield higher values than those estimated using WHO criteria. In this cohort, prevalence of dentin caries (WHO 1997 criteria) was found to be 30.30% at the baseline evaluation and 39.50% after six months. Even though this study presents limitations regarding representativeness and generalizability owing to its relatively small sample size and nature of our sample (as addressed in detail in the last section of this chapter), our findings are consistent with other reports. Previous national studies on ECC using the WHO diagnostic criteria have reported a broad range of prevalence rates and referred figures similar to ours, as 28.7% (C. M. Alves, 2009) and 34.6% (C. C. Martins, Pires, & Pereira, 2014), but also wider values, as 15% (Vasconcelos et al., 2004), and 48.1% (S. Mendes et al., 2009). With regard to international surveys, a recent systematic review has also revealed high variability of prevalence data on dentin caries, ranging from 23% to 90%, despite most of the available reports presented figures higher than 50% (K. J. Chen et al., 2019).

As to ECC epidemiological studies using ICDAS-II system, international investigations have also reported differing values, ranging from 33% in the United States of America (Finlayson, Siefert, Ismail, & Sohn, 2007) to 62.50% in Spain (Albert et al., 2016), 71.14% in Colombia (Martínez-Pabón, Ramírez-Puerta, Escobar-Paucar, & Franco-Cortés, 2010) or 80.84% in Italy (Majorana et al., 2014). Within our cohort, the percentages of ECC-afflicted children according to ICDAS-II criteria (40.10% and 46.50% at the first and second evaluation, correspondingly) were lower than the 56.4% rate registered by one Portuguese research involving a three- to five-year-old children sample, representative of Lisbon district (*n*=443) (Borralho, 2014; Sónia Mendes & Bernardo, 2015). Authors reported the prevalence of 56.4% was equivalent to a figure of 26% considering the WHO criteria. The fact that such value is much lower than the prevalence of dentin caries we reported (30.30% and 39.50%) may be justified by a higher proportion of children exhibiting initial carious lesions in their cohort (who were included when estimating ECC prevalence but excluded when considering only lesions in dentin). More recently, another national study exploring prevalence of dental caries in six-year-old subjects according to ICDAS-II diagnostic system stated the disease affected about 68.10% of the assessed children, a figure equivalent to 45.20% when considering only cavitated dentin lesions (cut-off point at ICDAS-II code 5, *distinct cavity with visible dentin*) (Calado, 2017; Direção-Geral da Saúde (DGS), 2015).

Assessment of disease severity was performed through scrutiny of the average dmft scores, since any child having experienced at least one caries lesion (or subjected to one dental treatment) was considered ECC-afflicted and, therefore, only analysis of the dmft values (the sum of all teeth affected by caries) allowed full comprehension of the extent to which the disease affected participants. In our cohort, mean $d_{A\text{-}6}$mft at baseline and after six months was 1.62 (± 2.52) and 1.54 (± 2.54), respectively. These severity figures were lower than that found by Mendes & Bernardo in 2014, of 2.50 (Borralho, 2014; Sónia Mendes & Bernardo, 2015), and than the 3.30 mean $d_{A\text{-}6}$mft reported in six-year-old Portuguese children (Calado, 2017; Direção-Geral da Saúde (DGS), 2015). Concerning $d_{4\text{-}6}$mft indexes, mean values of 1.05 (± 2.15) and 1.22 (± 2.28) were found in the present cohort at baseline and after six months, in contrast with the 0.9 value reported by Mendes & Bernardo (Borralho, 2014; Sónia Mendes & Bernardo, 2015).

With regard to S-ECC, prevalence rates of 11.30% and 13.20% were detected initially and at the six-month evaluation and although these rates comprise a variety of clinically distinct oral conditions (as they include both children presenting $d_{A\text{-}6}$mft values higher than six and those with only one cavitated lesion in a smooth surface of maxillary incisor, for instance), they refer to cases more severely disturbing the child's quality of life and often requiring complex dental care.

It should be taken in account that ECC cases reflect not only present experience of the disease, but also past ECC affliction, as AAPD's definition of the condition comprises children presenting teeth subjected to dental treatment and missing due to caries (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c). Analysis of the distribution of the dmft score components is, therefore, vital to interpret data. One of the most worrying findings arising from this screening is the elevated percentage of decayed teeth component, indicative of the high level of ECC lesions requiring effective dental treatment in the cohort. Initially, most of the ECC experience found within the cohort referred to decayed teeth (80.40%), whilst less than a fifth (19.60%) was relative to treated teeth. Lamentably, this finding is consistent with data from several international (Bissar et al., 2014; Campus et al., 2009; Ferro, Cecchin, & Besostri, 2010; T. Percival, Edwards, Barclay, Sa, & Majumder, 2019; Phantumvanit et al., 2018; X. Wang et al., 2017) as well as national reports (Borralho, 2014; S. Mendes et al., 2009; Sónia Mendes & Bernardo, 2015). In fact, in Portugal a considerably high 90.60% proportion of the decayed compo-

nent has been reported in preschoolers (Borralho, 2014; Sónia Mendes & Bernardo, 2015) and data on six-year-old children includes rates of 84.90% (Gomes, Mendes, & Bernardo, 2015). In the present study, after each examination all participants' tutors were informed on the oral health status of their offspring. Nonetheless, even though a decrease in the proportion of untreated lesions was verified throughout the study (mostly owing to an increase in filled teeth, rather than in teeth missing due to caries), a 63.40% rate of the decayed component was still registered at the six-month evaluation. Overall, the significant proportions of decayed teeth might possibly be attributed to several factors, including parental perceptions and attitudes towards primary teeth, limited accessibility to dental care and general lack of awareness concerning oral health primary prevention. The reported findings, along with the high prevalence of ECC found in both evaluation moments, highlight the severe impact of the disease and also the urge to implement appropriate prevention programs from the prenatal period, in particular attending to the WHO's goal of reducing caries experience among children until the year of 2020 (American Academy of Pediatric Dentistry (AAPD), 2016; World Health Organization (WHO), 1999).

From the baseline to the six-month evaluation, ECC and S-ECC prevalence rates increased, despite the decline of the disease severity ($d_{A\text{-}6}$mft scores). These findings can be explained considering the 21.23% dropout rate: both ECC-afflicted children who presented high $d_{A\text{-}6}$mft indexes and controls did not participate in the six-month assessment. In addition, by the time of the second evaluation, some of the initial controls had already developed ECC. Accordingly, loss of participants extensively affected by ECC and inclusion of new cases exhibiting few lesions led to such results.

No statistically significant associations were found in this cohort regarding ECC occurrence and enamel defects in any of the evaluations, a finding not consistent with the current available evidence. As previously described in Chapter 1 ('*Background*'), hypoplastic or hypomineralized defects have been suggested to be associated with caries development (American Academy of Pediatric Dentistry (AAPD), 2017c; Caufield et al., 2012; Gussy et al., 2006; Vadiakas, 2008) and, recently, a systematic review on ECC's risk factors demonstrated the presence such defects, particularly enamel hypoplasia, is one of the strongest risk factors of the disease (along with presence of dentin caries and high levels of Mutans Streptococci) (Kirthiga, Murugan, Saikia, & Kirubakaran, 2019).

In this cohort, none of the genders was positively associated with experiencing ECC. Regarding preschool location and type, no variable was found significantly associated to occurrence of ECC either. Mendes & Bernardo had reported a significant association between ECC prevalence and severity and the type of preschool, in particular lower disease rates in private preschools and ascribed this finding to a possible relationship between the type of preschool and the family's socioeconomic level (Borralho, 2014; Sónia Mendes & Bernardo, 2015). In fact, out of the many sociodemographic variables that have been explored in literature regarding ECC, gender (in particular males) and low family income have been the factors more frequently associated to ECC (S. S. Gao et al., 2018; Gussy et al., 2006; Kagihara et al., 2009; Khani-Varzegani et al., 2017; Kirthiga et al., 2019; Vadiakas, 2008; Zemaitiene et al., 2017).

3.3. ECC experience and oral health related behavior factors

3.3.1. Oral hygiene habits

The association between selected oral health related behavior parameters and the pattern and severity of ECC within this cohort was also assessed; the most meaningful findings arising from this analysis will be hereinafter presented. Regarding oral hygiene practices, the investigated variables included the age at which the child began toothbrushing, frequency of toothbrushing and type of dentifrice used.

In the present cohort, establishment of toothbrushing habits before the first year of life was inversely associated with occurrence of ECC, S-ECC and dentin caries (controls: CI 63.20 – 86.00%; ECC: CI 14.00 – 36.80%; S-ECC: CI 1.10 – 13.20%; dentin caries: CI 11.00 – 32.50%) (Table 3.4). Accordingly, the overall average dmft score of subjects who only began toothbrushing habits between the second and third years of life (mean d_{A-6}mft=3.00 3.46 and d_{4-6}mft=2.11 ± 2.60) was markedly higher than that of those who started toothbrushing before the first year of life (mean d_{A-6}mft=0.71 ± 1.78 and d_{4-6}mft=0.55 ± 1.64) (Table 3.4). The items "*after the third year of life*" and "*the child has never started toothbrushing habits*" were not chosen by any of the participants' legally authorized representatives.

	Controls % (*n*) [95% CI]%	**ECC** % (*n*) [95% CI]%	**S-ECC** % (*n*) [95% CI]%	**Dentin caries** % (*n*) [95% CI]%	mean d_{A-6}mft	mean d_{4-6}mft
Oral hygiene habits						
Age at which the child began toothbrushing habits						
Before the first year of life	76.50% (39/51) [63.20, 86.00]%	23.50% (12/51) [14.00, 36.80]%	3.90% (2/51) [1.10, 13.20]%	19.60% (10/51) [11.00, 32.50]%	0.71 (1.78)	0.55 (1.64)
Between the first and the second years of life	44.80% (13/29) [28.40, 62.50]%	55.20% (16/29) [37.50, 36.70]%	20.70% (6/29) [9.90, 38.40]%	34.50% (10/29) [19.90, 52.70]%	1.97 (2.72)	1.38 (2.38)
Between the second and the third years of life	31.60% (6/19) [15.40, 54.00]%	68.40% (13/19) [46.00, 84.60]%	31.60% (6/16) [15.40, 54.00]%	52.70% (10/19) [31.70, 72.70]%	3.00 (3.46)	2.11 (2.60)
After the third year of life	0	0	0	0	0	0
The child has never started toothbrushing habits	0	0	0	0	0	0
Toothbrushing frequency						
Seldom / never	0	0	0	0	0	0
Once a day	36.60% (15/41) [23.60, 51.90]%	63.40% (26/41) [48.10, 76.40]%	26.80% (11/41) [15.7, 41.90]%	46.30% (19/41) [32.10, 61.30]%	2.71 (3.21)	2.10 (2.89)
Twice a day	72.90% (35/48) [59.00, 83.40]%	27.10% (13/48) [16.60, 41.00]%	6.30% (3/48) [2.20, 16.80]%	18.80% (9/48) [10.20, 31.90]%	0.75 (1.76)	0.40 (0.96)
More than twice a day	80.00% (8/10) [49.00, 94.30]%	20.00% (2/10) [5.70, 51.00]%	0.00% (0/10) [0.00, 27.80]%	20.00 (2/10) [5.70, 51.00]%	0.30 (0.68)	0.30 (0.68)
Type of dentifrice used						
Dentifrice for children	59.10% (55/93) [49.00, 68.60]%	40.90% (38/93) [31.40, 51.00]%	14.00% (13/93) [8.40, 22.50]%	40.00% (28/93) [21.70, 40.10]%	1.53 (2.62)	1.09 (2.15)
Adult dentifrice	40.00% (2/5) [11.80, 76.90]%	60.00% (3/5) [23.10, 88.20]%	20.00% (1/5) [3.60, 62.40]%	40.00% (2/5) [11.70, 76.90]%	1.60 (2.51)	1.40 (2.61)

Table 3.4. Descriptive statistics for oral hygiene habits and bivariate associations with ECC, S-ECC, dentin caries, mean d_{A-6}mft and mean d_{4-6}mft. [95% CI]%, 95% confidence interval; dmft, decayed, missing, or filled teeth score.

Differences reaching statistical significance were also encountered in relation to twice daily toothbrushing: most of the participants who reported to toothbrush twice a day were controls (72.90%, CI 59.00 – 83.40%), contrasting with reported behaviors from children afflicted by ECC (27.10%, CI 16.60 – 41.00%), S-ECC (6.30%, CI 2.20 – 16.80%) and dentin caries (18.80%, CI 10.20 – 31.90%) (Table 3.4). Lower average dmft indexes were registered in subjects who reported to toothbrush more regularly (Table 3.4). None of the surveyed legally authorized representatives chose the option "s*eldom / never*" concerning toothbrushing frequency.
In relation to the type of dentifrice used, about 59.10% of the participants who used dentifrice "for children" were controls (CI 49.00, 68.60%), significantly differing from the lower percentage of children with S-ECC (CI 8.40, 22,50%) or with dentin caries (CI 21.70, 40.10%) who reported to do so (Table 3.4). No significant associations were detected with regard to ECC prevalence and use of "adult dentifrice".

Discussion

The establishment of toothbrushing habits before the first year of life was found inversely associated to ECC, S-ECC and dentin caries experience in the present cohort, as statistically significant associations were detected between these variables. Similar findings have been reported in several investigations, which overall highlight the relevance of establishing early preventive strategies to limit ECC (Chankanka et al., 2011; Ghazal et al., 2015; Gomes et al., 2015; Tsai, Chen, Li, Hsiang, & Hsu, 2006).

During the first year(s) of life, these preventive strategies are mainly related to anticipatory guidance, which concerns the process of providing practical and individualized parental counseling for ECC prevention, as well as the early identification of children at high risk for developing ECC (American Academy of Pediatric Dentistry (AAPD), 2017a, 2017c; Kagihara et al., 2009). The establishment of the dental home, that is, the ongoing relationship between the dentist and the child that includes comprehensive oral health care, within six months of eruption of the first tooth and no later than twelve months of age is critical to set a lifetime foundation for effective and opportune prevention and for oral health care (American Academy of Pediatric Dentistry (AAPD), 2017d, 2017a, 2017c). Following AAPD's recommendations, an individualized preventive program based on a caries-risk assessment should be timely established and counseling to caregivers should target specific factors known to increase children's risk to develop ECC, namely oral hygiene and dietary habits, Mutans Streptococci transmission and presence of enamel defects (American Academy of Pediatric Dentistry (AAPD), 2017a, 2017d; Caufield et al., 2012; J. Xiao et al., 2019).

Implementation of oral hygiene practices at most, by the time of the first primary tooth's eruption is, indeed, a crucial preventive measure (American Academy of Pediatric Dentistry (AAPD), 2017c; European Academy of Paediatric Dentistry (EAPD), 2008; Scottish Intercollegiate Guidelines Network (SIGN), 2014). Recommendations also state toothbrushing should take place at least twice daily, one of which before bedtime (Direção-Geral da Saúde (DGS), 2005; Scottish Intercollegiate Guidelines Network (SIGN), 2014). In the present study, a statistically significant association was, in fact, found regard twice daily toothbrushing habits and ECC, as only a minority of children affected by caries (either ECC, S-ECC and dentin caries) reported to do so.

Besides from the relevance of dental plaque's mechanical removal, strong evidence has shown toothbrushing at least twice a day with a fluoridate toothpaste is essential to prevent ECC, in both optimally-fluoridated and fluoride-deficient communities (American Academy of Pediatric Dentistry

(AAPD), 2017c, 2017e; European Academy of Paediatric Dentistry (EAPD), 2008; Scottish Intercollegiate Guidelines Network (SIGN), 2014; T. Walsh, Worthington, Glenny, Marinho, & Jeroncic, 2019). Regular toothbrushing with fluoride toothpaste is considered the most cost-effective homecare preventive measure and there is high-certainty evidence that the use of toothpaste containing 1000 or more ppm of fluoride is more effective preventing caries that non-fluoridated toothpaste (Direção-Geral da Saúde (DGS), 2005; Scottish Intercollegiate Guidelines Network (SIGN), 2014; T. Walsh et al., 2019). In this regard, despite a dose-response effect has been demonstrated, evidence on the impact of different fluoride concentrations is more limited (T. Walsh et al., 2019).

In the present research, no significant associations were detected between occurrence of ECC and the use of dentifrice for children or for adults. Although ambiguous, the designations '*children dentifrice*' and '*adults dentifrice*' given to the answer options served the purpose to assure one of the options ('*adults dentifrice*') likely included only dentifrices with fluoride content higher than 1000 ppm, as high variability of fluoride amount can be generally found in toothpastes commercially available in Portugal. It is conceivable that if caregivers were questioned directly about the toothpaste's amount of fluoride, they would possibly not be able to provide such detailed information. In general, current recommendations on the concentration of the fluoride toothpaste should be balanced against the individual's risk of ECC and of dental fluorosis, a defect in enamel's mineralization caused by an excessive amount of fluoride mainly during the secretory and maturation phases of the enamel formation (European Academy of Paediatric Dentistry (EAPD), 2009; Scottish Intercollegiate Guidelines Network (SIGN), 2014; T. Walsh et al., 2019). To ensure fluoride's protective effect is maximized while the risk of fluorosis is minimized, caution should be taken regarding the amount of used toothpaste (European Academy of Paediatric Dentistry (EAPD), 2009). In general, it is advised to use a smear or rice-sized amount of fluoridated toothpaste in children younger than three years old, while a pea-sized amount is often recommended for three to six-year old children (American Academy of Pediatric Dentistry (AAPD), 2017e). In addition, children should be encouraged to spit out any excess of toothpaste, instead of ingesting it, as well as not to rinse after brushing (European Academy of Paediatric Dentistry (EAPD), 2009; Scottish Intercollegiate Guidelines Network (SIGN), 2014).

3.3.2. Dietary habits

The effect of dietary factors, as prolonged breastfeeding and frequency of sweets ingestion, in the occurrence of ECC in this cohort was also analyzed. The majority of children who were never breastfed were controls, with a statistically significant difference having been detected between this subgroup and dentin caries subgroup (controls: CI 45.30 - 93.70%; dentin caries: CI 2.00 – 43.50%) (Table 3.5). Likewise, 62.8% of the subjects who were not breastfed after the first year of life were controls, as the difference between controls (CI 47.90 – 75.60%) and subjects with S-ECC (CI 3.70 – 21.60%) or dentin caries (CI 14.60 – 40.20%) also reached statistical significance (Table 3.5). Unexpectedly, the prevalence of breastfeeding after the first year of life was significantly higher among controls (CI 37.20 - 64.70%) than in subjects affected by S-ECC (CI 12.00 - 34.90%) (Table 3.5). Despite these conflicting results, a general increase in the average dmft index was verified when longer breastfeeding habits were taken in consideration.

	Controls % (*n*) [95% CI]%	ECC % (*n*) [95% CI]%	S-ECC % (*n*) [95% CI]%	Dentin caries % (*n*) [95% CI]%	mean d_{A-6}mft	mean d_{4-6}mft
Dietary habits						
Breastfeeding habits after the first year of life						
The child was never breastfed	77.80% (7/9) [45.30, 93.70]%	22.20% (2/9) [6.30, 54.70]%	0.00% (0/9) [0.00, 29.90]%	11.10% (1/9) [2.00, 43.50]%	0.67 (1.66)	0.56 (1.67)
No	62.8% (27/43) [47.90, 75.60]%	37.20% (16/43) [24.40, 52.10]%	9.30% (4/43) [3.70, 21.60]%	25.60% (11/43) [14.60, 40.20]%	1.30 (2.34)	0.86 (1.82)
Yes	51.10% (24/47) [37.20, 64.70]%	48.90% (23/47) [35.30, 62.80]%	21.30% (10/47) [12.00, 34.90]%	38.30% (18/47) [25.80, 52.60]%	1.87 (2.94)	1.40 (2.47)
Frequency of sweets consumption						
Up to once a week	82.80% (24/29) [65.50, 92.40]%	17.20% (5/29) [7.60, 34.50]%	6.90% (2/29) [1.90, 22.00]%	13.80% (4/29) [5.50, 30.60]%	0.83 (2.70)	0.66 (2.22)
Two to three times a week	59.10% (26/44) [44.40, 72.30]%	40.90% (18/44) [27.70, 55.60]%	11.40% (5/44) [5.00, 24.00]%	27.30% (11/44) [16.30, 41.80]%	1.21 (2.30)	0.93 (2.05)
Once a day	30.80% (8/26) [16.50, 50.00]%	69.20% (18/26) [50.00, 83.50]%	26.90% (7/26) [13.70, 46.10]%	53.80% (14/26) [35.50, 71.20]%	2.81 (2.61)	1.85 (2.11)

Table 3.5. Descriptive statistics for dietary habits and bivariate associations with ECC, S-ECC, dentin caries, mean d_{A-6}mft and mean d_{4-6}mft. [95% CI]%, 95% confidence interval; dmft, decayed, missing, or filled teeth score.

In this cohort daily consumption of sweets was positively associated with ECC with statistical significance (ECC: CI 50.00 - 83.50%; controls: CI 16.50 – 50.00%) (Table 3.5). Statistically significant differences were also found regarding weekly ingestion of sugary-foods: while 82.80% of the children who ingested sugary-foods up to once a week were controls (CI 65.50 - 92.40%), only 17.20%, 6.90% and 13.80% were subjects with ECC (CI 7.60 - 34.50%), S-ECC (CI 1.90 - 22.00%) and dentin caries (CI 5.50 - 30.60%), respectively (Table 3.5). The same propensity was detected regarding two to three times a week consumption: this habit was much reported by controls (CI 44.40 - 72.30%) than by children presenting S-ECC (CI 5.00 - 24.00%) or dentin caries (CI 16.30 - 41.80%). An overall trend towards higher dmft score values was also noticed when increasing frequency patterns of ingestion were considered (Table 3.5).

Discussion

In this study, analysis of the impact of prolonged breastfeeding on ECC experience did not yield consistent results. While a trend towards a general rise of the mean dmft indexes was seen with increasing duration of breastfeeding habits, all three answer options (that is, the child was never breastfed and the child was or was not breastfed after the first year of life) were significantly associated with ECC-free children.

Although strong and cumulative epidemiological research has demonstrated breastfeeding provides multiple general health, nutritional and developmental advantages (American Academy of Pediatric Dentistry (AAPD), 2017b; P. Moynihan et al., 2019; K.G. Peres et al., 2018; World Health Organization (WHO), 2018), several studies have explored the role of breastfeeding in the development of ECC (American Academy of Pediatric Dentistry (AAPD), 2017c; Avila, Pordeus, Paiva, & Martins, 2015; Cui et al., 2017; Kühnisch et al., 2016; Tham et al., 2015; Wulaerhan et al., 2014). Although many studies with high level of evidence have shown prolonged breastfeeding may be associated to ECC experience

when such practices occur after twelve (Cui et al., 2017), eighteen (Tham et al., 2015) or twenty four months of life (Karen Glazer Peres et al., 2017; K.G. Peres et al., 2018), a recent systematic review has stated breastfeeding up to 24 months of age does not increase ECC risk (P. Moynihan et al., 2019).

Despite exposure to fermentable carbohydrates being necessary to the disease's onset and development, infants are often subjected to several diet-related factors that can modify ECC's risk (Feldens, Rodrigues, de Anastácio, Vítolo, & Chaffee, 2018; Loveren, 2019; Naidoo & Myburgh, 2007). Dietary patterns and drinking habits may influence not only the type or the total amount of ingested carbohydrates, but more importantly, affects the frequency and timing of its consumption (Gussy et al., 2006; Loveren, 2019; Vadiakas, 2008). Regularity of carbohydrates intake has proven to be a critical issue on this matter, considering frequent consumption of such substrates enables prolonged contact between sugars in the ingested food/beverage, consequently supporting the establishment of cariogenic bacteria, maintaining oral pH values decreased during longer periods and shifting oral conditions towards demineralization more often (American Academy of Pediatric Dentistry (AAPD), 2017c; Gussy et al., 2006; Loveren, 2019; Vadiakas, 2008). Accordingly, regular in-between meal ingestion of sugary snacks or beverages, either sugar-sweetened (that is, beverages to which sugar has been added) or sugar-containing beverages (in which sugars are naturally present), is known to increase the risk of ECC (American Academy of Pediatric Dentistry (AAPD), 2017c, 2017b; Kühnisch et al., 2016). Although extreme caution should be taken when interpreting oral health related behavior metadata from the present cohort, our observations concerning sweets consumption are in agreement with this data, as a statistically significant association was detected among daily consumption of sweets and ECC experience.

In addition, regular bottle-feeding or use of sippy cups with any liquid other than water during night periods have also been found to be potentially cariogenic practices (American Academy of Pediatric Dentistry (AAPD), 2017c; Kagihara et al., 2009; Kühnisch et al., 2016; Meyer & Enax, 2018). Such nighttime feeding practices are particularly adverse, especially if an infant falls asleep while feeding, since salivary flow, oral movements and subsequently the oral clearance of cariogenic substrates are substantially reduced during sleep, hence fostering acid production for long periods (Kagihara et al., 2009; Meyer & Enax, 2018). Hence, frequent ingestion of sugary foods and/or beverages, in particular when such liquids are given in baby bottles or sippy training cups, as well as use of baby bottle after twelve to eighteen months of life, especially at nighttime, are also discouraged practices (American Academy of Pediatric Dentistry (AAPD), 2017c; European Academy of Paediatric Dentistry (EAPD), 2008).

Finally, a number of limitations inherent to overall findings should be addressed. This observational longitudinal study comprised a convenience sample of preschoolers from the same district followed through a period of six months. As a convenience sample, the inclusion of the participating preschools in the study was predominantly based on their accessibility and/or proximity to the research team and to the district school nurses who assisted in obtaining cooperation from the educational establishments. The alternative adoption of a probability sampling strategy would possibly have yielded an unbiased sample representative of the target population, again more likely ensuring the obtained results were both unbiased and generalizable. However, such strategy would present a significant challenge in terms of time and costs, possibly jeopardizing the study timeline and, consequently, compromising the execution of the study. Accordingly, some measures were implemented aiming to overcome the main potential limitation of the present non-probability sample: lack of representativeness of the study population. While selecting the participant preschools, efforts were performed in order to include both rural and urban preschools and to involve all types of educational establishments included in the national preschool education network. Even though our sample did not involve an equivalent number

of public, private and IPSS preschools, this attempt possibly contributed to include participants from distinct ethnic and socioeconomic backgrounds.

Findings of the present longitudinal study should also be interpreted with caution attending to its relatively limited sample size. In fact, despite all the efforts made to enhance preschools' cooperation and parental adherence to the study (as described in detail in Chapter 2 - '*Experimental procedure*s'), the initially planned minimum sample size (about 240 children) was not achieved. Although inclusion of additional preschools and repetition of evaluations to include children who did not attend school in the scheduled evaluation periods could have been performed, these methodological options were not feasible given the available logistical, human and time resources.

Several limitations inherent to conducting dental research in field settings should also be addressed. Although oral observations were performed by a previously calibrated and trained dentist and following the WHO recommendations (World Health Organization (WHO), 2013), to ensure the reliability and consistency of the obtained data, diagnosis of ECC in epidemiological settings can constitute a complex process, particularly regarding initial lesions. Presence of white spot lesions and loss of discontinuity of enamel's surface is usually better assessed under artificial light; moreover, cavitated lesions in proximal surfaces can often remain undetected without the use of an artificial light source and/or radiographic examination, seldom carried out in such screening situations. Limited visualization may additionally have hindered detection of highly esthetical tooth-colored restorations. It is thus possible that, overall, these limitations led to an underestimation of disease experience and influenced the results towards lower ECC prevalence rates and dmft scores.

Interpretation of results arising from oral health related behavior metadata of the cohort should be carried out carefully, as such data was not obtained through application of a validated questionnaire. To ensure the validity and reliability of a survey, the instrument must be subjected to a series of rigorous testing. Regarding research on dietary habits, it is much complex to clarify the concepts investigated by each item of the instrument; in this particular case, for instance, one may speculate that the perception of the concept of 'sweet' foods possibly differed significantly among caregivers. Moreover, validity and reliability of parental report is questionable, since they may not be completely aware or able to report the child's behaviors or may tend to underreport facts they consider less acceptable. Other methodological issues relate to the fact that both diet and oral hygiene practices involve complex individual patterns and, accordingly, additional variables should had been taken in account to fully depict these dynamics (e.*g.* timing of toothbrushing or breastfeeding habits).

Chapter 4. Characterization of saliva by NMR spectroscopy

Chapter 4. Characterization of saliva by NMR spectroscopy

This chapter describes the results that enabled the characterization of children saliva composition to be achieved through 1D and 2D NMR experiments. For this purpose, a concise explanation of the basic principles inherent to the general strategy used to identify metabolites by NMR spectroscopy is firstly presented. A typical 1D ^{1}H NMR spectrum of children saliva is then shown and interpreted with basis on existing NMR databases. Finally, 2D NMR experiments of saliva are presented and discussed in order to complete the interpretation of the ^{1}H NMR spectrum and produce a complete list of metabolites identified in children's saliva by NMR.

4.1. Basic principles of NMR spectroscopy

^{1}H NMR spectroscopy is a technique based on the magnetic properties of some atomic nuclei, with the property named nuclear spin which enables them to adopt particular orientations when subjected to a strong magnetic field (B_0) and, in such conditions, absorb electromagnetic radiation in the radiofrequencies range. Examples of nuclei having spin include the proton (^{1}H), ^{13}C, ^{14}N, ^{15}N, and many others. This property is the basis of NMR and a valuable source of detailed information on chemical structure and nature of molecules (S. Barnes et al., 2016). In a high external magnetic field, protons resonate between spin nuclear energy states absorbing radiation. The excess of absorbed energy is measured in the form of a free induction decay (FID) signal which corresponds to energy as a function of time (time domain). The FID is then Fourier transformed so it is converted to an energy function as a function of frequency (frequency domain), thus producing the typical 1D NMR spectrum (S. Barnes et al., 2016; Lindon et al., 2007).

Each nucleus is surrounded by electrons, which create a local magnetic field, usually opposed in direction to the external field B_0. Consequently, a shielding effect is produced and the effective magnetic field (B_{eff}) experienced by the nucleus is given by:

$$B_{eff} = B_0 (1-\sigma),$$

where σ is the shielding constant. This constant reflects the extent to which the electron cloud around the nucleus (for instance a proton) shields it from the magnetic field (Banwell, 1972; Lindon et al., 2007). Within a molecule, the chemical environment and location of protons, *i.e.* the nature of neighbouring atoms and the type of chemical bonds, affect the extent to which they are magnetically shielded. Depending on these factors, each nucleus will resonate at distinctive frequencies (ν, expressed in hertz (Hz)), corresponding to one characteristic absorption signal at a particular position in the NMR spectrum (Abraham et al., 1987; S. Barnes et al., 2016). The obtained resonance frequency of each nucleus is then usually normalized using a reference compound (*e.g.* TSP, as aforementioned), divided by the spectrometer frequency (500 MHz, in the present work) and multiplied by 10^6. The resulting value is designated as chemical shift (δ, expressed in ppm), thus defining a constant frequency scale regardless of the operating frequency of the particular spectrometer used to acquire spectra (Lindon et al., 2007). The nucleus proximity to electronegative atoms (*e.g.* nitrogen (N) or oxygen (O)) or unsaturated

moieties (*e.g.* double or triple bonds, aromatic rings) within the molecular structure modifies its B_{eff} and, consequently, affects its specific resonance frequency and chemical shift. Neighbouring atoms with high electronegativity will de-shield the nuclei, lowering their electronic density and causing them to resonate at higher frequencies, located in the higher ppm scale (left side of a spectrum, also known as downfield region). On the other hand, nuclei without electronegative atoms nearby, will be more shielded and resonate at lower resonance frequencies, their signals appearing at lower chemical shift (right side of a spectrum or upfield region) (Dona et al., 2016; Lindon et al., 2007). In addition, protons within an aromatic ring will resonate at higher chemical shifts and a similar effect occurs to protons near unsaturated groups, depending not only on the type of multiple bonds but also on the molecular geometry (Dona et al., 2016; Lindon et al., 2007).

Besides chemical shift, further spectral parameters present in the ^{1}H NMR spectra, such as the spin-spin coupling pattern (signal multiplicity) and peak intensity can provide valuable information concerning molecular structure (C. J. Silwood et al., 1999). In liquids, the interaction between neighbouring nuclei occurs via the electrons in chemical bonds, through a process called scalar coupling (or *J*-coupling) and this creates a peak splitting. For instance, for a given pair of nuclei, A and X, with spin I, this process depends on the quantum number of nuclei (m_A and m_x), as well as on a parameter that quantifies the strength of the interaction, known as the coupling constant J_{AX}. As a result, the NMR signal splits into more than one signal, with multiplicity (*i.e.* the number of peaks in the split signal) given by 2nI + 1 (where n refers to the number of neighbour equivalent nuclei) (Dona et al., 2016). For instance, when a methyl group (CH_3) is attached to a carbon atom not linked to a further proton, a single resonance peak (singlet) is produced. However, if the second carbon atom is attached to a single proton, the methyl resonance will be split into two signals, forming a doublet (S. Barnes et al., 2016). More complex splitting patterns are observed in molecules where the adjacent carbon contains more protons, in distinct chemical environments.

Finally, the area under each NMR peak is proportional to the number of nuclei present in the sample, that is, to compound concentration (Lindon et al., 2007). Under specific experimental conditions, NMR spectroscopy can be a quantitative method. However, in order to provide an absolute quantification of metabolites, longer acquisition times need to be used and a reference compound (such as TSP) is added to the sample. Long acquisition times are a drawback of particular relevance in this study, and in metabolomics in general, due to the high number of samples to be analyzed (Weljie, Newton, Mercier, Carlson, & Slupsky, 2006). Moreover, in a complex fluid such as saliva signal, the reference compound will tend to bind to sample components and this will render its reference area non-quantitative (Lindon et al., 2007). Furthermore, extensive signal overlapping can originate difficulties in metabolites assignment and in the identification of the integration area for a specific signal, thus limiting the accuracy of the quantification results. Considering these aspects, semi-quantitative conditions were used in this study. These allow determination of relative metabolite concentrations (that is, it enables the measurement of variation ratios between metabolites) rather than the absolute concentrations.

In a standard 1D spectra, both large and small molecules originate peaks with large and small bandwidths (*i.e.* broad and narrow signals), since bandwidth is related to molecular mobility. Another type of 1D experiment, named the CPMG experiment, enables the peaks arising from protons of macromolecules to be suppressed, originating a spectrum dominated by well resolved narrow peaks arising from small molecules (Lindon et al., 2007). For this reason, CPMG experiments have been reported as a useful contribution for spectral simplification and characterization of the metabolome of salivary samples (Aimetti et al., 2012; Fidalgo et al., 2015, 2013; Mikkonen et al., 2012; Neyraud et al., 2013; Singh et al., 2017). However, some signal loss may occur and, in this study, we have opted for registering standard ^{1}H NMR spectra of saliva samples.

The use of 2D NMR methods is also of utmost importance to aid peak assignment, in addition to simple comparison to database 1D spectra of metabolite standards (Alonso et al., 2015). 2D NMR experiments rely on the scalar coupling between nuclei and result in the spread of spectral information in two dimensions, thus significantly reduced spectral overlap problems and metabolite scalar coupling patterns that identify each metabolite unambiguously. Depending on the type of nuclei involved, 2D experiments can be homonuclear (nuclei from the type, usually ^{1}H) and heteronuclear (two different types of nucleus, e.g. ^{1}H and ^{13}C) experiments (Alonso et al., 2015). The 2D ^{1}H-^{1}H TOCSY spectrum is often a useful tool to provide molecular information for the assignment of complete spin systems, since it generates data of coupled protons belonging to the same spin-system, separated by up to five chemical bonds (Lindon et al., 2007). In fact, this technique allows the transfer of magnetization from a ^{1}H nucleus bonded to one specific carbon atom to one or more magnetically distinct nuclei (located 2 or more carbon positions further along the molecular chain) (Alonso, Marsal, & Julià, 2015). The use of ^{1}H–^{13}C HSQC methods can also be helpful in spectral assignment, particularly in the case of singlet resonances that have no scalar correlation and therefore cannot be observed in TOCSY experiments (Lindon et al., 2007). These experiments generate a 2D heteronuclear chemical shift correlation map between ^{1}H directly attached to ^{13}C. Each signal in a HSQC spectrum represents a proton bounded to a C atom and provides information about the chemical shift of ^{13}C correlated with the ^{1}H chemical shift of the directly bound proton (Lindon et al., 2007). Examples of the above experiments will be given in the text below for the specific case of saliva samples.

4.2. Typical 1D ^{1}H NMR spectrum of saliva

While the salivary proteome has been comprehensively explored in the last decade (with more than 3000 different proteins already identified) (Amado et al., 2013), the wide range of small molecules in saliva has not been particularly well-characterized (Fidalgo et al., 2015; K. Wang, Zhou, Li, & Zhang, 2019). However, despite most studies having targeted only specific classes of salivary metabolites, such as amino acids or organic acids, saliva's chemical diversity is believed to be comparable to those of human serum and cerebrospinal fluid metabolomes (Dame et al., 2015). In fact, it is believed that salivary metabolites comprise 112 distinct chemical classes, from carboxylic acids and derivatives, steroids and steroid derivatives, to quaternary ammonium salts and polyketides (Dame et al., 2015).

A typical 1D ^{1}H NMR spectrum of saliva presents hundreds of peaks arising from its broad range of composing metabolites. An average 1D ^{1}H NMR spectrum of USS from CF children from our pilot study cohort (*n*=23), acquired at 500 MHz, is shown in Figure 4.1, with several of the resonances identified. As described in detail in Chapter 2 ('*Experimental procedures*'), resonance assignments were performed based on the analysis of spectral parameters such as chemical shift, coupling constant and splitting pattern of metabolites (measured both by 1D and 2D NMR) and their comparison with previously reported data and particular existing databases (Dame et al., 2015; C. J. L. Silwood et al., 2002; Takeda et al., 2009; Wishart et al., 2018).

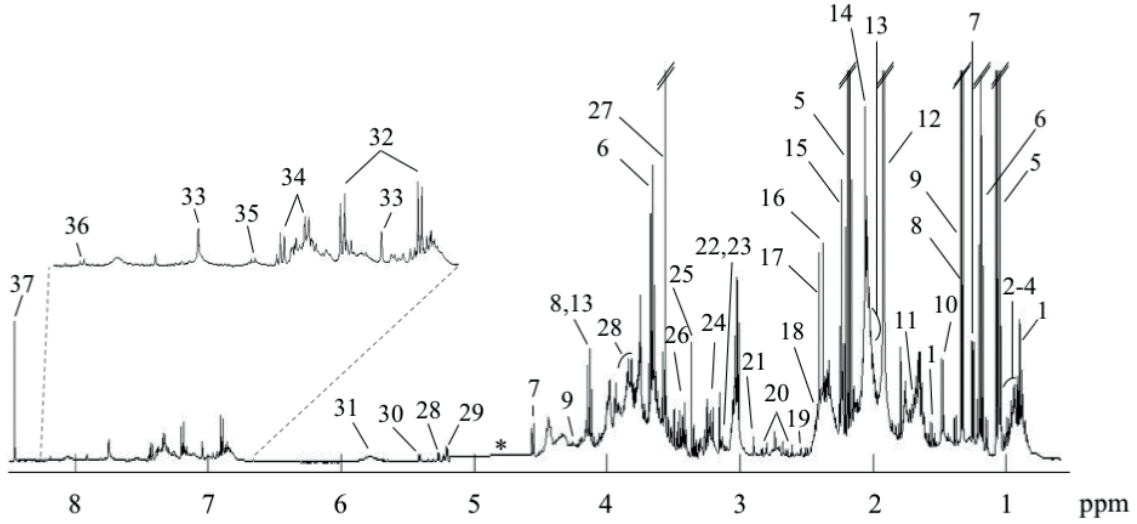

Figure 4.1. Average ^{1}H-NMR 500 MHz spectra of USS samples from CF children. Peak numbering identifies the main assignments: (1) butyrate, (2) isoleucine, (3) leucine, (4) valine, (5) propionate, (6) ethanol, (7) fucose, (8) lactate, (9) threonine, (10) alanine, (11) lysine, (12) acetate, (13) proline, (14) *N*-acetyl of glycoproteins, (15) acetone, (16) pyruvate, (17) succinate, (18) glutamine, (19) dimethylamine, (20) aspartate, (21) trimethylamine, (22) dimethylsulfone, (23) arginine, (24) choline, (25) methanol, (26) taurine, (27) glycine, (28) galactose, (29) glucose, (30) sucrose, (31) urea, (32) tyrosine, (33) histidine, (34) phenylalanine, (35) uracil, (36) hypoxanthine, (37) formic acid. *Excluded region (water) prior to multivariate analysis. Remaining assignments are shown in Table 4.1.

As it can be observed in Figure 4.1, a complete average 1D ^{1}H NMR salivary spectrum ranges from 0.4 to 8.5 ppm, excluding the water region (from 4.57-5.19 ppm), as previously mentioned in Chapter 2 ('*Experimental procedures*') and as commonly performed in NMR studies of human biofluids (A.-H.

Emwas et al., 2018). Like other biofluids, saliva contains a high water content (about 99.5%) (Hemadi et al., 2017) and, consequently, a large water signal would dominate the spectrum at approximately 4.8 ppm. This signal would interfere with the peaks of other metabolites lying below, hampering their clear observation. For this reason, a suitable suppression technique for the water resonance was applied in spectral acquisition (*noesypr1d* pulse sequence) and, afterwards, during spectra pre-processing, this region was excluded from multivariate analysis as the spectra trace in that region may vary considerable between spectra (A.-H. Emwas et al., 2018; Lindon et al., 2007).

A peak corresponding to TSP, which was added to salivary samples prior to spectral acquisition, corresponds to the reference signal at 0.0 ppm (Figure 4.1). In fact, TSP is a common internal standard in NMR experiments of biological samples, since it produces a single high-intensity and well-resolved resonance that can be set to 0.0 ppm and is used for chemical shift referencing of all the remaining compounds within the sample (Lindon et al., 2007).

A significant number of narrow peaks arising from small molecules were detected superimposed on broader resonances, as previously reported (Dame et al., 2015; Grootveld, Algeo, Silwood, Blackburn, & Clark, 2006; C. J. L. Silwood et al., 2002; Takeda et al., 2009). These broader signals are due to the presence of macromolecules, as peak broadness reflects the metabolite's mobility and size. In general, bigger molecules (such as lipids or proteins, for *e.g.*) are less mobile, thus producing broader resonances. In saliva spectra, these signals were assigned to the molecularly mobile portions of certain macromolecules, mostly glycoproteins (C. J. L. Silwood et al., 2002). The numerous sharp and prominent signals in the standard 1D salivary spectrum in Figure 4.1 originate from the different types of protons of low molecular weight components, such as short-chain organic acids (acetate, lactate, acetoacetate, butyrate, propionate, pyruvate and citrate), some amino acids (glutamine, proline, alanine, valine, methionine, leucine, threonine, tyrosine and phenylalanine), carbohydrates (such as glucose, galactose, fucose) and amines (trimethylamine, dimethylamine) (Grootveld et al., 2006; C. J. L. Silwood et al., 2002). Three different expanded spectral regions, namely aliphatic (0.4-3.1 ppm), sugars (3.1-5.8 ppm) and aromatic (5.8-8.5 ppm) regions are presented in Figures 4.2, 4.3 and 4.4, respectively. In these expansions, the presence of the mentioned broader signals can be more distinctively observed comparing to the complete spectrum in Figure 4.1.

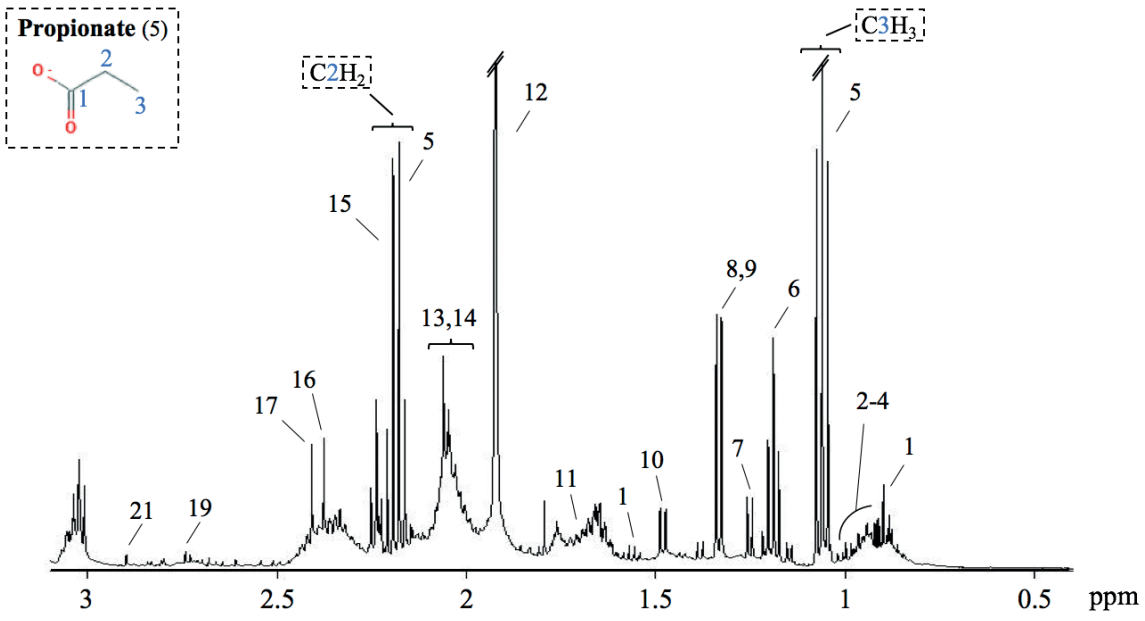

Figure 4.2. Expansion of the aliphatic region (0.4-3.1 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1. The molecular structure of propionate with indication and assignment of its different types of protons is also included.

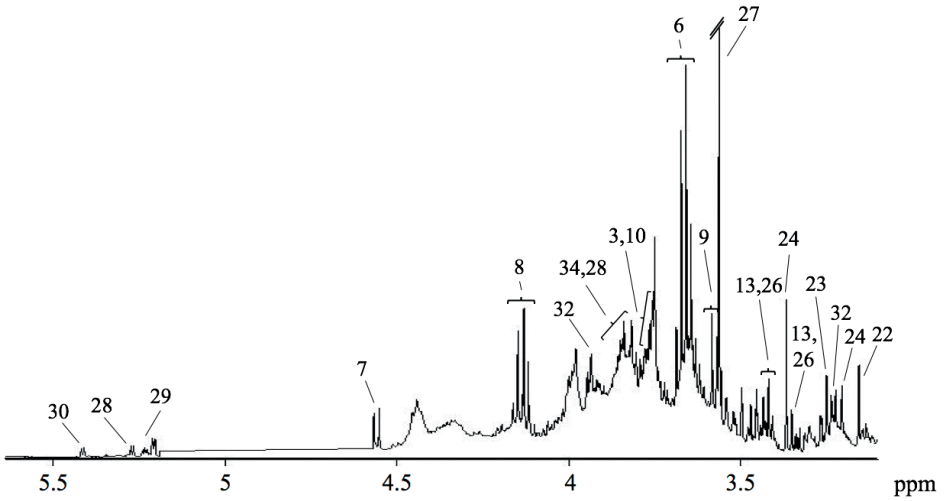

Figure 4.3. Expansion of the sugars' region (3.1-5.8 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1.

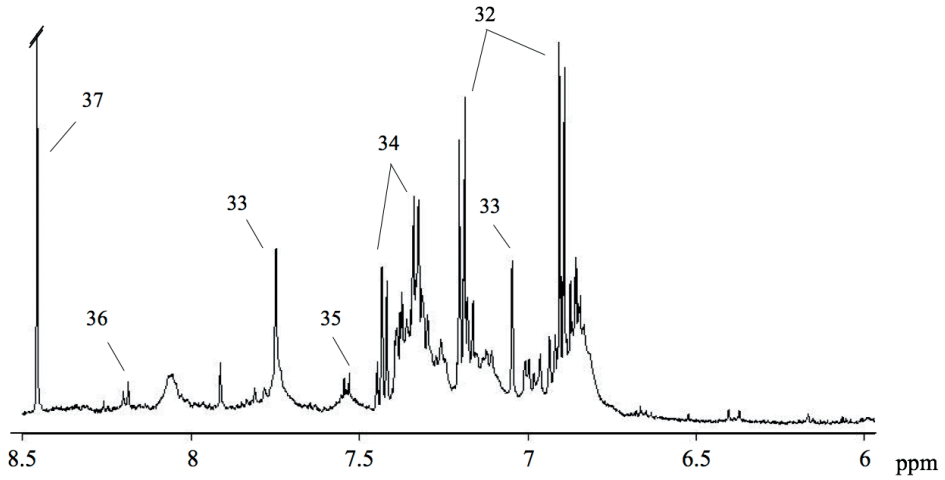

Figure 4.4. Expansion of the aromatic region (5.8-8.5 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1.

The aliphatic region exhibits many sharp signals mostly associated to protons from more shielded methyl (-CH_3) and methylene (-CH_2) functional groups. Without any electronegative atoms or complex chemical bonds nearby, these protons resonate at lower frequencies. CH_3 group protons from ethanol, organic acids including lactate, acetate and pyruvate and from amines (dimethylamine and trimethylamine) are, therefore located in this region of the ppm scale. Protons from CH_2 linked to CH_2 functional groups also can exhibit peaks in this region, as it occurs with hydrogens from the CH_2 functional group of succinate. Additionally, a broad spectral region corresponding to the acetoamide methyl group protons (-NHC-OCH_3) of *N*-acetylsugars is exhibited at 2.06 ppm. These *N*-acetylsugars are believed to be located in the molecularly mobile branching carbohydrate side-chains of glycoproteins, like salivary mucins.

Aiming to clearly exemplify the metabolite assignment process, the molecular structure of propionate, a common salivary organic acid, is shown in Figure 4.2, with indication of its different hydrogen environments (1, 2 and 3). In this particular case, protons from the CH_3 group (*i.e.* C3H_3) resonate at lower chemical shift (1.04 ppm) than the protons from the C2H_2 group (2.17 ppm), which are nearer to electronegative atoms (oxygen) and to a double bond. Moreover, taking in account the number of neighbour equivalent nuclei, signal multiplicity is easily understood: protons from the C3H_3 group are linked to a carbon which is attached to 2 protons, originating one triplet (2 equivalent neighbour nuclei + 1; on the other hand, protons from the C2H_2 group are split their signal into a quartet (3 equivalent neighbour nuclei + 1). The sugar region, ranging from 3.1-5.8 ppm (Figure 4.3, presents peaks from different types of more de-shielded protons, due to the proximity of electronegative atoms such as N, as in the case of the $N(CH_3)_3$ protons from choline (at 3.20 ppm) or in the CH_2 group from glycine (at 3.65 ppm). Similarly, the presence of O atoms affects the chemical shifts of CH_2 protons from ethanol (which presents an –OH group), as well as the methine (-CH) proton from lactate (peak at 4.11 ppm). This region is characterized by a crowded region of resonances from several sugars (*e.g.* glucose, fucose and galactose) and amino acids (including arginine, tyrosine, histidine and glycine). Finally, the region at δ 5.8-8.50 (aromatic region - Figure 4.4) is dominated by resonances arising from aromatic amino acids (such as tyrosine, phenylalanine and histidine), nucleosides (uracil and hypoxanthine) and formate. Tyrosine, whose CH group protons resonances had been assigned in the sugar region (multiplet and doublet at 3.0 and 3.83 ppm, respectively), also exhibit peaks in the aromatic region, owing to the chemical shift of aromatic protons. Likewise, distinct peaks from the CH ring protons of phenylalanine can be found at 7.32, 7.38 and 7.43 ppm. Histidine, on the other hand, presents a ring structure containing two N-containing groups and, therefore, exhibits multiplets at even higher chemical shifts due to the de-shielding effect of both N and double bonds present in the ring. A similar effect occurs in uracil, whose chemical structure includes an aromatic ring with an N group, also generating a signal in this region (doublet; 7.52 ppm).

In addition to the acquisition of standard 1D NMR spectra, some CPMG experiments were performed on selected samples, however, in the present study, CPMG experiments did not provide any relevant additional data in comparison to the remaining spectra (data not shown).

4.3. Complementary NMR assignment strategies (2D experiments)

After performing the initial approach to assign saliva NMR signals, through analysis of 1D spectral parameters, 2D NMR experiments, namely 2D ^{1}H-^{1}H TOCSY and ^{1}H–^{13}C HSQC, were recorded to confirm and resolve ambiguous assignments. The spectral complexity encountered in saliva samples, particularly the extensive overlap in several spectral regions (clearly visible in expanded spectra regions, Figures 4.2 to 4.4) justified the need to obtain further data from 2D NMR spectroscopy on selected samples. Expansions of a TOCSY and a HSQC spectra of saliva are shown in Figures 4.5 and 4.6, respectively.

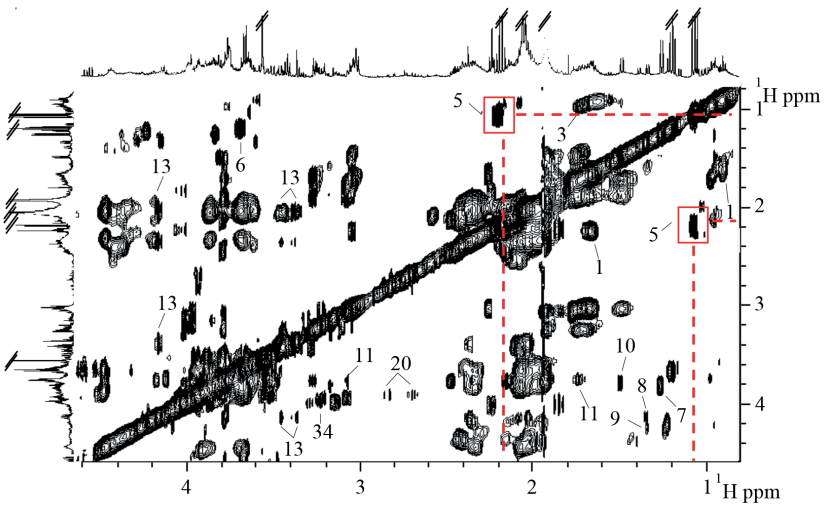

Figure 4.5. Expansion (0-4.6 ppm) of the 2D NMR TOCSY spectra of control (CF) saliva. Some peak assignments are shown and numbering corresponds to that shown in Figure 4.1. In red: illustration of TOCSY use in the confirmation of the propionate assignment (metabolite number 5).

As shown in the TOCSY spectra in Figure 4.5, spin systems of metabolites can be readily observed as rows of cross-signals correlating the chemical shifts of all protons comprising a spin system. In this spectra some peak assignments are indicated and, as an example, the spin system of propionate (triplet at 1.04 ppm and quartet at 2.17 ppm) is identified (red squares).

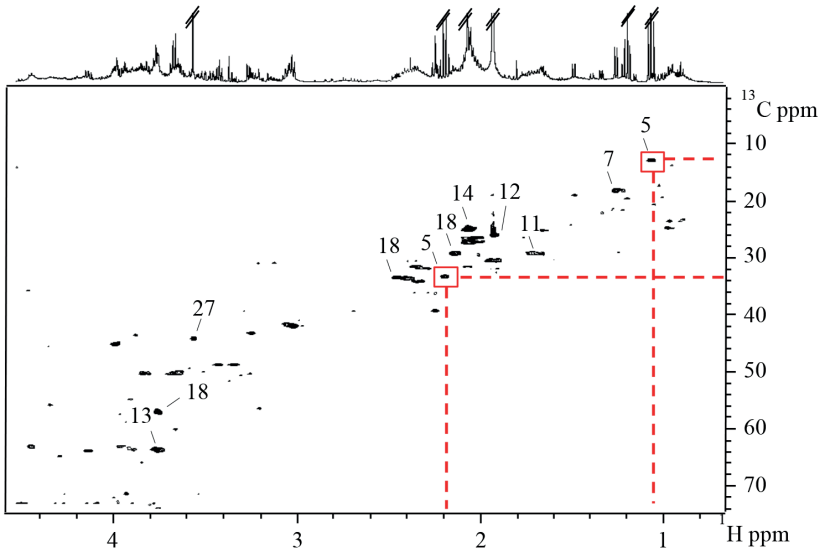

Figure 4.6. Expansion (0-4.6 ppm) of the 2D NMR HSQC spectra of control (CF) saliva. Some peak assignments are shown and numbering corresponds to that shown in Figure 4.1. In red: illustration of HSQC use in the confirmation of the propionate assignment (metabolite number 5).

In Figure 4.6. an expansion of an HSQC spectra is presented: the ^{1}H spectrum is shown on the horizontal axis, while the ^{13}C spectrum is shown on the vertical axis. In this figure, the propionate assignment is, once again, confirmed: the red squares show the direct cross peaks of propionate's CH_3 (1.04 ppm in ^{1}H axis and 12.9888 ppm in ^{13}C axis) and CH_2 groups (2.17 ppm in ^{1}H axis and 33.4802 ppm in ^{13}C axis).

Overall, through the combination of several assignment strategies, a total of 47 metabolites were identified in this study, all of which had already been previously reported in literature, mostly for adult subjects (Dame et al., 2015; C. J. L. Silwood et al., 2002; Takeda et al., 2009). The full list of assignments presented here is, to our knowledge, the most comprehensive list obtained for children saliva. Table 4.1 lists all the compounds assigned in the present study, as well as the corresponding ^{1}H chemical shifts, multiplicity, assignment and HMDB identifier (HMDB ID). The level of assignment according to the Metabolomics Standard Initiative (Salek et al., 2013) and the confirmation of some assignments through 2D experiments are also included in Table 4.1. Even though no further new compounds were assigned when compared to the available literature on human saliva, a detailed catalogue on children's salivary metabolites was obtained and this wide data set revealed fundamental for the preciseness of the following metabolomic tasks performed in this thesis.

Metabolite	HMDB IDs	Assignment level	Confirmed by ^{1}H NMR (n/y)	Observed in 2D NMR [d]	$\delta_{H\ ppm}$ (multiplicity, assignment)
4-Hydroxy-phenyl-lactate [a]	HMDB0000755	2	y	n	2.87 (dd), 3.04 (dd), 4.35 (dd), 6.85 (d), 7.17 (d)
5-Aminopentanoate [a]	HMDB03355	1	y	y2	1.64 (m), 2.22 (m), 3.00 (m)
Acetate [a, b, c]	HMDB0000042	1	y	y1	1.92 (s)
Acetone [a, b, c]	HMDB0001659	1	y	y1	2.23 (s)
Alanine [a, b, c]	HMDB0000161	1	y	y1, y2	1.48 (d), 3.76 (q)
Allantoin [c]	HMDB0000462	2	y	n	5.39 (s)
Arginine [a,b]	HMDB0000517	1	y	y2	1.68 (m), 1.90 (m), 3.23 (t), 3.76 (t)
Ascorbate [c]	HMDB0000044	2	y	n	3.76 (m); 4.01 (m); 4.52 (d)
Aspartate [a, c]	HMDB0000191	1	y	y2	2.69 (dd), 2.83 (dd), 3.90 (dd)
Butyrate [a, c]	HMDB0000039	1	y	y2	0.89 (t), 1.56 (m), 2.14 (t)
Choline [a, c]	HMDB0000097	1	y	y1,y2	3.20 (s), 3.52 (m), 4.07 (m)
α-Galactose [a, c]	HMDB0000143	1	y	y1,y2	3.48 (dd), 3.64 (dd), 3.72 (m), 3.82 (m), 3.92 (d), 3.96 (d), 4.07 (t), 5.27 (d)
α-Glucose [a, b, c]	HMDB0000122	1	y	y1,y2	3.23 (dd), 3.39 (m), 3.45 (m), 3.52 (dd), 3.72 (m), 3.82 (m), 3.88 (dd), 4.63 (d), 5.24(d)
Dimethyl sulfone [a]	HMDB0004983	2	y	n	3.15 (s)
Dimethylamine [a, c]	HMDB0000087	1	y	y1	2.73 (s)
Ethanol [a, b, c]	HMDB0000108	1	y	y1,y2	1.19 (t), 3.65 (q)
Formate [a, b, c]	HMDB0000142	1	y	n	8.46 (s)
α-Fucose [a]	HMDB0000174	1	y	y1, y2	1.25 (d), 3.45 (dd), 3.64 (dd), 3.79 (m), 4.56 (d)
Glutamate [a, c]	HMDB0003339	1	y	y2	2.04 (m), 2.11 (m), 2.34 (m), 3.74 (dd)
Glutamine [a, c]	HMDB0000641	1	y	y1,y2	2.12 (m), 2.44 (m), 3.76 (t)
Glycine [a, b, c]	HMDB0000123	1	y	y1	3.56 (s)
Histidine [a, b, c]	HMDB0000177	1	y	y2	3.02 (m,), 3.15 (s), 7.05 (s), 7.75 (s)
Hypoxanthine [a, c]	HMDB0000157	1	y	y2	8.17 (s), 8.20 (s)
Isoleucine [a, c]	HMDB0000172	1	y	y1, y2	0.92 (t), 0.99 (d), 1.24 (m), 1.45 (m), 1.96 (m), 3.66 (d)
Lactate [a, b, c]	HMDB0000190	1	y	y2	1.32 (d), 4.11 (q)
Leucine [a, b, c]	HMDB0000687	1	y	y2	0.94 (t), 1.7 (m), 3.72 (m)
Lysine [a, c]	HMDB0000182	1	y	y1, y2	1.46 (m), 1.71 (m), 1.89 (m), 3.02 (t), 3.74 (t)
Methanol [a, b, c]	HMDB0001875	1	y	y1	3.36 (s)
Methylamine [a, c]	HMDB0000164	1	y	y1	2.61 (s)
Methylguanidine [a, c]	HMDB0001522	2	y	n	2.83 (s), 3.36 (s)
N-acetyl-glycoproteins [c]		2	y	y1	2.06 (s)
Phenylalanine [a, b, c]	HMDB0000159	1	y	y2	3.19 (m), 3.98 (dd), 7.32 (d), 7.36 (m), 7.42 (m)
Proline [a, c]	HMDB0000162	1	y	y1,y2	1.99 (m), 2.06 (m), 2.35 (m), 3.33 (dt), 3.41 (dt), 4.16 (dd)
Propionate [a, b, c]	HMDB0000237	1	y	y1,y2	1.06 (t), 2.18 (q)
Propylene glycol [a, b]	HMDB0001881	1	y	y2	1.15 (d), 3.43 (dd), 3.53 (dd), 3.87 (m)
Pyruvate [a, b, c]	HMDB0000243	1	y	y1	2.38 (s)
Sarcosine [a, b, c]	HMDB0000271	2	y	n	2.74 (s), 3.60 (s)
Succinate [a, c]	HMDB0000254	1	y	y1	2.41 (s)
Sucrose [b, c]	HMDB0000258	1	y	y1,y2	3.46 (t), 3.55 (dd), 3.67 (s), 3.75 (t), 3.82 (m), 3.87 (dd), 3.89 (dd), 4.04 (t), 4.21 (d), 5.42 (d)
Taurine [a, b, c]	HMDB0000251	1	y	y1,y2	3.27 (t), 3.42 (t)
Threonine [a, c]	HMDB0000167	1	y	y2	1.32 (d), 3.57 (d), 4.26 (m)

Metabolite	HMDB IDs	Assignment level	Confirmed by ^{1}H NMR (n/y)	Observed in 2D NMR [d]	$\delta_{H\ ppm}$ **(multiplicity, assignment)**
Trimethylamine [a, c]	HMDB0000906	2	y	n	2.89 (s)
Tyrosine [a, b, c]	HMDB0000158	1	y	y2	3.06 (dd), 3.12 (dd), 3.95 (dd), 6.90 (d), 7.20 (d)
Uracil [a]	HMDB0000300	1	y	y2	5.79 (d), 7.52 (d)
Urea [a, b]	HMDB0000294	1	y	n	5.78 (br)
Valine [a, c]	HMDB0000883	1	y	y2	0.99 (d), 1.05 (d)
Xylose [a]	HMDB0000098	1	y	y1,y2	3.23 (dd), 3.33 (dd), 3.42 (t), 3.53 (dd), 3.63 (m), 3.93 (dd), 4.58 (d), 5.21 (d)

Table 4.1. Peak assignments in the ^{1}H NMR spectrum of children saliva. s: singlet, d: doublet, t: triplet, q: quartet, dd: doublet of doublets, dt: doublet of triplets, m: multiplet, br: broad signal. [a,b,c] compounds identified in unstimulated saliva of healthy adults in Dame *et al.* 2015, Takeda *et al.* 2009 and Silwood *et al.* 2002, respectively. [d] n: not observed, y1: observed in HSQC, y2: observed in TOCSY. Compounds are identified according to Salek *et al.* 2013 and Wishart *et al.,* 2018. Assignment level according to the Metabolomics Standard Initiative (Salek et al., 2013): level 1, identified metabolites; level 2, putatively annotated compounds.

Additionally, the results presented in this chapter allowed the acquisition of crucial metabolomic skills by the doctorate student, a pediatric dentist. In fact, the identification of metabolites through NMR approaches relies on the individual skills of the researcher, who classifies a compound as a metabolite, based on his knowledge on saliva components, their chemical structure and interpretation of spectra. Undoubtedly, the complexity of spectral data, along with the chemical diversity of the salivary metabolome warranted the attainment of theoretical background in chemistry, as long with practical expertise on sample preparation techniques and several computational skills.

Chapter 5. Optimization of saliva collection protocol for metabolomics

Chapter 5. Optimization of saliva collection protocol for metabolomics

This chapter describes a pilot study which was carried out with the aim of establishing a suitable protocol for collection of saliva for ^{1}H-NMR-based research in pediatric populations. For the first time to our knowledge, the impact of stimulation on saliva metabolome was assessed through comparison of metabolic profiles of SS and USS, collected from a pilot cohort of children, using ^{1}H-NMR spectroscopy. After having demonstrated the absence of significant differences between SS and USS metabolomes and considering that the latter is produced in resting conditions, thus representing more faithfully the oral cavity baseline status, USS was used for subsequent studies. In light of the difficulties often encountered during USS collection using PD in young children, the suitability of a commercially available saliva absorbing device, SCS (Salimetrics, State College, PA, USA), was subsequently evaluated. For this purpose, a comparative analysis of USS composition of a small group of children, who donated saliva by both PD and SCS methods, was performed. The significant variations in sample composition induced by SCS collection were then presented and discussed.

Although saliva metabolomics is currently a promising and emerging research field concerning medical diagnosis, with particular interest for pediatric populations, several problematic gaps have been recognized. Few studies have explored children saliva through NMR-based approaches (typically of a holistic nature) and, in addition, studies performed in adults present a high degree of variability concerning multiple methodological steps. In fact, numerous disparities among sampling conditions are noted in currently available reports *e.g.* while some studies on adults have explored USS, others have used SS, obtained through distinct stimulation methods. To date, the most suitable saliva collection method for metabolomic studies in children has not been established, particularly since to our knowledge no study has clarified the existence of eventual differences between the SS and USS metabolomes. In addition, although the uncomplicated and non-invasive nature of USS collection obviously contributes to much of the interest of saliva as a diagnostic biofluid, its collection through PD in young children is unexpectedly difficult and time-consuming (Beale et al., 2016; Granger et al., 2007; Nunes et al., 2015). Difficulties gathering sufficient sample volumes for assays can lead to reduced sample sizes and missing values on data sets, as well as to potential unexpected delays in the completion of planned saliva collections. These potential PD limitations may justify the use of USS absorbing methods in children, however, the suitability of these devices for ^{1}H-NMR studies has not been addressed yet, constituting another scientific void to be explored.

The existence of such critical lacks on current literature not only hinders comparison of existing evidence, but also hampers the development of further research. Several reports have highlighted the relevance of performing studies involving saliva under well-controlled conditions, mostly regarding sample collection method and timing (A.-H. M. Emwas et al., 2013; Granger et al., 2007; Hanrahan et al., 2006; Harmon, Hibel, Rumyantseva, & Granger, 2007; Topkas et al., 2012). Conducting pilot studies for sample quality control is, therefore, advisable whenever saliva collection is to be employed in a new research field and/or in particular populations (Harmon et al., 2007), thus justifying the present pilot study.

5.1. Effects of stimulation

Aiming to fill in some of the currently existing gaps regarding analytical issues on saliva collection in children for NMR studies, the effect of stimulation on saliva metabolome was firstly investigated, in comparison to USS metabolome. The average ^{1}H-NMR spectra of children´s USS and SS are presented in Figure 5.1 (a and b, respectively). Only some of the most relevant assignments are indicated in Figure 5.1 a), all of which had already been identified in the preceding chapter (for complete assignment of saliva spectra please consult Chapter 4). Each of the average spectra shown in Figure 5.1 was calculated using spectral data from a cohort subset: CF children matched for gender and dentition stage (females in mixed phase of dentition), who donated both USS and SS samples. Although this matching process certainly limited the number of subjects in each group (n=4 in each group), it enabled interpretation of spectral changes solely with basis on saliva stimulation, rather than having to consider other variables such as gender, age or presence of dental caries. The average spectra shown in Figure 5.1 a) are, therefore, not the same as the USS average spectra presented in Figure 4.1 of Chapter 4, which corresponded to all CF children who provided USS samples (through PD).

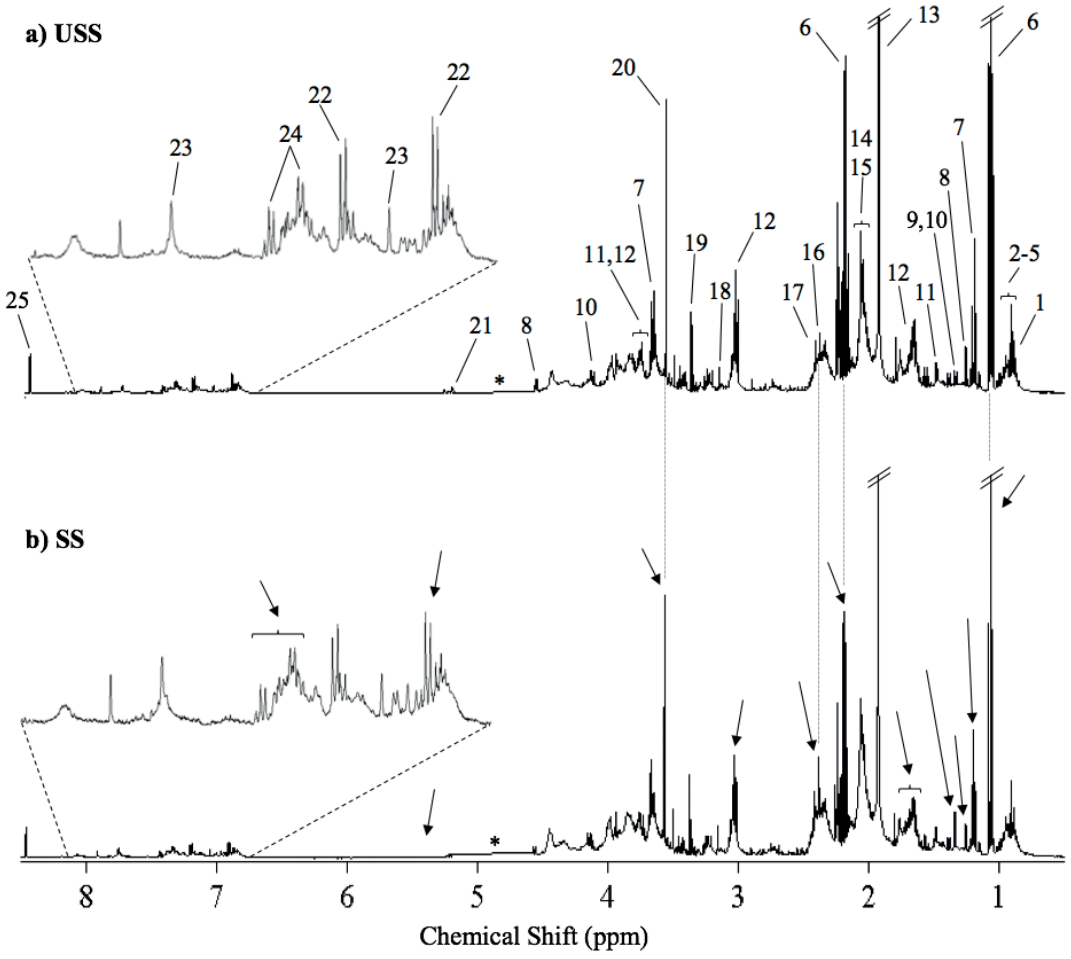

Figure 5.1. Average 500 MHz ^{1}H-NMR spectra of a) USS and b) SS from the same subset of CF children, matched for gender and dentition stage. Some assignments are noted on a) and major visual changes between spectra are indicated in b) (see arrows). Legend: 1, Butyrate; 2, 0.90(s); 3, Isoleucine; 4, Leucine; 5, Valine; 6, Propionate; 7, Ethanol; 8; Fucose; 9, Threonine; 10, Lactate; 11, Alanine; 12, lysine; 13, acetate; 14, Proline; 15, *N*-acetyl glycoproteins; 16, Pyruvate; 17, Succinate; 18, Dimethylsulfone; 19, Methanol; 20, Glycine; 21, Glucose; 22, Tyrosine; 23, Histidine; 24, Phenylalanine; 25, Formate.

As expected, both average spectra in Figure 5.1 revealed a significant number of narrow peaks (arising from small molecular weight compounds), superimposed on broader resonances, which arise from macromolecules, mostly glycoproteins. Visual comparison of these spectra allowed the detection of some differences in the average composition of saliva, indicated through arrows in Figure 5.1 b). Mostly located in the aliphatic region, from 0.4 to 3.1 ppm, some of the most distinct visual differences included decreases in propionate, ethanol, fucose and lysine, and increases in threonine, lactate and pyruvate levels in SS samples. In the sugars region, only glycine and glucose levels seemed to change, appearing to be decreased in SS samples. Lastly, in the aromatic section of spectra (5.8 – 8.5 ppm), an apparent decrease was noted for tyrosine and phenylalanine in the presence of stimulation.

However, in order to assess the statistical relevance of the apparent visual spectral changes, MVA methods, namely PCA and PLS-DA, were performed using the NMR spectra, after excluding the water region (4.57 - 5.19 ppm), which is particularly variable due to acquisition conditions. As described in detail in the Experimental procedures Chapter, the children cohort composing SS (n=30) and USS groups (n=38) was considered approximately matched for age, gender, dentition stage and dental caries status. As an unsupervised method, the PCA carried out to compare both groups did not consider any information on sample class, that is, SS or USS, in this case. The PCA model revealed no group separation between SS and USS groups (Figure 5.2 a). In this score scatter plot, a potential outlier, *i.e.* a sample obviously deviating on PCA (corresponding to a USS sample from a CA male in permanent dentition), was identified. Since the presence of this sample may determine group separation, the model was repeated after exclusion of this sample. Nonetheless, the overall sample groups remained largely overlapped in the second PCA and no separation trends between them were detected (data not shown). Likewise, in the corresponding PLS-DA model (Figure 5.2 b), performed with basis on class definition, no robust group separation occurred since no predictive power was found (Q^2=0.07-0.16, Table 5.1), indicating that no significant changes in metabolite levels could be detected between SS and USS.

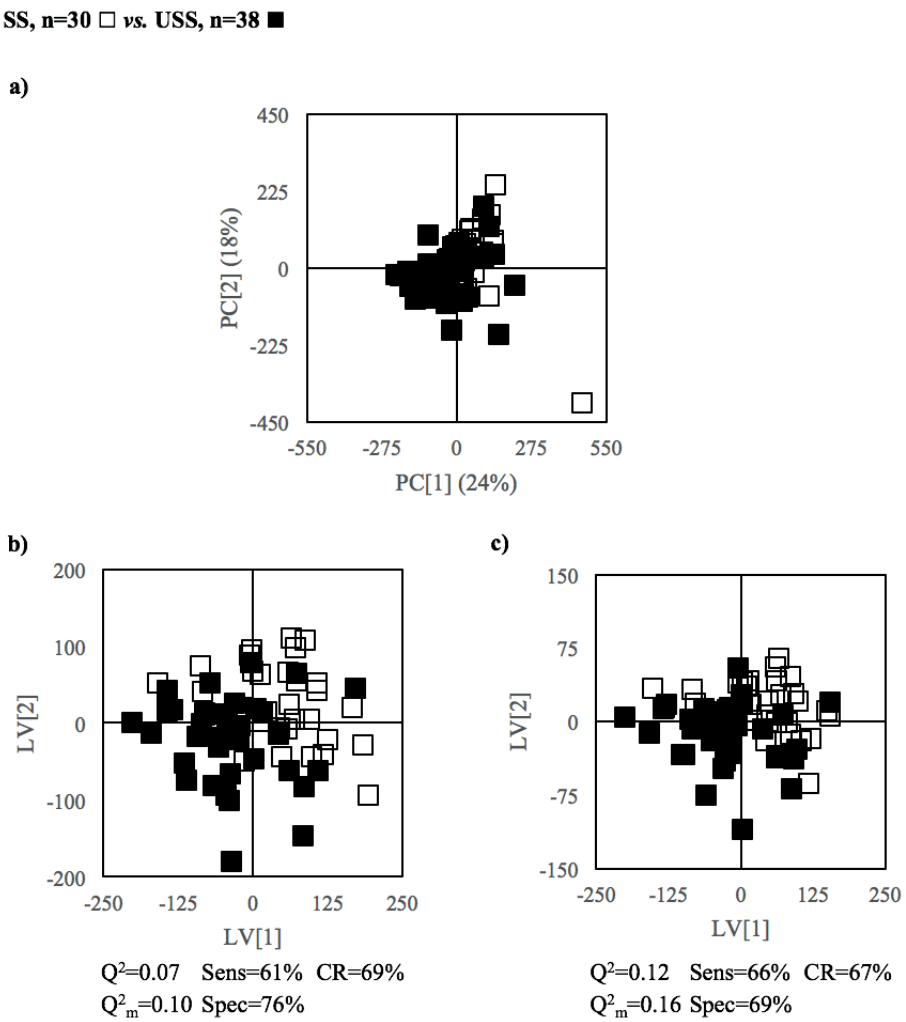

Figure 5.2. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra for saliva stimulation comparison: SS (□, *n*=30) and USS (■, *n*=38). Q^2 value and MCCV results are shown for each model.

Group		Dataset	% full resolution dataset	R^2X	R^2Y	Q^2	Q^2_m	CR (%)	Sens (%)	Spec (%)
Collection method	SS, *n*=30 *vs.* USS, *n*=38	Full resolution	–	0.32	0.40	0.07	0.10	69	61	76
		VS	34	0.54	0.37	0.12	0.16	67	66	69
	PD, *n*=5 *vs.* SCS, *n*=5 [a]	Full resolution	–	0.55	0.96	0.80	0.52	79	76	81

Table 5.1. Q^2 (predictive power) values and MCCV parameters obtained for the PLS-DA models of saliva collection methods corresponding to full resolution datasets and datasets obtained after variable selection. Q^2, cumulative predictive power obtained by cross-validation; Q^2_m, median Q^2 obtained by MCCV; CR: classification rate; Sens, sensitivity; Spec, specificity; SS, stimulated saliva; USS, unstimulated saliva; PD, passive drool; SCS, SalivaBio's Children's Swab®, Salimetrics, State College, PA, USA. [a] comparison carried out using USS.

VS of the NMR data was then applied to the spectra, aiming to select spectral regions with stronger correlation to class definition (in this case, presence or absence of stimulation), and the resulting PLS-DA score plot is presented in Figure 5.2 c). As displayed in Table 5.1, although a slight increase of the Q^2 value was registered after performing this procedure, the corresponding PLS-DA still maintained a poor performance (Q^2=0.12) and no group trends were visualized in the score plot, confirming no substantial differences were observable between SS and USS in our cohort. In view of these results, none of the apparent alterations in specific metabolites initially detected through visual (and therefore subjective) comparison of average spectra were statistically significant.

Based on the literature, stimulation is expected to affect saliva composition to some extent, since USS is secreted predominantly from the submandibular and sublingual glands, whereas in the presence of a stimulus the parotid glands are the major contributors to saliva, producing more than 50% of whole saliva (Mahvash Navazesh & Kumar, 2008; Neyraud et al., 2013). Previous studies have shown that each salivary gland secretes a characteristic type of secretions, as saliva from submandibular glands is more viscous and secretions from parotids tend to be more serous (Neyraud et al., 2013; Pfaffe et al., 2011; Takeda et al., 2009). In general, while some authors note that stimulation increases the amount of water output, diluting the concentration of analytes (Chiappin et al., 2007; Cuevas-Córdoba & Santiago-García, 2014; Malamud, 2011; F. Xu, Laguna, & Sarkar, 2019), others refer that, along with a higher flow rate, an increase in protein secretion occurs due to stimulation (though it is partially masked by the increase of secretions amount) (Neyraud, Bult, & Dransfield, 2009). The reported differences have been mainly characterized with respect to the proteomic signature (Neyraud et al., 2006, 2013; Pfaffe et al., 2011; Quintana et al., 2009; Schipper et al., 2007; Takeda et al., 2009) and distinct protein profiles indicative of each gland type have been suggested (Pfaffe et al., 2011; F. Xu et al., 2019).

To date, previous reports on adults' saliva metabolome performed through ^{1}H-NMR spectroscopy have found significant differences between USS and SS, though some contradictory variations have been observed (Figueira et al., 2017; Neyraud et al., 2013; Takeda et al., 2009). Neyraud *et al.* noted, in SS collected through paraffin chewing, an overall level increase in some organic acids (*e.g.* acetate, lactate), amino acids and fatty acids, the latter viewed by MS metabolomics, while taurine and propionate were the only metabolites to decrease in SS, under the same conditions (Neyraud et al., 2013). Increased concentrations of some amino acids, namely glutamine, lysine and proline, were considered a potential reflection of higher PRPs degradation products levels, which account for the majority of the proteins secreted by parotids. On the other hand, the increased amount of fatty acids in SS was attributed to either lipids produced by parotid glands or originated from the cell layer of the oral mucosa due to paraffin chewing. According to the authors, paraffin chewing might also have been the cause for the increased levels of acetate and lactate, the main organic acids commonly found in dental plaque, since the mechanical effect of paraffin chewing may cause partial removal of plaque adhered to dental surfaces (Motisuki et al., 2005). More recently, another study explored the stimulation effect in the saliva metabolome of one subject, through both ^{1}H-NMR and LC-MS, and found higher levels of lactate and glucose in SS, having however detected a decrease in acetate concentration (Figueira et al., 2017).

The use of citric acid solution as a stimulation method has also been studied in adults through ^{1}H-NMR spectroscopy (Takeda et al., 2009). Under these stimulation conditions, the overall levels of salivary metabolites decreased, though some to a lesser extent than others. Metabolites such as acetate, acetone, formate, glucose, glycine, succinate, sucrose, phenylalanine, propionate, taurine and tyrosine were found to decrease more extensively in comparison to alanine, dimethylamine, lactate and pyruvate. However, sampling conditions may have caused the observed variations. Indeed, the use of different

types of stimuli has been noted as potentially inducing different metabolite variations, as well as pH alterations (Hanrahan et al., 2006; Neyraud et al., 2013; Nunes et al., 2015). Owing to this possibility of introducing bias several reports have, therefore, suggested stimulants should be used with caution (Beale et al., 2016; Granger et al., 2007; Voegtline & Granger, 2014).

Considering the literature account given above, to our knowledge, comparison between metabolome of USS and SS has not been reported for children saliva and our results suggest that, at earlier ages, the metabolic composition of SS and USS may not be that significantly different. Though our results contrast with published data on adults, caution should be taken when comparing children and adult populations. Some authors have previously pointed out age-dependent variations in saliva composition, mainly related to increase in total protein concentration with age (Hemadi et al., 2017; Pappa et al., 2018), as well as increase of both SS and USS flow rates (Bretz et al., 2001; Torres et al., 2006). This may reflect the impact of developmental alterations related to salivary gland size and/or maturation and hormonal, weight, height changes, from early childhood to adulthood (Ben-Aryeh, Fisher, Szargel, & Laufer, 1990; Hemadi et al., 2017; Pappa et al., 2018), thus perhaps explaining the lack of significant differences between SS and USS in our young-aged cohort. Subsequently, and taking into account the absence of significant discrepancies found in the present work, we selected USS for the following studies, given that it is believed to represent a neutral sample, more reflective of the natural oral environment (Granger et al., 2007).

5.2. Effects of using an absorbing/absorbent device

The suitability of SCS, a saliva absorbing device, for USS collection in young children for ^{1}H-NMR spectroscopy was investigated for the first time, to our knowledge, in a small subset of the abovementioned cohort, comprising four-year old children who donated saliva through PD (n=5) and SCS (n=5).

Comparison between saliva collected through PD and SCS was firstly assessed through a PCA model (Figure 5.3 a)), which showed a clear separation trend between groups. In fact, in relation to PD, SCS collection clearly induced sample separation towards negative PC1 in PCA scores plot (Figure 5.3 a), left). A PLS-DA score scatter plot of the same model was then performed (Figure 5.3 b)) and a distinct separation trend between PD and SCS samples was confirmed (in particular, SCS induced separation towards negative LV1 in PLS-DA, as shown in Figure 5.3 b), right). Indeed, this separation between classes was supported by the Q^2 value of 0.80 (Table 5.1). Model robustness, assessed by MCCV, yielded a CR of 79%, sensitivity 76% and specificity 81%, along with a median Q^2 value of 0.52, reflecting the good predictive power of this model (quality parameters summarized in Table 5.1).

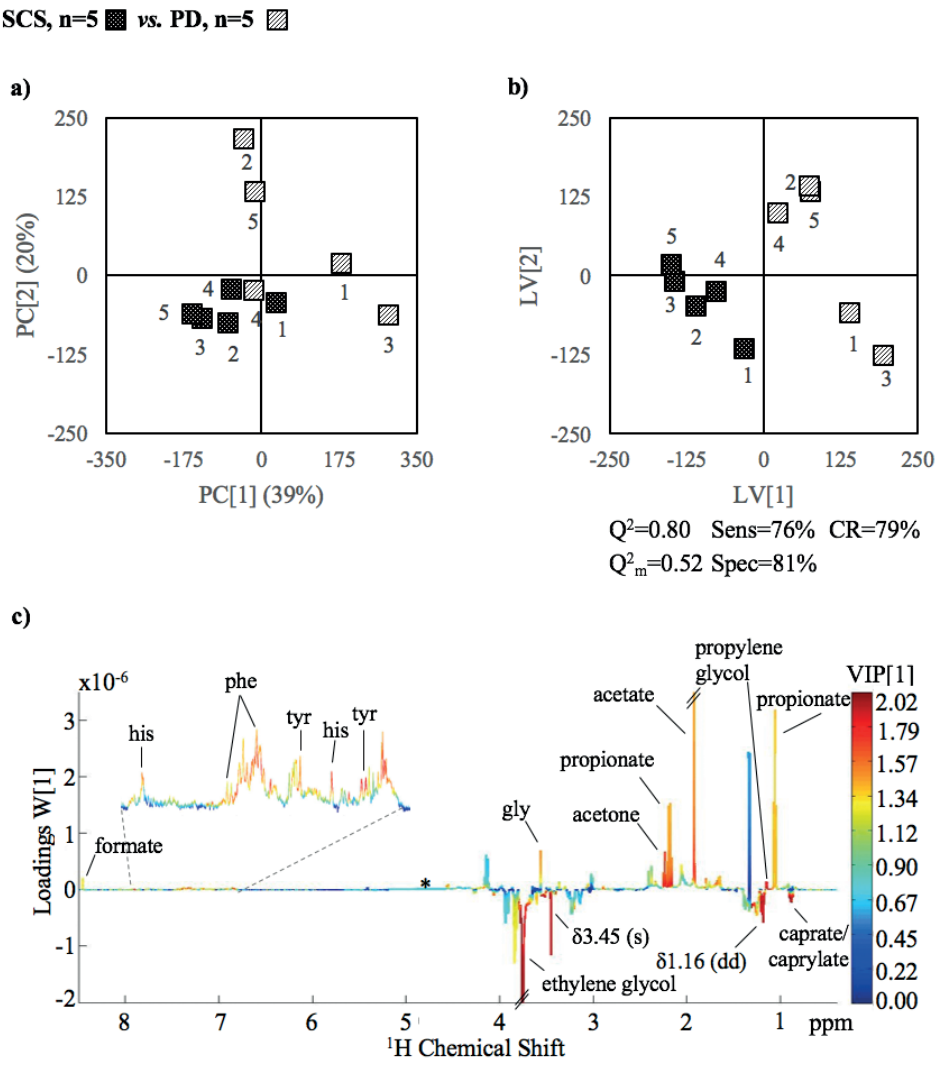

Figure 5.3. a) PCA and b) PLS-DA scores scatter plots of ^{1}H-NMR spectra of USS from children collected through PD (stripes pattern square, n=5) and SCS (Salimetrics, State College, PA, USA) (dots pattern square, n=5). In both scatter plots, each participating child is indicated by a number code. Q^2 value and MCCV results are shown for each model. (c) Loadings plot corresponding to the PLS-DA model shown in (b). The loadings plots are colored according to VIP and some assignments are identified. The three-letter code is used for amino acids; *excluded region (water) prior to MVA.

Inspection of the corresponding loading weights plot (Figure 5.3 c)), which is colored so that it reflects the extent that each NMR peak contributes to class separation in the PLS-DA model, enabled the detection of the compounds varying between groups. Analysis of the red positive peaks in the corresponding LV1 PLS-DA loadings (Figure 5.3 c)) revealed that some metabolites, including propionate, propylene glycol, acetate, acetone, glycine, formate and aromatic amino acids (histidine, phenylalanine and tyrosine), appeared to present decreased levels in SCS-collected saliva. On the other hand, negative red peaks in the loading weights plot suggested SCS samples had increased levels of some unassigned resonances: triplet at 0.89 ppm, doublet of doublets at 1.16 ppm, singlet at 3.45 ppm and singlet at 3.71 ppm. Through comparison of 1D spectra with spectral databases (Bruker Biorefcode and Chenomx, contained in the Chenomix NMR Suite 8.4. software (Chenomx Inc, Alberta, Canada)), the unassigned resonance at 0.89 ppm was suspected to be relative to caprate/caprylate, while the singlet at 3.71 ppm appeared to possibly belong to ethylene glycol. Given the chemical structure of the three suspected compounds (Figure 5.4) and after consulting the chemical shifts and multiplicity of their signals in HMDB database (Wishart et al., 2018), assignment to fatty acids caprate and caprylate was confirmed.

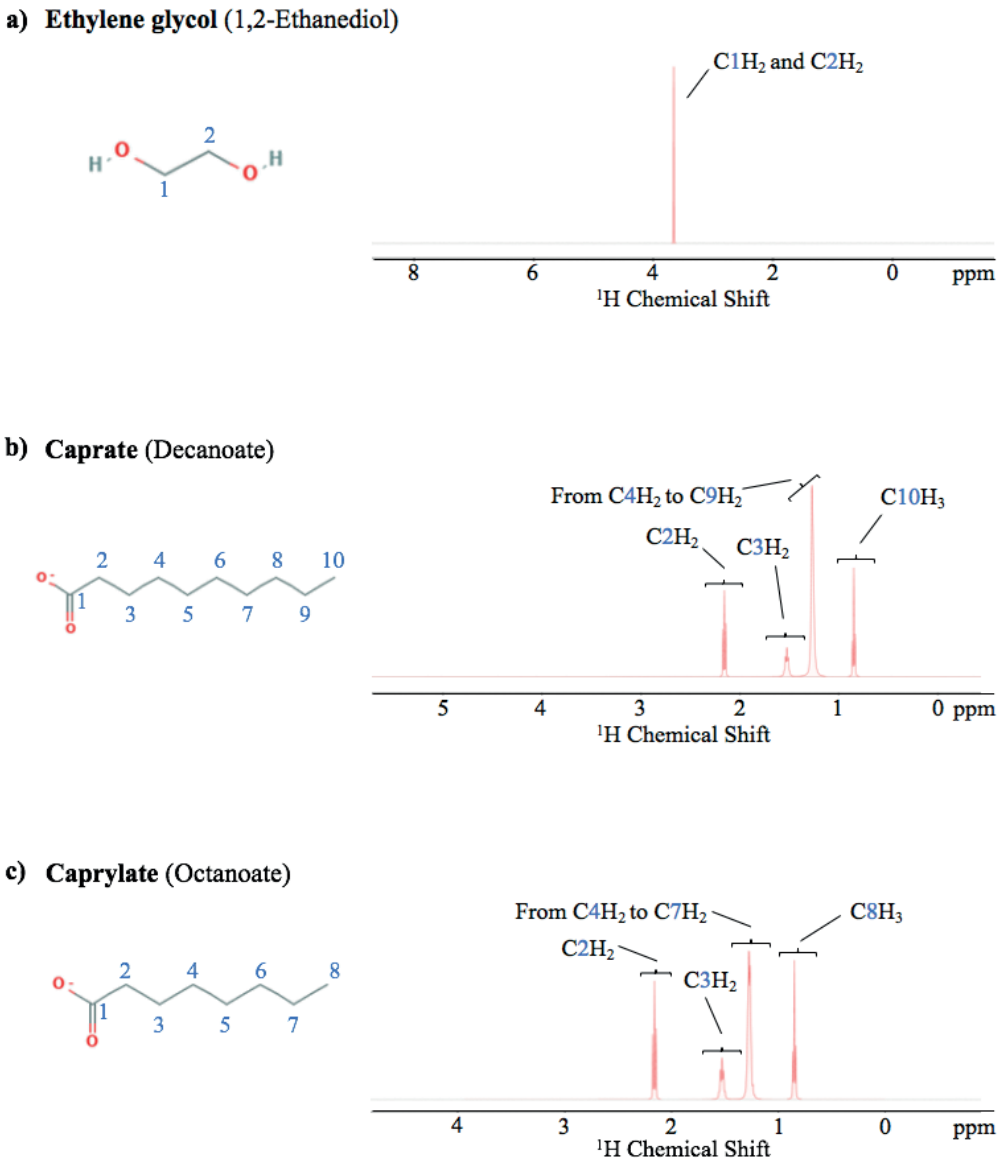

Figure 5.4. Chemical structures and NMR spectra of a) ethylene glycol (1,2-Ethanediol), b) caprate (Decanoate) and c) caprylate (Octanoate). Chemical structures illustrations were adapted from the website and 1D NMR spectra of the compounds are available at http://www.hmdb.ca/.

As shown in Figure 5.4, the chemical structures of caprate (Figure 5.4 b)) and caprylate (Figure 5.4 c)) are similar. Compared to caprylate, caprate presents 2 additional methylene groups (-CH_2) in the carbon chain. However, since these additional protons present the same chemical environment, they are considered equivalent and, therefore, caprate and caprylate exhibit very similar ^{1}H-NMR profiles. In relation to the unknown signal at 3.71 ppm, considering the spectral region in which the resonance was detected (sugar region) is complex due to the overlapping signals with distinct multiplicities, mainly arising from compounds such as sugars and amino acids, the presence of that particular singlet was possibly indicative of the existence of a compound with a simpler molecular structure compared to the previous compound families. After consulting the abovementioned spectral databases and considering the chemical structure of ethylene glycol, we strongly suspected the resonance was in fact attributable to ethylene glycol. Nonetheless, and considering ethylene glycol is not usually detected in human saliva, in an attempt to confirm that assignment, STOCSY was performed for the resonance at 3.71 ppm (Figure 5.5). In this experiment, an NMR spectrum of saliva is colored accordingly to the correlation coefficient of the peak at 3.71 ppm (indicated by the arrow) with the whole spectral region. Highly correlated resonances (r=1, colored in red) comprised signals at 0.89, 1.16, and 5.20 ppm, of which two resonances had already been assigned (0.89 ppm to caprate and caprylate and 5.20 ppm to xylose). Such results revealed the unassigned compound probably shared similarity with the molecules giving rise to highly correlated resonances in terms of atomic structure (reassuring the unassigned compound was indeed composed by carbon, oxygen and hydrogen atoms), and thus the assignment of ethylene glycol was confirmed. Taken these results together, it is clear that considering the mentioned unassigned compounds, along with ethylene glycol and caprate and/or caprylate, were found in increased levels in SCS samples, in comparison to PD samples from the same children, such compounds must have derived from the material the absorbing device is made from.

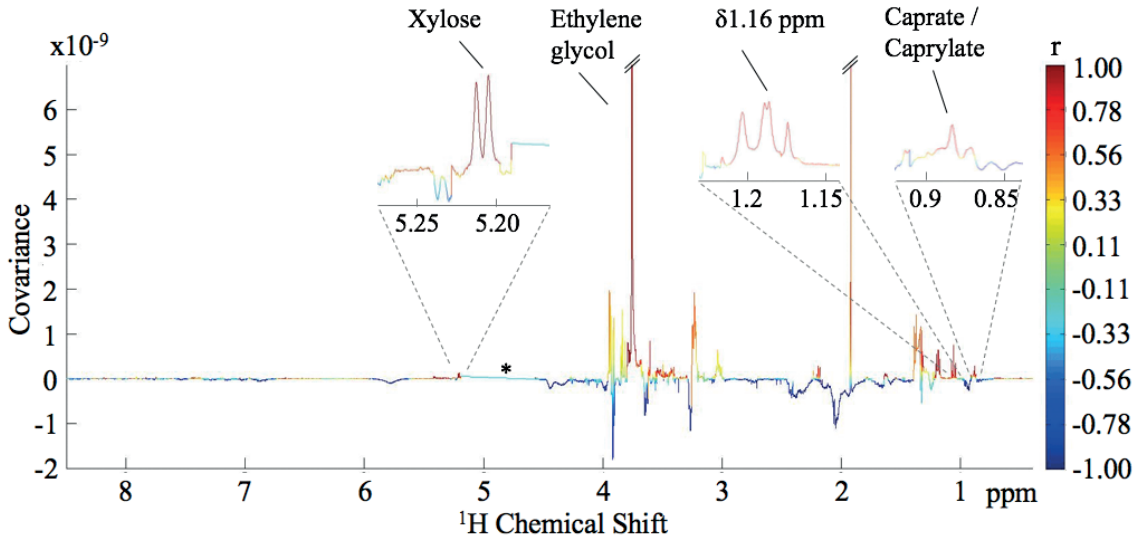

Figure 5.5. 1D STOCSY obtained using the peak at 3.71 ppm (ethylene glycol) as the root peak (see the arrow), with color scale expressing the correlation (r) value and expansions of the 1.16 ppm and 5.20 ppm regions (r=1).

Though analogous attempts to identify the remaining unassigned resonances (at 1.16 and 3.45 ppm) were performed (data not shown), these peaks remained unidentified.

The metabolites found to contribute the most in discrimination between PD and SCS groups in the loading plot, were then further evaluated by spectral integration and tested through univariate comparison for statistical (p-value < 0.05) and biological (effect size) significance. Significant metabolite variations, along with their corresponding effect sizes and p-values), are listed in Table 5.2.

Family	Compound	δ_H/ppm (multiplicity)	ES	*p-value*
Amino Acids	Propionate	2.18 (q)	↑ 2.23 ± 1.58	2.39E-02
Organic Acids	Acetate	1.92 (s)	↑ 1.81 ± 1.47	4.08E-02
Other compounds	Acetone	2.24 (s)	↑ 2.54 ± 1.66	7.94E-03
	Caprate / Caprylate	0.89 (t)	↓ -6.81 ± 3.23	5.29E-06
	Ethylene glycol	3.71 (s)	↓ -6.83 ± 3.24	7.94E-03
Unassigned Compounds	–	1.16 (dd)	↓ -5.44 ± 2.69	9.75E-04
	–	3.45 (s)	↓ -3.74 ± 2.06	8.56E-04

Table 5.2. List of metabolite variations in the saliva collected through PD, compared to saliva collected using SCS, as viewed by NMR, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and p-value. s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. Only variations with p-value < 0.05 are shown. U*i*: unassigned spin system *i*, numbered by chemical shift.

Significant variations included higher amounts of caprate and/or caprylate (p-value 5.3E-6), ethylene glycol (p-value 7.9E-3), and of the two unassigned compounds at δ 1.16 (p-value 9.8E-4) and δ 3.45 (p-value 8.6E-4) in SCS samples, in comparison to PD. Conversely, acetate (p-value 4.1E-2), acetone (p-value 7.9E-3) and propionate (p-value 2.4E-2) were found to be decreased in SCS-collected saliva. The qualitative tendencies for decreased levels of aromatic amino acids, propylene glycol, glycine and formate were not confirmed, since statistical significance was not observed.

Overall, in this study, comparison between PD and SCS groups revealed several important differences in the composition of the samples collected. The increase in ethylene glycol, caprate and/or caprylate and the remaining unassigned compounds in SCS samples most probably originate from the swab manufacturing process, the nature of which was unknown to us. Although we have established contact with Salimetrics, the company that produces SCS, details on its composition were not disclosed. On the other hand, acetate, acetone and propionate were retained in the absorbing SCS material and hence had decreased levels in SCS-collected saliva. These results indicate that, if SCS is to be employed to collect saliva, significant inherent compositional changes in metabolome of saliva are to be expected, probably depending on SCS material and its source.

As reported previously in other research fields, the use of swab devices such as SCS for saliva sampling constitutes an alternative USS collection method with good acceptability among children (O'Farrelly & Hennessy, 2013) rendering, in theory, adequate sample volumes in most participants. To our knowledge, this device had not been used in ^{1}H-NMR-based studies yet and, therefore, we gauged its suitability for these contexts in a subset of this pilot cohort. The importance of such analytical assessments has been referred in literature, attending to the need of assuring new saliva collection devices do not interfere with analytes' concentrations, compromising the validity of the study or the interpretation of its' results (A.-H. M. Emwas et al., 2013; Harmon et al., 2007; Topkas et al., 2012).

Even though comparative researches similar to this pilot study had not been reported yet, the adsorption of salivary metabolites to swabs had been already referred in literature (Chojnowska et al., 2018; Granger et al., 2007; Harmon et al., 2007; Michishige et al., 2006; Nunes & Macedo, 2013; Sugimoto et al., 2013; Voegtline & Granger, 2014). Several studies stated cotton-based materials could bind or retain analytes, such as cortisol (Harmon et al., 2007) or some proteins (Michishige et al., 2006), which were not recovered from the fiber network after compression or centrifugation, leading to false lower results in assays. Additionally, other researchers have reported cotton swabs cause interference in immunoassays of salivary biomarkers, through the release of substances from the cotton (Granger et al., 2007). Despite such limitations concerned cotton swabs and SCS is composed of an unknown polymeric material, our results suggest this device presents similar drawbacks and may not be a reliable collection device for metabolome analysis through NMR. Furthermore, concerns have been expressed in relation to the possibility of swab methods, regardless of device composition, introducing some degree of salivary stimulation, since there is physical contact between the device and the oral cavity of the participant during sampling procedures (Bhattarai, Kim, & Chae, 2018; Michishige et al., 2006; Sugimoto et al., 2013). Another reported limitation of salivary swabs relates to the possibility of not recovering sufficient volume of saliva whenever the amount of available sample is small comparing to the capacity of the absorbing material (Granger et al., 2007; Harmon et al., 2007). Nonetheless, in this cohort, such limitation did not occur and adequate sample volumes for ^{1}H-NMR spectroscopy were obtained from all subjects using SCS.

In sum, in the present chapter, an effort was made to define the most suitable saliva collection protocol for NMR-based studies. Our work has established that children USS does not differ significantly from SS in its metabolome composition, as viewed by NMR, whereas PD collection is recommended as preferable over SCS collection, which seems to cause both release of contaminants and retention of several saliva metabolites. USS collection through PD was, therefore, selected as the preferable method for the subsequent NMR-based studies.

Chapter 6. Pilot study: preliminary evaluation of relative impact of dental caries on saliva metabolome

Chapter 6. Pilot study: preliminary evaluation of relative impact of dental caries on saliva metabolome

In the present chapter the relative impacts of gender, dentition stage and presence of dental caries on the USS metabolome of a pilot cohort of four- to sixteen-year-old children were assessed through ^{1}H-NMR spectroscopy. Firstly, the effects of gender and dentition on the salivary metabolite signature were gauged, as these physiological variables could potentially mask the impact of dental caries on metabolome. Subsequently, after having demonstrated the absence of detectable effects related to gender or dentition stage alone in this pilot cohort, the role played by dental caries in the metabolome of afflicted children was evaluated. The presence of dental caries was found to significantly affect salivary metabolome, inducing differences in the levels of 21 metabolites. The identified metabolomic profile was consistent with protein hydrolysis and deglycosylation and comprised mainly variations in amino acids and monosaccharides, observed for the first time to our knowledge regarding dental caries in children. These results unveiled the appealing possibility of establishing a dental caries metabolic salivary phenotype potentially useful as a biomarker.

6.1. Impact of gender

The convenience sample recruited for the pilot study involved 38 healthy children, as thoroughly described in the Experimental procedures Chapter. Aiming to investigate gender-related effects on the metabolome of USS collected by PD, subgroups of 22 males (primary/mixed/permanent staging: 6/8/8); CA/CF=10/12) and 16 females (staging: 3/8/5; CA/CF=5/11) were considered. PCA was initially performed for gender pairwise comparison, considering the original spectra. However, no separation trends were detected between male and female groups in neither of the PCA scores plots (Figure 6.1 a)).

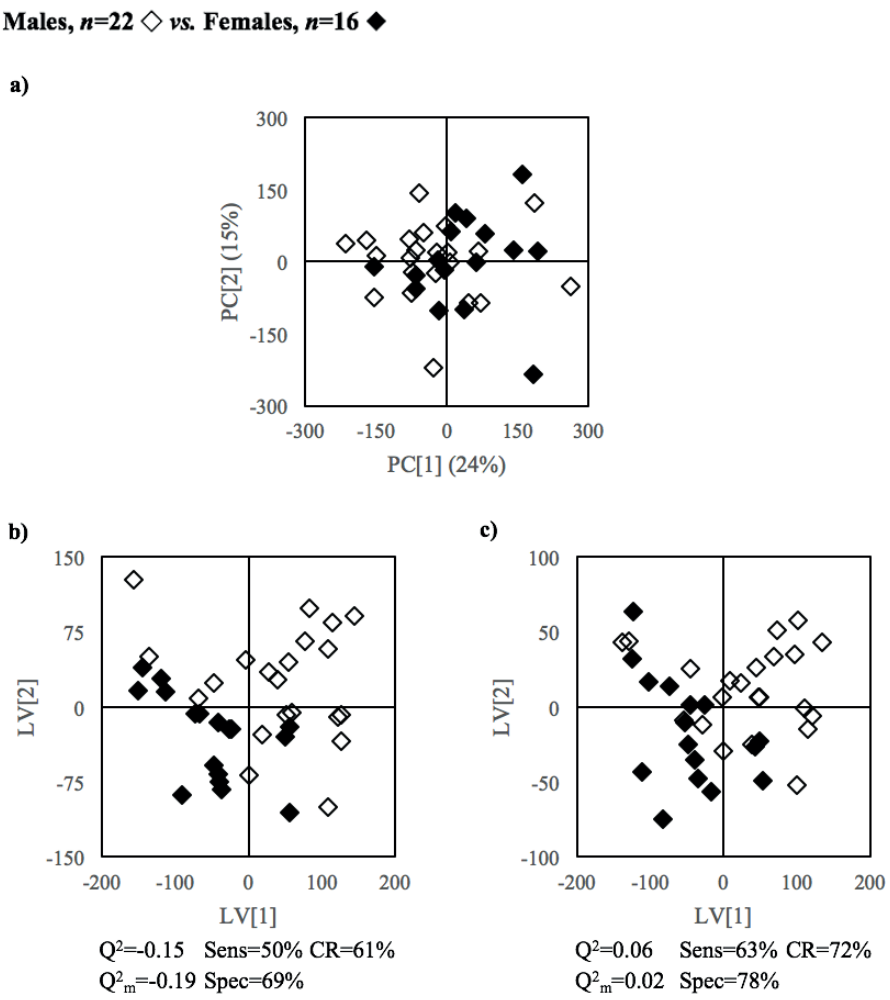

Figure 6.1. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR for gender comparison: males, *n*=22 (◇); females, *n*=16 (◆). Q^2 value and MCCV results are shown for each model.

PLS-DA was then carried out (Figure 6.1 b)) and, although a slight separation between groups was noticed in comparison to the significant dispersion of samples previously seen in PCA models, the model was found to have no robustness, considering the negative Q^2 value of -0.15 and supported by results yielded by MCCV (Table 6.1). In Table 6.1 all of the Q^2 values, MCCV validation results and number of selected variables obtained for each of the PLS-DA models performed throughout this chapter are presented. The PLS-DA model was recalculated upon application of VS methods (Figure 6.1 c)) but still the resulting scores plot did not show evident distinction between groups. Even though an increase in the corresponding Q^2 (0.06) was registered (Table 6.1), the model maintained a poor performance, confirming that no significant variations in metabolite levels were detected between male and female salivary samples in this cohort.

Group		Dataset	% full resolution dataset	R^2X	R^2Y	Q^2	Q^2_m	CR (%)	Sens (%)	Spec (%)
Gender	Males, *n*=22 *vs.* Females, *n*=16	Full resolution	–	0.32	0.46	-0.15	-0.19	61	50	69
		VS	34	0.54	0.42	0.06	0.02	72	63	78
Dentition Stage	Primary + mixed, *n*=25 *vs.* permanent, *n*=13	Full resolution	–	0.19	0.60	-0.21	-0.30	55	20	73
		VS	27	0.46	0.38	0.06	-0.14	57	28	72
	Primary, *n*=9 *vs.* mixed + permanent, *n*=29	Full resolution	–	0.31	0.48	-0.21	-0.37	67	7	86
		VS	31	0.30	0.60	0.12	-0.05	60	25	79
Dental Caries	**CA, *n*=15 *vs.* CF, *n*=23**	Full resolution	–	0.26	0.69	0.14	0.22	74	66	79
		VS	31	0.40	0.65	0.39	0.29	83	94	65

Table 6.1. Q^2 (predictive power) values and MCCV parameters obtained for the gender, dentition stage and dental caries PLS-DA models corresponding to full resolution datasets and datasets obtained after VS. Q^2, predictive power obtained by cross-validation; Q^2_m, median Q^2 obtained by MCCV; CR: classification rate; Sens, sensitivity; Spec, specificity.

Many reports have explored variations in multiple saliva characteristics associated to gender (P. D. Almeida et al., 2008; Dame et al., 2015; Davidopoulou et al., 2012; Dezan et al., 2002; Granger et al., 1999; Hemadi et al., 2017; Jafarzadeh et al., 2010; Netherton, 2004; Pfaffe et al., 2011; Prodan et al., 2015; S. Sun et al., 2014; Tulunoglu et al., 2006; Watanabe et al., 2009; Zaura et al., 2017), as inherent gender-specific differences in gene expression, hormonal metabolism and salivary gland size are expected to impact on gland function (Grimaldi et al., 2018; Li-Hui et al., 2016; Pedersen et al., 2018; Sugimoto et al., 2013). Regarding NMR metabolomics earlier reports, all on adults´ saliva, have registered either no significant changes (Bertram et al., 2009) or possible changes in some metabolites (Takeda et al., 2009). The latter study reported discrimination between male (*n*=20) and female (*n*=20) USS metabolomes to some extent, based on fluctuations in the levels of several organic acids (namely acetate, formate, lactate, propionate, pyruvate and succinate), as well as of taurine, glycine, propylene glycol and methanol (Takeda et al., 2009). In general, the significant variations reported by the authors related to an increase of salivary metabolites in adult males, in comparison to female participants (Takeda et al., 2009). In our pilot study, however, no significant differences were found between the salivary metabolome of the two groups, leading us to postulate that different results may be expected between adult and children cohorts, and/or that, in this children cohort, any possible effects of gender were either absent or of comparable magnitude to the other inter-subject factors (dentition stage, dental caries or other uncontrolled variables such as nutritional habits). In fact, although our convenience cohort presented subgroups comparable in size to the groups compared in Takeda *et al.* research (which were matched for oral health status), our subgroups were not perfectly matched in terms of other variables as dentition stage and oral health status, which may have possibly acted as confounders and masked any existing gender-related effects.

6.2. Impact of dentition stage

After establishing the lack of strong effects in the salivary metabolome due to gender, potential differences associated to dentition stage were assessed, as this cohort presented four- to sixteen-year-old children, thus comprising participants in the three dentition phase stages. In order to explore the influence of this variable, two subsets were defined within the cohort as follows: children in primary and mixed stages were grouped into one same class due to clinical similarity, resulting in 25 subjects at primary/mixed stages (M/F=14/11; CA/CF=10/15) and 13 subjects at the permanent stage (M/F=8/5; CA/CF=5/8).

The PCA initially performed to compare spectra from participants in primary/mixed to those in permanent stage did not reveal any clustering trends, a finding further confirmed through PLS-DA, as shown in the corresponding score plots in Figure 6.2 a) and b). Indeed, the obtained PLS-DA was considered a non-predictive model given its negative Q^2 value and low quality MCCV results (Q^2=-0.21; CR 55%, Sens. 20%, Spec. 73%; Table 6.1).

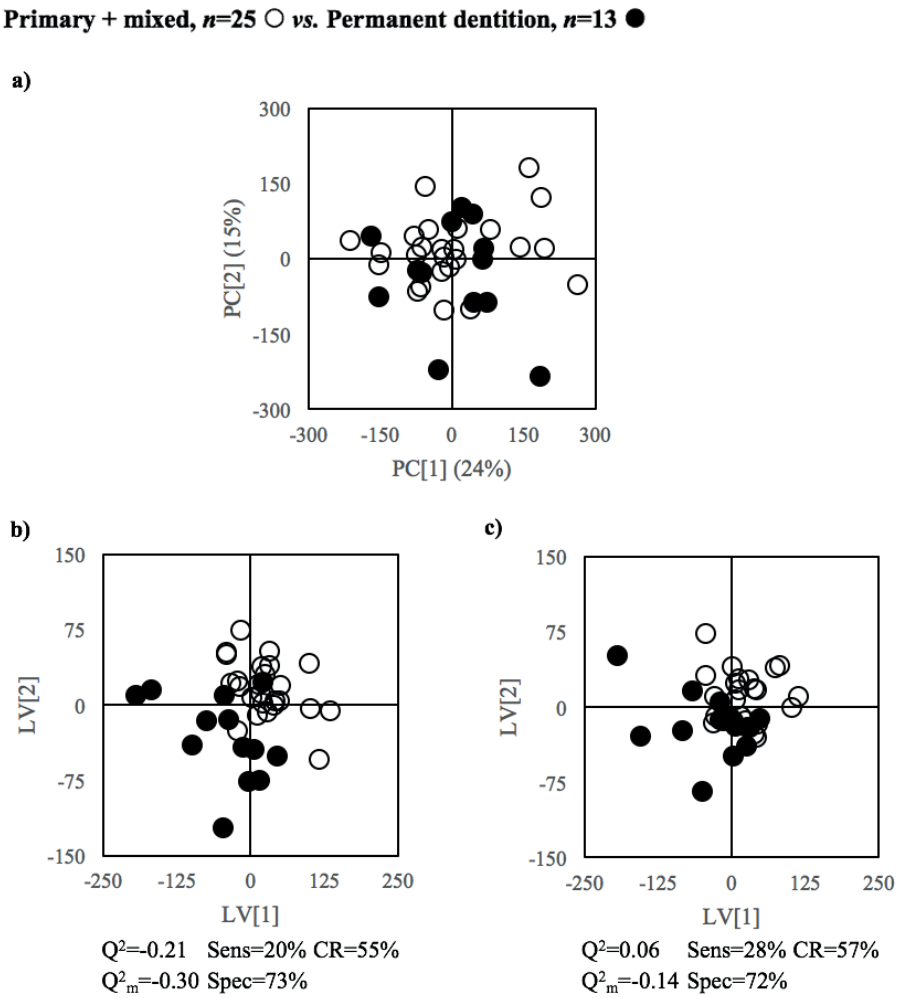

Figure 6.2. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR for dentition stage comparison: primary and mixed phase dentition, *n*=25 (○); permanent dentition, *n*=13 (●). Q^2 value and MCCV results are shown for each model.

When comparing the two subsets after applying VS to the spectra, the subsequent PCA still did not unveil any trend towards group separation (not shown). Similarly, the PLS-DA model performed after VS (Figure 6.2 c)) did not display separation between primary/mixed and permanent stages classes. Despite a slight improvement on the PLS-DA Q^2 value after VS, the model still presented no predictive power (Q^2=0.06) (quality parameters summarized in Table 6.1), indicating that no significant differences were observable between groups.

In an attempt to further confirm such observations, the cohort was regrouped into two different subsets: primary dentition group (*n*=9; M/F=6/3; CA/CF=2/7) and the mixed/permanent dentition stage group (*n*=29; M/F=16/13; CA/CF=13/16), even though these subsets presented more discrepant sample sizes and probably less similarities regarding oral conditions, given that mixed phase dentition most likely resembles to primary than to permanent dentition. MVA methods were re-applied to spectra, however the definition of these new classes did not yield significantly distinct results concerning group separation, as none of the PCA and PLS-DA (Q^2=-0.21 considering full resolution dataset and Q^2=0.12 after VS) models indicated the existence of differences (only the predictive power values and MCCV parameters are shown, in Table 6.1). The fact that no robust separation was observed in none of the performed approaches (that is, through the selection of spectral regions with stronger correlation to class definition with VS or through the definition of different sample classes) indicates that no significant changes in metabolite levels could be detected between groups.

Age-dependent impact on several specific salivary features has been reported by a large number of researchers (Dame et al., 2015; Hassaneen & Maron, 2017; P. Pandey et al., 2015; Pappa et al., 2018) and, recently, MS-based proteomic studies have outlined how the proteome of saliva evolves during infant development, particularly with concentrations of PRPs, histatins, statherin and cystatins increasing from birth to the first months and years of life (Manconi et al., 2013; Messana et al., 2015). Despite this interest, the influence of age on the salivary metabolome remains briefly addressed in the literature to date. Fidalgo *et al.* findings have supported the existence of changes from early childhood to adolescence in USS composition, viewed by NMR spectroscopy, as they have suggested a distinction between stages based on levels of acetate, propionate and saccharides increasing with dentition evolution, from primary to permanent dentition, in CF children (Fidalgo et al., 2013). Authors have reasonably proposed that such disparities may be due to the hormonal and physiological normal development occurring until the pre-pubertal period, together with the progressive alterations in the oral cavity as permanent teeth succeedingly erupt (Fidalgo et al., 2013). Another factor possibly accounting for these findings relates to differences in oral hygiene and dietary habits usually verified among differently aged children. Nonetheless, the comparison between primary/mixed and permanent stages performed in the present pilot study differed from Fidalgo *et al.* results, as such reported changes in the salivary metabolome were absent or of a weak nature in our cohort. The reasons for this discrepancy may be attributable to the fact that our cohort also comprised CA children and/or that staging may possibly impact differently on the salivary composition of different cohorts, perhaps due to nutritional or environmental factors.

6.3. Impact of dental caries

After verifying that no strong isolated effect of the physiological variables gender or dentition stage were observable in saliva spectra this work aimed at determining the impact of dental caries on the salivary metabolome of this children cohort. For this purpose, two subgroups comprising 15 CA (staging: 2/8/5; M/F=10/5) and 23 CF subjects (staging: 7/8/8; M/F=12/11) were defined.

USS spectra of the two subsets were initially compared by PCA and PLS-DA to the full resolution proton spectra. While no evident grouping trend was visualized in the PCA model (Figure 6.3 a)), some degree of separation between CA and CF classes was seen on the PLS-DA scores plot (Figure 6.3 b)). However, in spite of the apparent separation obtained in the PLS-DA model, such trend was not meaningful regarding the existence of differences between sample groups, as given by the Q^2 value (0.14) of this relatively weak model (Table 6.1).

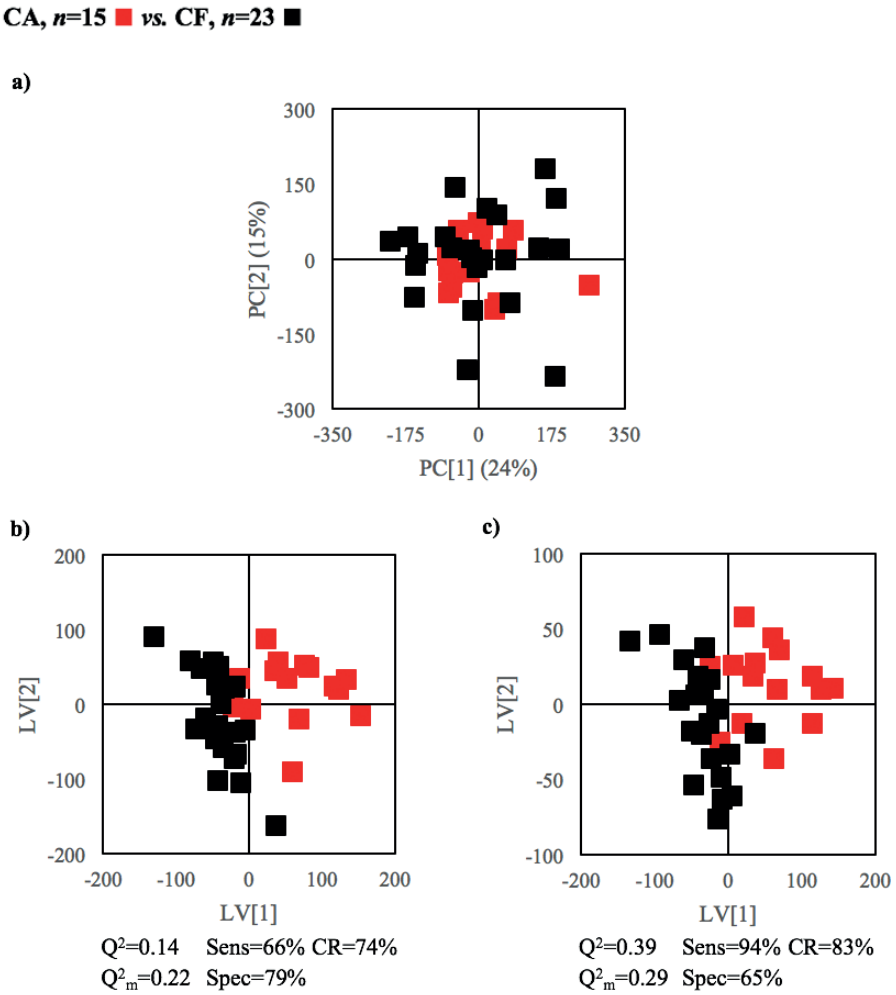

Figure 6.3. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of CA, *n*=15 (■) *vs.* CF children, *n*=23 (■). Q^2 value and MCCV results are shown for each model.

As VS methodology was applied, group separation was visible in the PCA model (data not shown) and the robustness of the PLS-DA model increased significantly (Figure 6.3 c)). Group separation was indeed supported by a satisfactory predictive power of the model (Q^2=0.3-0.4) and corresponding MCCV parameters: high classification rate and sensitivity, 83% and 94%, respectively, and lower specificity, 65% (Table 6.1). While the sensitivity yielded by the MCCV was high, meaning the PLS-DA model is able to correctly classify a subject as CA, its low specificity rate, that is, its ability of accurately classifying a control as disease-free, was lower, translating into a high rate of false positives. The increased PLS-DA predictive power, compared to those obtained for gender and dentition stage, suggested a relatively more important role played by dental caries on saliva metabolome composition. In fact, visual inspection of an average spectra composed by salivary spectra of this cohort's CA children (data not shown) had also suggested several possible changes (namely in several aliphatic amino acids and tyrosine (+), ethanol and lactate (-), uracil and hypoxanthine (+)), however such apparent spectral changes obviously require verification through PLS-DA interpretation and univariate statistical analysis, as described below.

To better interpret the PLS-DA model in Figure 6.3 c), its corresponding loadings plot was analyzed, to identify metabolites contributing the most to the discrimination between the two classes (Figure 6.4).

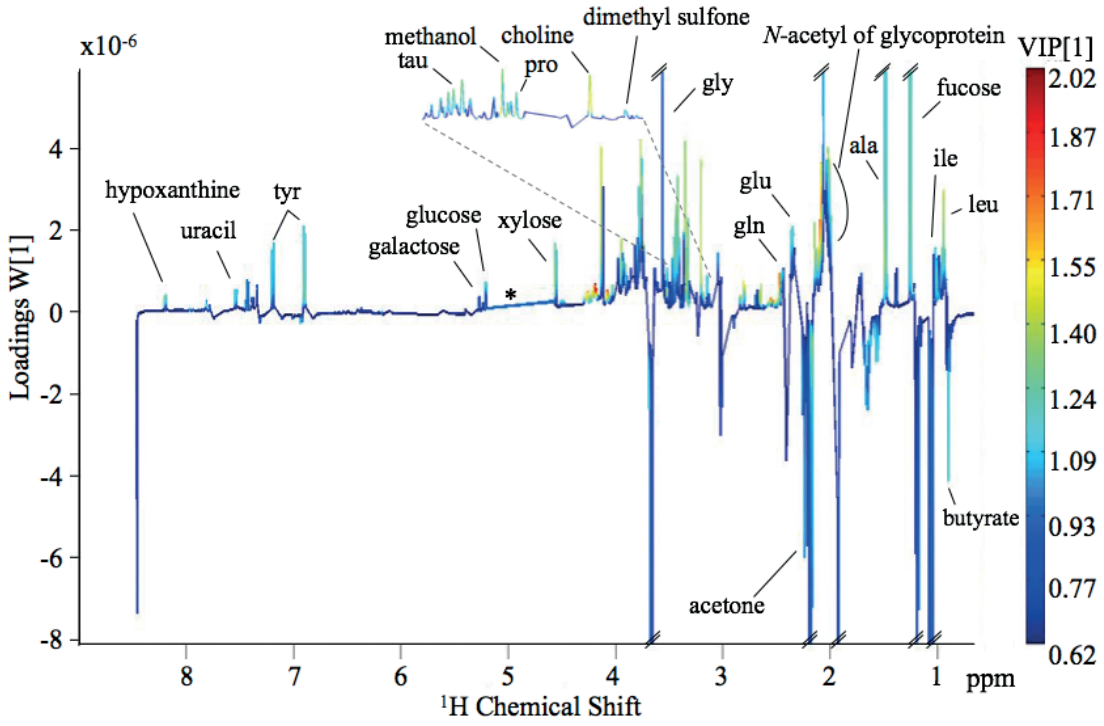

Figure 6.4. Loading plot corresponding to PLS-DA after VS scores scatter plot of CA, *n*=15 *vs.* CF children, *n*=23. The loadings plots are colored according to VIP and some assignments are identified. The three-letter code is used for amino acids; *excluded region (water) prior to MVA.

Brief inspection of the loadings suggested several metabolites, corresponding to the yellow/turquoise positive resonances in the plot, were apparently presenting higher levels in CA samples (which were seen mostly in the positive LV1 of the PLS-DA model in Figure 6.3 c). These metabolites included several amino acids and monosaccharides, as well as free choline, dimethylsulfone, hypoxanthine, methanol, *N*-acetyl groups of glycoproteins and uracil. Conversely, compounds relative to the yellow/turquoise negative peaks in the loadings plot, in particular acetone and butyrate, seemed to be increased in spectra of CF children. All of the inspected apparent variations between CA and CF groups were then assessed through integration of spectra signals and univariate statistical analysis, to gauge the statistical and biological significance of these observations. The set of significant metabolite variations found to characterize CA saliva in comparison to CF saliva is listed in Table 6.2, along with the metabolite's effect sizes and *p*-values.

Family	Compound	δ_H/ppm (multiplicity)	ES	*p-value*
Amino Acids	Alanine [a)]	1.46 (d)	↑ 1.04 ± 0.69	3.28E-03
	Aspartate [a)]	2.83 (d)	↑ 0.73 ± 0.67	2.01E-02
	Glutamine [a)]	2.42-2.48 (m)	↑ 1.21 ± 0.71	1.18E-03 [b)]
	Glycine [a)]	3.56 (s)	↑ 0.74 ± 0.68	2.80E-02
	Isoleucine [a)]	0.94 (t)	↑ 1.12 ± 0.70	2.94E-03
	Leucine [a)]	0.96 (t)	↑ 1.14 ± 0.70	9.00E-03
	Proline [a)]	3.33 (m)	↑ 1.21 ± 0.70	4.05E-03
	Taurine [a)]	3.43 (t)	↑ 1.01 ± 0.69	1.08E-02
	Tyrosine [a)]	7.20 (d)	↑ 0.89 ± 0.69	3.03E-02
Organic Acids	Butyrate [c)]	1.58 (q)	↓ -0.89 ± 0.67	1.88E-03
Carbohydrates and derivates	Fucose (α) [a)]	1.25 (d)	↑ 1.01 ± 0.69	9.88E-03
	Galactose [a)]	5.27 (d)	↑ 0.66 ± 0.67	4.78E-02
	Glucose (α) [a)]	5.23 (d)	↑ 0.84 ± 0.68	5.52E-03
	Xylose [a)]	4.58 (d)	↑ 0.89 ± 0.69	1.42E-02
Other compounds	Acetone [a)]	2.23 (s)	↓ -0.76 ± 0.67	2.19E-02
	Choline [a)]	3.20 (s)	↑ 1.33 ± 0.71	7.43E-03
	Dimethylsulfone [a)]	3.15 (s)	↑ 0.89 ± 0.68	5.52E-03
	Hypoxanthine [a)]	8.19 (s)	↑ 0.66 ± 0.67	4.12E-02
	Methanol [a)]	3.35 (s)	↑ 1.24 ± 0.71	6.73E-03
	N-acetyls [a), d)]	2.06 (s)	↑ 0.79 ± 0.67	1.55E-02
	Uracil [a)]	7.54 (d)	↑ 0.80 ± 0.67	7.43E-03
Unassigned Compounds	U1	0.84 (s)	↓ -0.80 ± 0.68	3.54E-02
	U2	1.66 (m)	↓ -0.77 ± 0.67	1.55E-02
	U3	2.14 (s)	↑ 1.18 ± 0.70	3.65E-03
	U4	2.35 (s)	↑ 0.68 ± 0.67	2.58E-02
	U5	2.64 (s)	↑ 1.31 ± 0.71	1.04E-03 [b)]
	U6	3.51 (s)	↑ 0.80 ± 0.68	4.78E-02
	U7	3.80 (s)	↑ 0.56 ± 0.66	4.05E-03
	U8	4.50 (s)	↑ 0.97 ± 0.69	8.18E-03
Unassigned Spectral Regions	region δ 1.22-1.23		↑ 0.77 ± 0.67	3.28E-02
	region δ 1.50-1.51		↑ 0.86 ± 0.68	3.28E-02
	region δ 2.53-2.55		↑ 0.89 ± 0.68	1.70E-02
	region δ 2.55-2.60		↑ 1.31 ± 0.71	9.22E-04 [b)]
	region δ 3.52-3.54		↑ 0.68 ± 0.67	2.80E-02
	region δ 6.92-6.94		↓ -0.78 ± 0.67	1.42E-02
	region δ 7.82-7.86		↑ 0.69 ± 0.67	3.28E-02

Table 6.2. List of metabolite variations in the saliva of CA children, compared to CF children, as viewed by NMR, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and *p*-value. s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. [a)] Metabolite variation to our knowledge not reported in previous studies on children; [b)] Alterations remaining significant after Bonferroni correction, with cutoff *p*-value 1.39E−03 (0.05 divided by 36 resonances); [c)] Metabolite variation reported before in relation to dental caries, but in the opposite direction (Fidalgo et al., 2015, 2013); [d)] resonance arising from *N*-acetyls in sugars, either free or glycoprotein-linked. Only variations with *p*-value < 0.05 are shown. U*i*: unassigned spin system *i*, numbered by chemical shift.

Bonferroni correction was then used to adjust the obtained *p*-values for multiple comparisons (by dividing the significance cut-off value, 0.05, by the number of hypothesis being tested simultaneously, in this case 36 resonances). Although glutamine (*p*-value 1.18E-03), the unassigned U5 peak (singlet at δ 2.64; *p*-value 1.04E-03) and the 2.55-2.60 ppm region (*p*-value 9.22E-04) were the only variations that remained significant upon Bonferroni correction ([b] in Table 6.2), statistically relevant changes (*p*-value < 0.05) were found for 21 identified metabolites, along with several important still unassigned peaks and regions. Main observations in CA group included changes in a) 9 amino acids (leucine, isoleucine, alanine, aspartate, glutamine, glycine, proline, taurine and tyrosine; (+)), b) butyrate (-), c) several monosaccharides (fucose, xylose, glucose, galactose; (+)) and d) a set of other variations: acetone (-), free choline, dimethylsulfone, hypoxanthine, methanol, N-acetyl groups of glycoproteins and uracil (+ for all). The most significant variations (*p*-value < 0.01) are shown in boxplot form for identified metabolites (Figure 6.5) and for unassigned resonances (Figure 6.6). Some of the above-mentioned apparent changes suggested merely by visual inspection of the average spectra, namely decreases of ethanol, lactate and formate, were found not to be statistically relevant.

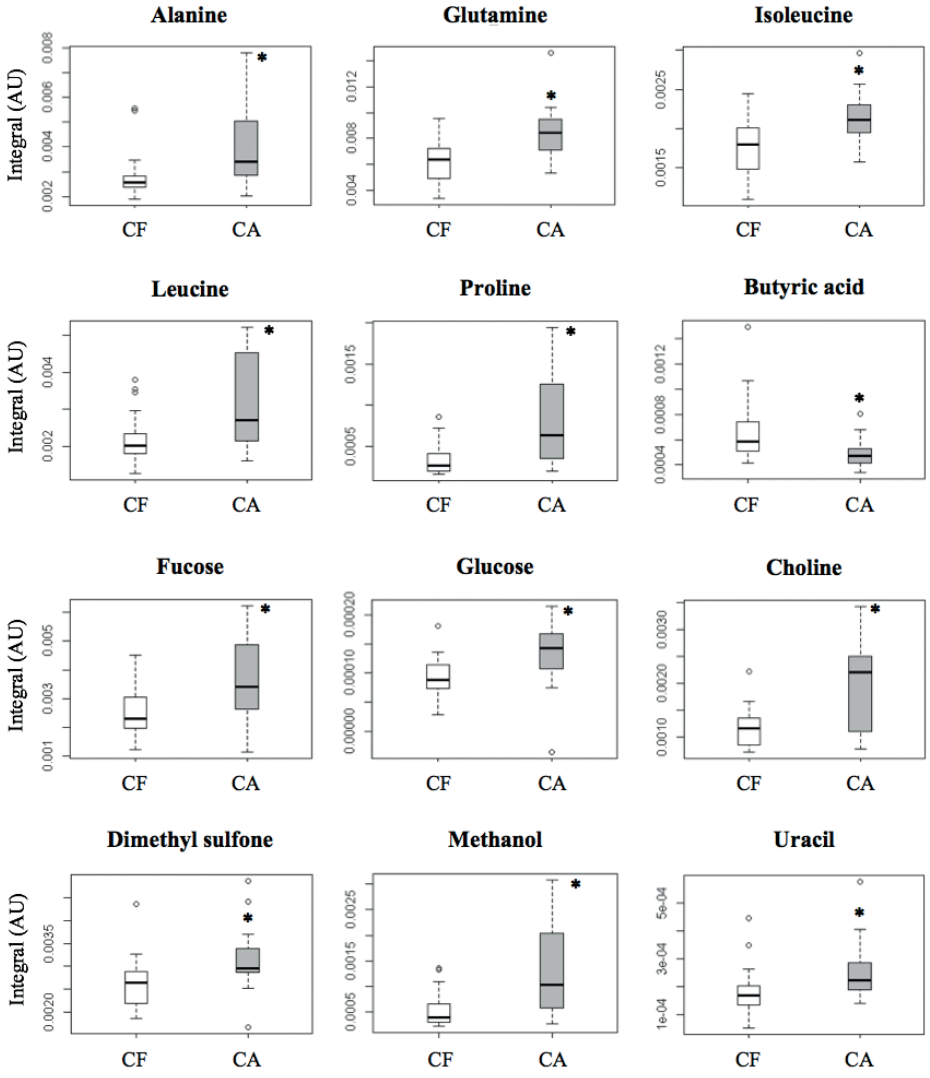

Figure 6.5. Boxplot representations of the main metabolite variations found statistically relevant, with * *p*-value < 0.01, in CA subjects, compared to CF children. Glutamine variation remained significant upon Bonferroni correction.

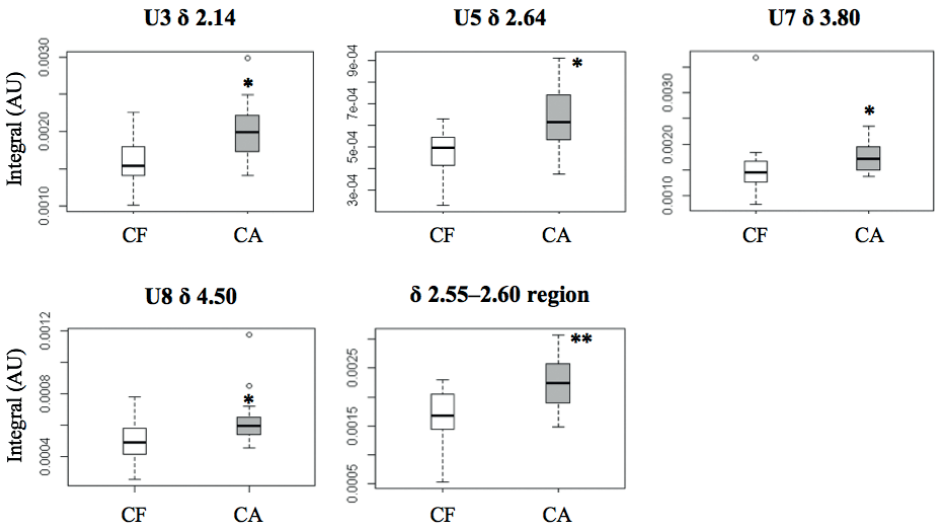

Figure 6.6. Boxplot representations of the unassigned compound variations found statistically relevant in CA subjects, compared to CF children. * indicates *p*-value < 0.01; ** indicates *p*-value < 0.001. U5 and 2.55-2.60 ppm region remained significant upon Bonferroni correction.

In this cohort, significant metabolite variations were identified as characterizing CA saliva, compared to CF saliva. A remarkable result emerging from this data is that, taking into account the overall findings of this pilot study, the effect of dental caries on saliva metabolome was observed to be stronger compared to those of gender and dentition stage. A set of metabolites and resonances were put forward as a salivary metabolic signature of children dental caries, comprising 21 identified metabolites and important contributions from still unidentified compounds and spectral regions of overlapped signals. One of the main observations was a clear increase in 9 amino acids in the salivary metabolome of CA children (Table 6.2). Our study did not confirm earlier reports in relation to histidine, previously seen to increase in ECC (histidine-rich peptides) (Ao et al., 2017; Hemadi et al., 2017; X. Sun et al., 2016), or to phenylalanine levels, which had been found to decrease in CA children in Fidalgo *et al.* study (Fidalgo et al., 2013). Nonetheless, the higher salivary levels of proline found associated to the presence of dental caries in this study are in agreement with earlier findings correlating the elevated concentrations of this amino acid with the risk of children experiencing ECC (Fonteles et al., 2009). As proposed before, it is possible that higher amounts of free proline in saliva originate from the degradation of salivary PRPs (Fonteles et al., 2009; Neyraud et al., 2013), major salivary glycoproteins composed by repeated sequences of proline, glutamine, lysine and glycine, the extended chain conformation of which is due to proline rich sequences (Levine, 2011; Neyraud et al., 2013). Cumulative evidence has reported that elevated levels of PRPs may be significantly associated to increased ECC incidence (Bhalla et al., 2010; T. R. Ribeiro et al., 2013) and the potential use of these proteins as ECC predictors has been hypothetically suggested (Hemadi et al., 2017). High degradation of PRPs would thereby also explain the elevated glutamine and glycine concentrations found in our CA cohort, though no significant variations regarding lysine were detected. On the other hand, a recent study has identified glutamine and glutamate, increased in the saliva of our CA group and usually present in dental plaque, as the main source of ammonia production, thus contributing to pH homeostasis (Washio et al., 2016). The role of amino acid metabolism in pH regulation has not, however, been completely clarified (Nascimento et al., 2017; Washio et al., 2016) and further investigations should be undertaken in order to accurately interpret such results. Such a

marked increase in free amino acids in dental caries subjects is, to our knowledge, here reported for the first time in relation to dental caries, and may be indicative of protein hydrolysis as a reflection of bacterial activity (Dame et al., 2015; Fonteles et al., 2009; Neyraud et al., 2013). Indeed, our results are in agreement with previous research on periodontal disease, which revealed that elevated amounts of dipeptides and free amino acids in saliva may originate from bacteria with proteolytic activity, as a result of tissue degradation and the interaction between host and oral flora (V M Barnes et al., 2011; Virginia M. Barnes et al., 2010; Liebsch et al., 2019; Mikkonen et al., 2016). In addition, bacterial degradation of collagen from dental matrix has also been shown to occur in dental caries lesions in dentin and this process could possibly explain, at least partially, the observed increased content of amino acids in saliva (Aimetti et al., 2012). These interpretations of our findings should be, however, considered cautiously, as bacteria with proteolytic capacity produce organic acids, for instance acetate, proprionate, butyrate and succinate, as metabolic byproducts (Aimetti et al., 2012), and these metabolites were not found in higher concentrations in our cohort.

As far as organic acids are concerned, an unexpected decrease in butyrate salivary levels was the only statistically relevant change noted in this pilot study (Table 6.2). Interestingly, this metabolite has been reported to increase in the saliva of CA children, possibly indicating a higher colonization of micro-organisms with consequent bacterial fermentative processes (Fidalgo et al., 2015, 2013). The fact that it is here seen to vary in the opposite direction may, again, mean that butyrate levels perhaps reflect the contribution of other variables (besides gender or dentition stage, since these were found not to give rise to significant butyrate changes), thus indicating that its levels should be interpreted with care. Other reports have noted higher levels of lactate (Fidalgo et al., 2013), acetate and fatty acids (Fidalgo et al., 2015, 2013), as well as lower propionate levels (Fidalgo et al., 2013) in CA children, variations not observed in our cohort. In fact, in contrast to such previous results, only weak decreasing tendencies without statistical significance were noted here for lactate and formate. In general, organic acids have been acknowledged to present a role in dental demineralization, as they decrease pH levels and are considered an evident reflection of diet or oral microflora activity, namely carbohydrate metabolism (Aimetti et al., 2012; Dame et al., 2015; Fidalgo et al., 2013; C. J. L. Silwood et al., 2002; C. J. Silwood et al., 1999). Accordingly, such aspects may need to be closely controlled or monitored for a more definite interpretation of organic acids profile associated to dental caries in children to be achieved. Also, the fact that a recent study has proposed that lactate may not only be originated by food fermentation in oral cavity, but also constitute a host-derived metabolite arising in saliva from plasma, demonstrates the difficulty in interpreting collected data in this context (Gardner et al., 2019).

To the best of our knowledge marked increases in fucose, galactose, glucose and xylose (Table 6.2) found in our CA cohort have not been specifically reported before. Actually, these findings differ significantly from those of Fidalgo *et al.,* which identified a decreasing tendency in saccharides in the saliva of CA in comparison to CF children (Fidalgo et al., 2013). Nonetheless, in the subsequent longitudinal work by the same research group, authors reported decrease on the salivary concentration of saccharides upon dental treatment (Fidalgo et al., 2015), which apparently contradicts the earlier suggestion. This has led the authors to suggest that ingested carbohydrates and/or different subjects´ oral habits may possibly contribute to the measured carbohydrate levels. While low levels of saccharides in saliva of children with dental caries might be considered as a consequence of the use of these metabolites as a substrate in bacterial energetic metabolism, higher saccharide concentrations in CA children could relate to the possibility that irregularities presented in dental surfaces with dental caries lesions boost plaque retention and delay oral clearance of metabolites from food ingestion, maintaining saccharides in lesions for longer periods. A possible explanation for our specific observations is consistent with an

early report that bacterial enzymes promote hydrolysis of salivary glycoprotein linkages to their carbohydrate moieties, as shown by a noted increase in fucose (an important constituent of glycoproteins (Mikkonen et al., 2018)), in adults with periodontal disease (Shetty & Pattabiraman, 2004) . It is noted that some of the released sugars may be *N*-acetylated, thus contributing to the peak δ 2.06 (Figure 6.4), usually assigned to *N*-acetylated sugars linked to glycoproteins. Therefore, the apparent increase in glycoproteins based on that peak (Table 6.2) may include a contribution from increased levels of free *N*-acetylated sugars. Indeed, although the presence of some glycoproteins (PRPs) has been associated to dental caries, preliminary results reported in the literature have suggested a negative correlation should be expected between salivary mucins (also glycoproteins) levels and ECC (Hemadi et al., 2017), thus possibly supporting the hypothesis that the increase δ 2.06 peak may reflect, at least in part, an increase of free *N*-acetylated sugars, rather than just of glycoproteins. The relative contributions of free and protein-bound *N*-acetylated sugars is, therefore, an interesting subject for further investigation in the context of the effect of dental caries on saliva composition.

Regarding other metabolite changes, putative interpretations of the variations in acetone (-), choline, methanol and uracil (+) levels (Table 6.2) are difficult to advance at this stage. However, it may be hypothesized that the higher M/F ratio characterizing our CA group could be presumably contribute for the increased methanol and glycine concentrations detected in those subjects, considering higher levels of both metabolites have been previously found in male saliva of a NMR study involving an adult cohort (Takeda et al., 2009). As for dimethylsulfone (+) levels (Table 6.2), literature has shown it may originate from dietary sources or bacterial metabolism, as noted previously in relation to periodontal disease (Figueira et al., 2016). Still in relation to the same disease, increased hypoxanthine levels have been demonstrated too (V M Barnes et al., 2011), suggesting accelerated purine degradation from oxidative stress and inflammation inherent to the physiopathology of the condition. One likely explanation for this result is that a similar process may occur in dental caries process as a possible consequence of inflammatory response, leading to higher hypoxanthine levels and possibly to increased uracil, although there is no evidence, as far as we are aware, that pyrimidine pathways may also be affected.

Despite the fact that this pilot study's framework constituted a starting point towards the development of further research targeting the assessment and validation of a metabolic signature of ECC, this work entails some shortcomings which need to be addressed. To begin with, in general, our group sizes were relatively small, as preliminary power calculations indicated that about 20-30 samples per group should had been included to enable the full set of comparisons to be performed (namely the effect of salivary stimulation in Chapter 5 ('*Optimization of saliva collection protocol for metabolomics*') and the impact of physiological conditions and dental caries in the present chapter). Nonetheless, the sample sizes used (roughly approximating the calculated sample size) were those realistically available within our convenience sample and, in fact, these group sizes were comparable to previous NMR reports (Fidalgo et al., 2015, 2013). As this small cohort comprised four- to sixteen-year old children attending a pediatric dentistry clinic, groups used to gauge the magnitude of each variable were not perfectly matched either, thus it is plausible that this limitation also possibly influenced the obtained results. The alternative use of matched groups in subsequent work involving larger cohorts, as described in the following chapter, might disclose new metabolic information for detection and classification of ECC, which would however be expected to include any effects observed in the present study. In order to prevent additional confounding factors from contributing significantly to the resulting data, children presenting systemic diseases, under medication that could affect saliva secretion or who had received antibiotics or used antiseptic mouthwash/toothpaste in the preceding two weeks were excluded from this study. Furthermore, signs of marked gingivitis or periodontal disease were considered

in the exclusion criteria, not only to avoid the increased risk of blood contamination on salivary samples, but also to exclude the possibility of effects related with periodontal conditions being detected on the salivary metabolome of the cohort. Children wearing orthodontic devices or presenting crying or restless behavior during procedures were also excluded, due to the potential influence of these factors in saliva secretion rate and composition. However, despite all of these efforts to minimize the occurrence of bias, another possible source of error relates to the fact that this convenience cohort was composed of healthy children who attended a pediatric dentistry clinic seeking for regular dental care. Inevitably, it is possible that the same concern with oral health also translated into differences in the oral hygiene and dietary habits of the cohort, in comparison to population it aimed to represent. Several other efforts were carried out to standardize procedures and minimize the effect of confounding variation in the research. All salivary samples were collected in the midmorning period before any dental treatment was performed, only in patients who had not ingested food/drinks or used oral hygiene products within the 120 minutes prior to evaluation. As stated in the Experimental procedures Chapter, even though this pilot study was conducted in the settings of a pediatric dentistry office, oral conditions diagnosis was performed in each subject without using any dental chair equipment nor radiographic examination, in an attempt to reproduce the conditions that would be found in preschools in the main study. Finally, regarding the study´s NMR-based methodology, it should be underlined that the several important unassigned peaks and regions found varying significantly along with the 21 identified metabolites, were not possible to assign in spite of all of our efforts, a usual limitation of untargeted metabolomic studies (either NMR- or MS-based).

In sum, evidence from this pilot study suggests that dental caries seems to affect saliva metabolome more strongly than gender or dentition stage, the latter corresponding to physiological variables often believed to be confounding and rather challenging to control or match within the design of this type of research. The CA metabolic signature found here is consistent with pronounced protein hydrolysis and sugar release from glycoproteins. Organic acids and sugars levels seem to be somewhat inconsistent between different studies, perhaps reflecting their dependence on dietary/oral hygiene habits, whereas hypoxanthine and dimethylsulfone may reflect inflammation and bacterial growth. Most of these observations are novel to our knowledge and, together with important unassigned peaks in the NMR spectra, compose a putative saliva metabolic signature of childhood dental caries, which may become useful as a biomarker.

Chapter 7. Observational study: impact of ECC on saliva metabolome in a large cohort

Chapter 7. Observational study: impact of ECC on saliva metabolome in a large cohort

This chapter outlines the metabolomic NMR study of USS collected through PD from a large five-year-old cohort, aspiring to disclose the effects of ECC on children's salivary metabolome. In the first two sections of this chapter, the isolated impacts of gender and dentition on the cohort's metabolomic profile were assessed. After excluding the presence of significant variance due to those physiological features on this cohort, a salivary metabolic signature of ECC was sought. After performing multiple comparisons between samples from ECC-afflicted children and those from healthy controls, alanine levels were found to vary significantly between the two groups, whereas none of the variations detected in the previous pilot study could be confirmed, at this point, in this larger cohort. The possible reasons for these observations are discussed.

Although the design of this observational study involved data collection at two different time points (initially and after six months), in the present thesis only the 1H-NMR results corresponding to USS samples collected in the first evaluation are presented, since the six-month evaluation samples are still subject of our ongoing work (publication in preparation). Eventually comparison between first and second collection cohorts may enable further studies of a longitudinal nature which may disclose interesting new ECC metabolic signatures, as will be discussed later. Out of the 143 participants who were present at baseline evaluation, only 122 adequate USS samples were collected, as 4 children were not able to provide sufficient amount of saliva, 1 participant refused to donate saliva and 16 spectra presented significant line distortion in the water region (probably due to difficulty in suppressing the water peak adequately), which thus led to their exclusion from further statistical analysis. The detailed description of sample characteristics and diagnostic criteria can be found in section 2.1.2 of Chapter 2 ('*Experimental procedures*').

7.1. Impact of gender

To investigate the source of variability associated to gender in the salivary metabolome of this cohort in the baseline evaluation, MVA was performed to compare the ^{1}H-NMR spectra of male (n=62) and female (n=60) subjects, irrespective of ECC presence or dentition stage. PCA considering the total spectra of the two groups revealed no separation trends and the corresponding PLS-DA model (scores plot not shown) had poor performance, as given by the Q^2 of -0.13 shown in Table 7.1. In Table 7.1 all of the Q^2 values and number of selected variables obtained for each of the PLS-DA models carried out within the scope of this chapter are presented. PCA and PLS-DA were then carried out considering the aliphatic (0.4-3.1 ppm) and aromatic (5.8-8.5 ppm) regions separately, as this methodology enables specific spectral regions to be individually assessed in order to evaluate whether a particular region indeed contributes significantly to differences among samples, without the presence of potential noisy variables originating in the remaining spectral regions. However, poor results were still obtained in the PLS-DA models considering the aliphatic (Q^2=0.00) and the aromatic (Q^2=-0.06) regions separately (data not shown), suggesting that, indeed, no strong gender-differences could be detected in the spectral profiles of saliva of the full cohort. This means that gender alone impacts less or at a comparable level to other variables affecting the cohort, namely ECC and dentition stage.

Group	Dataset	% full resolution dataset	R^2X	R^2Y	Q^2
Gender: Males, *n*=62 *vs.* Females, *n*=60	Full resolution	–	0.17	0.40	-0.13
	VS				
Gender in controls: Males, *n*=39 *vs.* Females, *n*=38	Full resolution	–	0.24	0.41	-0.03
	VS	33	0.40	0.40	0.17
Dentition stage in controls: Primary dentition, *n*=46 *vs.* mixed phase, *n*=31	Full resolution	–	0.23	0.42	-0.01
	VS	16	0.38	0.33	0.08
ECC impact: Controls, *n*=77 *vs.* ECC-afflicted, *n*=45	Full resolution	–	0.21	0.29	-0.09
	VS	26	0.20	0.37	0.11
After outlier exclusion: Controls, *n*=76 *vs.* ECC-afflicted, *n*=45	Full resolution	–	0.20	0.32	-0.12
	VS	21	0.17	0.46	0.19
Controls (*n*=77) *vs.* ECC-dentin (*n*=28)	Full resolution	–	0.13	0.41	-0.21
	VS	28	0.20	0.41	0.11
ECC severity: d_{A-6}mft ≤5 (*n*=114) *vs.* d_{A-6}mft ≥6 (*n*=8)	Full resolution	–	0.19	0.30	-0.15
	VS	26	0.24	0.41	0.05
ECC severity: 1< d_{A-6}mft ≤5 (*n*=37) *vs.* d_{A-6}mft ≥6 (*n*=8)	Full resolution	–	0.23	0.51	-0.21
	VS	29	0.33	0.60	0.24
Number of ECC lesions: d value=0 (*n*=83) *vs.* d value ≥1 (n=39)	Full resolution	–	0.17	0.32	-0.19
	VS	22	0.22	0.40	0.22
Number of ECC lesions: d value≤5 (*n*=116) *vs.* d value ≥6 (*n*=6)	Full resolution	–	0.17	0.32	-0.19
	VS	28	0.25	0.36	-0.05
Number of ECC lesions: 1< d value ≤5 (*n*=33) *vs.* d value ≥6 (*n*=6)	Full resolution	–	0.24	0.57	-0.06
	VS	30	0.30	0.69	0.33
Disease prediction: Children who remained controls, *n*=49 *vs.* Children who developed ECC, *n*=5	Full resolution	–	0.21	0.51	-0.07
	VS	20	0.33	0.53	0.25

Table 7.1. Q^2 (predictive power) values obtained for the gender, dentition stage, ECC impact, ECC severity, number of ECC lesions and disease prediction PLS-DA models corresponding to full resolution datasets and datasets obtained after VS. Q^2, cumulative predictive power obtained by cross-validation; Q^2_m, median Q2 obtained by MCCV; CR, classification rate; Sens, sensitivity; Spec, specificity; d_{A-6}mft, decayed, missing, or filled teeth score; d value, number of teeth with untreated carious lesions per child. Model robustness and predictive power was assessed by MCCV only for the disease prediction model after VS: Q^2_m 0.25, CR 91%, Sens 0%, Spec 100%.

In addition, group matching was carried out aiming at excluding the potential effect of ECC. Accordingly, the impact of gender on the salivary metabolome was assessed through MVA (PCA and PLS-DA) only considering control samples. The PCA score plot comparing spectra of male (*n*=39) and female controls (*n*=38) still did not show any discrimination between groups (Figure 7.1 a)), whereas PLS-DA revealed an apparent slight separation trend (Figure 7.1 b)) in comparison to the model previously performed including the whole cohort. However, the lack of robustness of such PLS-DA model was still clearly expressed by its negative Q^2 value (-0.03). PCA and PLS-DA models were then developed upon VS (Figure 7.1 c) and d), respectively), to filter out possible random variability not related to sample groups, but still no model showed the existence of significant variances between males and females' samples, despite the subtle improvement verified in the PLS-DA (Q^2 =0.17; Table 7.1).

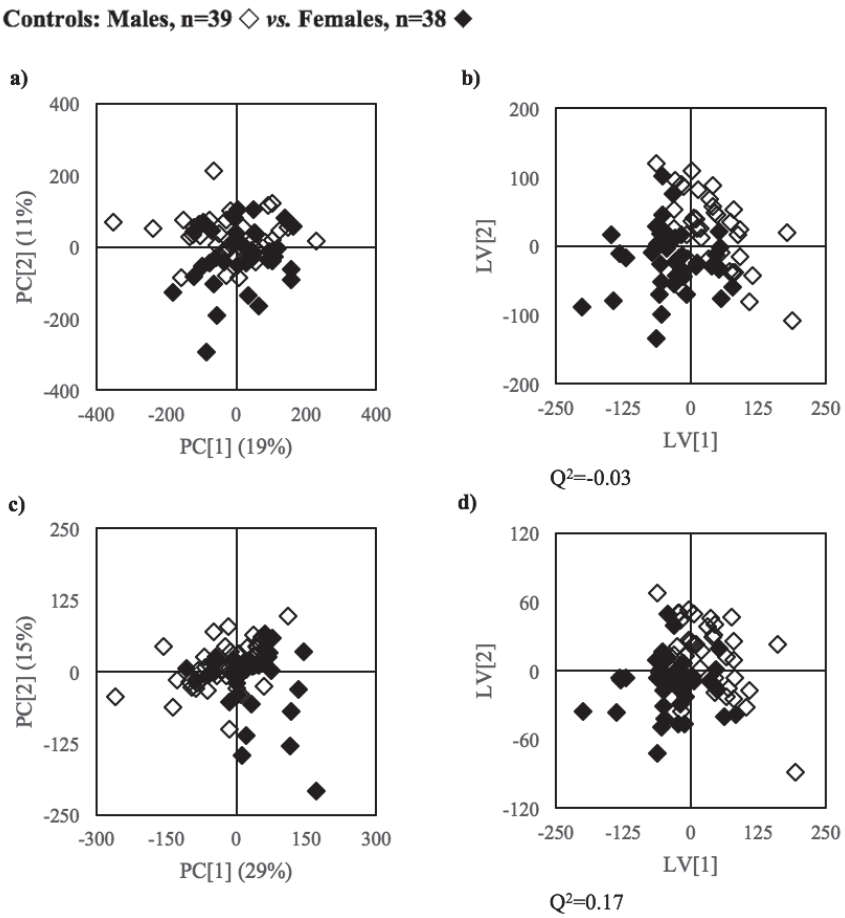

Figure 7.1. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR for gender comparison in controls: males, *n*=39 (◇); females, *n*=38 (◆). Q^2 value is shown for each model.

Again, the comparison between male and female controls was further performed considering only specific spectral regions, nonetheless that strategy did not improve significantly the resulting PLS-DA models of aliphatic (Q^2=0.01) and aromatic (Q^2=0.11) regions (data not shown). From these results, it was evident that no meaningful differences could be detected by ^{1}H-NMR spectroscopy in the saliva of male and female children who composed this cohort in the baseline evaluation moment.

The present findings corroborate our pilot study results, in which gender-related effects were, either absent, or presented similar magnitude to other inter-individual variables. In addition to possible unknown factors impacting on the metabolome (including dietary or oral hygiene habits, for instance), in the pilot study specific known variables (dentition stage and presence of dental caries) were known to vary among the unmatched groups used for gender comparison. Indeed, the fact that the previous study comprised a small cohort (*n*=22 males and *n*=16 females) covering a wide age range prevented us from defining perfectly matched subsets. The larger cohort composing the main study (*n*=62 male and *n*=60 female five-year old subjects) allowed the definition of appropriately sized subgroups matched in terms of ECC absence (*n*=39 male and *n*=38 female controls). The lack of significant variations in metabolite levels between male and female salivary samples is, therefore, a remarkable result emerging from this main study data, particularly considering that our matched cohort was nearly twice the size of the adult cohort participating in Takeda *et al.* research, to our knowledge the only study which reported gender-related variations in the USS metabolome (Takeda et al., 2009). Though it is conceivable that the absence of differences results from the possibility that gender may not impact significantly on salivary metabolome at such early ages, our findings are, nonetheless, consistent with another previous study in adults which showed gender had no detectable effect on the salivary metabolite profile (Bertram et al., 2009).

7.2. Impact of dentition stage

With the purpose of verifying the potential influence of dentition stage on the salivary composition, two subgroups comprising controls in primary dentition (*n*=46) and in mixed phase (*n*=31) were defined within the cohort. PCA and PLS-DA models were initially built for the whole spectra (0.4-8.5 ppm). Figure 7.2 a) and b) shows the scores scatter plots obtained for the NMR data, evidencing no clear distinction between classes could be visually detected. Indeed, the PLS-DA Q^2 of -0.01 (Table 7.1) substantiated the absence of differences among samples expressed by the plots.

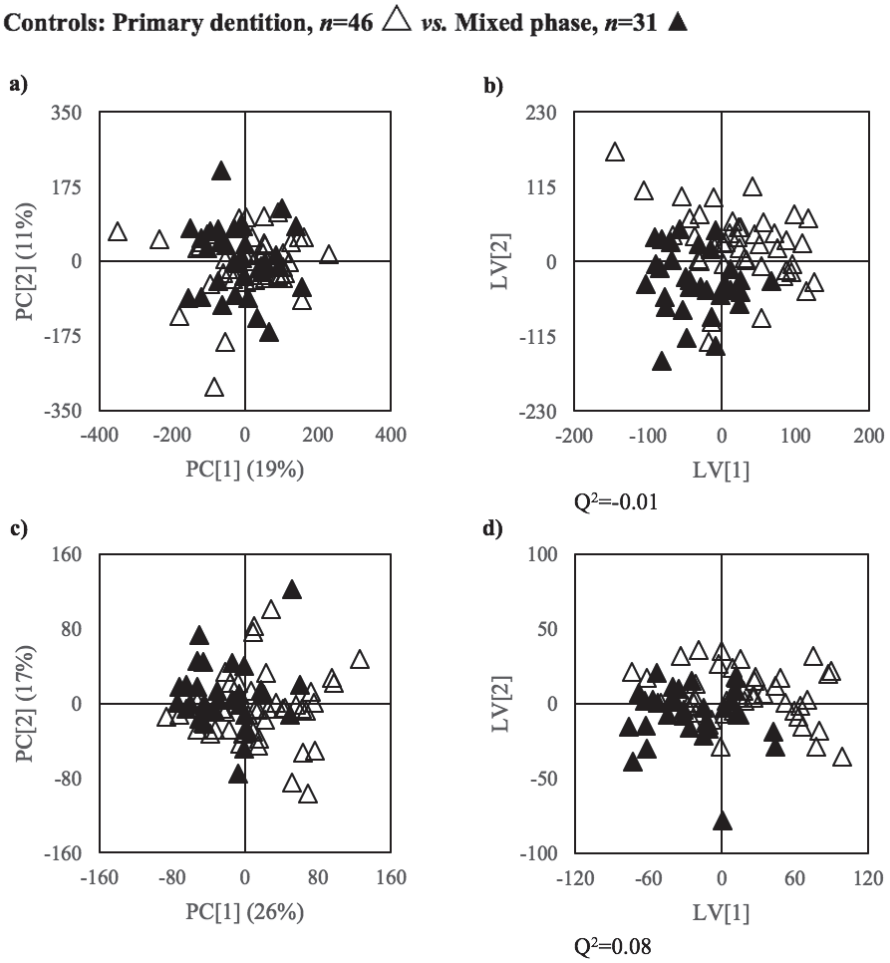

Figure 7.2. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR for dentition stage comparison in controls: primary, *n*=46 (Δ) *vs.* mixed phase dentition, *n*=31 (▲). Q^2 value is shown for each model.

Similarly to the strategy employed for gender analysis in this chapter, VS was then applied to the spectra, in an effort to extract information more efficiently from saliva spectra. Upon VS application, PCA and PLS-DA were recalculated using the resulting subsets of data and the subsequent plots are shown in Figure 7.2 c) and d), respectively. The new PCA model maintained the initial trend towards

sample dispersion and, likewise, the PLS-DA did not enable class classification or prediction, as given by the Q^2 value of 0.08 (Table 7.1). Finally, MVA analysis considering only the aliphatic (Q^2=-0.02) and aromatic (Q^2=0.11) spectral regions of the groups did not produce any major improvements on the performance of the models (plots not shown), thus confirming that no separation tendency due to different dentition stages occurs between groups.

Though the lack of meaningful differences between dentition stage groups in this main cohort lends support to our previous pilot study findings, data interpretation should be performed with caution. Unlike the dentition stage comparison conducted in the pilot research, the subgroups analyzed here were matched for oral health status and gender and involved a larger number of samples (*n*=46 controls in primary dentition, M/F=23/23 *vs.* *n*=31 controls in mixed dentition phase, M/F=16/15), aspects that most likely favor the strength of the present findings.

However, it should be emphasized that, in comparison to the pilot investigation, our large cohort subgroups exhibited minimal variations regarding the number and type of teeth present in the subjects' oral cavity, since all participants were five years old and, accordingly, subjects in mixed dentition phase only presented one or a few permanent teeth erupted, at most, in comparison to the remaining children. By comparing our results to those reported by Fidalgo e*t al.*, we may hypothesize that as the small CF children groups they compared substantially differed in terms of age (*n*=15 primary dentition, mean age=4.27±1.27; *n*=18 mixed phase, mean age=7.94±2.09 and *n*=17 permanent dentition, mean age 10.88±1.05), more significant disparities related to physiological and behavioral features could be detected impacting on the USS metabolome (Fidalgo et al., 2013).

It is thus possible that salivary metabolomic changes may in fact occur as children grow and their dentition evolves, however such was not detected in our cross-sectional study. This means that large cross-sectional cohorts may not me suitable to unveil underlying metabolic differences, if existing, due to probable large variability between subjects in terms of dietary habits and oral routine hygiene habits. To investigate that these variables do indeed have a masking effect, a longitudinal analysis would be advised, as will be carried out in future studies comparing the same subjects between first and second saliva collections. Longitudinal studies will thus minimize inter-subject variability due to diet, lifestyle, and hygiene and then truly reveal any potential markers of dentition stage.

7.3. Impact of ECC

7.3.1 Full unmatched cohorts

For the study of the metabolic effect of ECC on saliva ^{1}H-NMR profiles of samples from children afflicted by ECC as defined by the AAPD, *i.e.* children presenting $d_{A\text{-}6}mft > 0$ according to ICDAS-II criteria in the baseline evaluation, were compared to those of controls (children whose $d_{A\text{-}6}mft = 0$ in the same time period) (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c), using the full unmatched cohorts *i.e.* assuming that any potential ECC impact would superimpose on weaker/absent effects of gender and dentition stage (based on the pilot study). Characterization of the main study cohort and metadata used for group definition concerning disease prevalence, characteristics and severity are presented in detail in *Chapter 2 ('Experimental procedures')*. The PCA scores plot corresponding to controls (*n*=77) *vs.* ECC-afflicted (*n*=45) disclosed a large overlap among samples from distinct groups, also visible in the PLS-DA plot, consistently with the yielded Q^2 value of -0.09 (Table 7.1; plots not shown). When reducing the dataset through VS, no relevant changes regarding group clustering were noticed on either PCA or PLS-DA (Q^2=0.11; Table 7.1) models (data not shown). However, visual inspection of both PLS-DA plots (*i.e.* considering the whole dataset and upon VS) unveiled a clear outlier sample from a control subject. Subsequent outlier exclusion did not improve controls *vs.* ECC-afflicted children (*n*=76 *vs.* *n*=45) comparison (Figure 7.3 a) and b)) (Table 7.1).

Moreover, models were rebuilt considering only aliphatic and aromatic spectral regions, still not resulting in any meaningful group separation (Q^2=-0.19 and 0.09, respectively; data not shown). Following VS application, the absence of discrimination between controls and ECC samples remained (Figure 7.3 c), in spite of the PLS-DA obtained after VS (Figure 7.3 d)) having showed a slight tendency towards group separation but still without robustness (Q^2=0.19; Table 7.1). Taken as a whole, these findings suggested that no relevant differences were noticeable between the salivary metabolic profiles of controls and ECC-afflicted children in the baseline evaluation of this cohort.

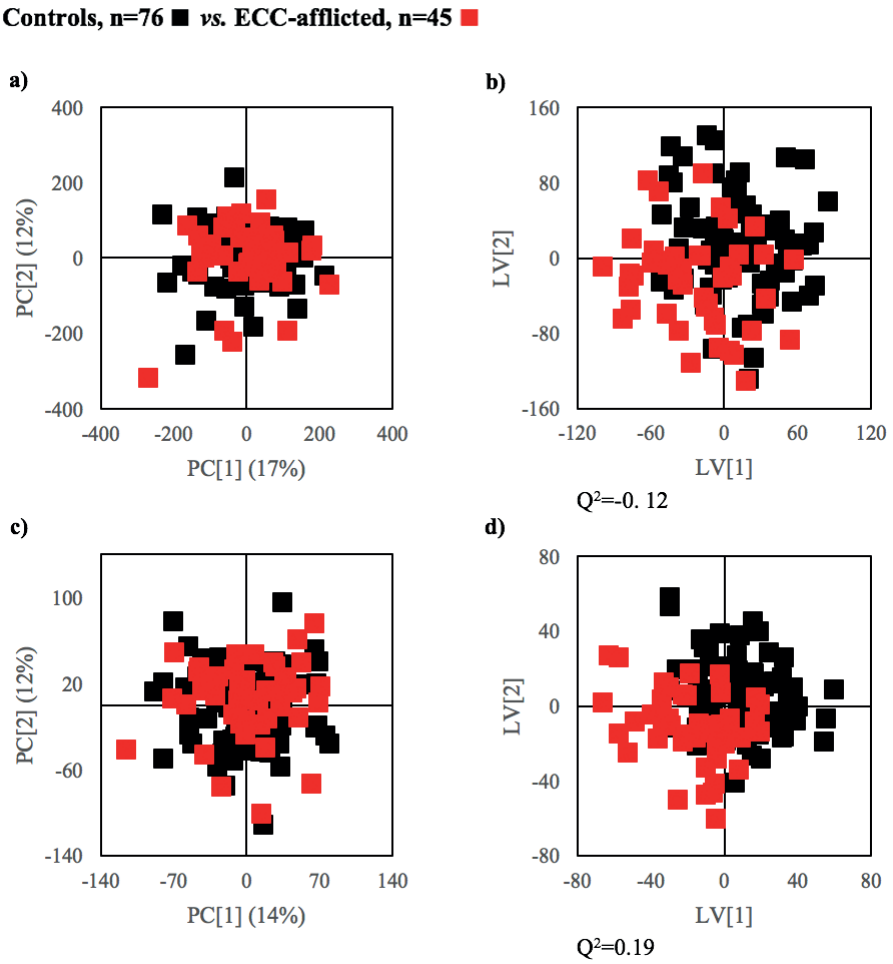

Figure 7.3. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of controls, *n*=76 (■) *vs.* ECC-afflicted children, *n*=45 (■). Q^2 value is shown for each model.

Notwithstanding, still aiming to explore the saliva composition as a function of ECC presence, and considering the aforementioned ECC-afflicted group was defined according to the AAPD definition of the disease and, therefore, was composed not only by children presenting one or multiple ECC lesions (from initial white spot to severe cavitated lesions), but also by participants who had already been subjected to dental treatment(s) (*i.e.* who had extracted teeth or presented dental restorations due to caries), a new subgroup was defined for MVA purposes, the *ECC-dentin* group. This group comprised only subjects who presented at least one carious lesion involving dentin (ICDAS dental caries code ≥ 4), that is, who were ECC-afflicted due to a decayed component (d value, the number of teeth with untreated carious lesions *per* child) > 0 according to the WHO 1997 criteria. Initial PCA of the full spectra of controls (*n*=77) vs. ECC-dentin children (*n*=28) was unable to separate samples from controls and from affected children, as did its corresponding PLS-DA (data not shown), which produced a very low Q^2 value (-0.21; Table 7.1). Distinction between controls and ECC-dentin samples was neither observed when only the aliphatic (Q^2=-0.04) and aromatic (Q^2=0.04) spectral regions were analyzed through PLS-DA (data not shown). Lastly, application of VS method resulted in PCA and PLS-DA models (Q^2=0.11; Table 7.1; plots not shown) exhibiting similar random sample scattering and hence suggested the absence of significant differences between the salivary metabolome from controls and ECC-afflicted children in this study.

Further attempts were performed to gauge the possible NMR metabolic signature of ECC using multiple group comparisons either as a function of the d value or of ECC severity. Regarding the latter, groups were defined according to the d_{A-6}mft score and subjected to the following two comparisons: 1) children presenting d_{A-6}mft ≤ 5 (*n*=114) *vs.* those with d_{A-6}mft ≥ 6 (*n*=8) and 2) children presenting 1 ≤ d_{A-6}mft ≤ 5 (*n*=37) *vs.* d_{A-6}mft ≥ 6 (*n*=8). As to analysis of samples according to the subjects' d value according to ICDAS-II criteria, several comparisons were performed: 1) d value=0 *vs.* d value ≥ 1; 2) d value ≤ 5 *vs.* d value ≥ 6 and 3) 1 ≤ d value ≤ 5 *vs.* d value ≥ 6, as summarized in Table 7.1. However, most of the resulting MVA models, performed in the full dataset, after VS or specifically on particular regions of the spectra, did not retrieve any meaningful differences among groups based on visual inspection of the plots (data not shown) or on their predictive power (Table 7.1). Interestingly, within these models, PLS-DA of the 1 ≤ d value ≤ 5 (*n*=33) *vs.* d value ≥ 6 (*n*=6) comparison was the only one in which an apparent separation pattern was detected between classes, in full dataset and as well as upon VS analysis. However, despite the apparent sample discrimination visualized on PLS-DA plots, only VS led to more acceptable predictive power (Q^2=0.33, in comparison to Q^2=-0.06 obtained when analyzing the whole data).

As previously mentioned, in this observational study, although salivary samples were collected from the cohort at baseline and after six months, due to the large number of collected samples and the time constraints of the research, only baseline samples were subjected to a systematic MVA analysis, as the six-month specimens are currently subject of ongoing work. Accordingly, and even though no data arising from the ^{1}H-NMR spectra of the six-month collection were included in the present thesis, since we were able to identify through metadata which children were controls at the baseline period and after six months had developed ECC, a disease prediction model could still be performed.

The disease prediction model compared the baseline samples from children who remained controls throughout the whole study (*n*=49) to baseline samples of children who developed ECC in the time period between the two evaluations (*n*=5). The resulting PCA model (Figure 7.4 a)) did not show any sample group discrimination although the corresponding PLS-DA plot evidenced slight group separation (Figure 7.4 b)). The tendency detected visually in the scatter plot was not, however, robust, as expressed by the model's predictive power of $Q^{2=}$-0.07 (Table 7.1). PLS-DA analysis of the aliphatic region alone did not improve significantly the model (Q^2=-0.21; data not shown).

When the aromatic spectral regions of both classes were compared individually, the model improved its predictive power (Q^2=0.28), although considerable data dispersion and several outliers were detected in the plot (data not shown) thus not justifying further model analysis. After VS application, subtle tendencies towards class clustering were seen on PCA and PLS-DA models (Figure 7.4 c) and d)) and the latter also improved its predictive power (Q^2 = 0.25) (Table 7.1). MCCV evaluation provided a median Q^2 of 0.25, 0% sensitivity, 100% specificity and 91% classification rate. This sensitivity rate expressed the model's complete inability to correctly identify the true positive cases, *i.e.* samples belonging to children who developed ECC throughout the study (*n*=5), whereas its specificity rate of 100% revealed the model was able to properly classify all controls as disease-free subjects (*n*=49). Therefore, these results probably only reflected the large discrepancy between group sizes and hence the model's robustness could not be confirmed at this stage, requiring larger more balance cohorts.

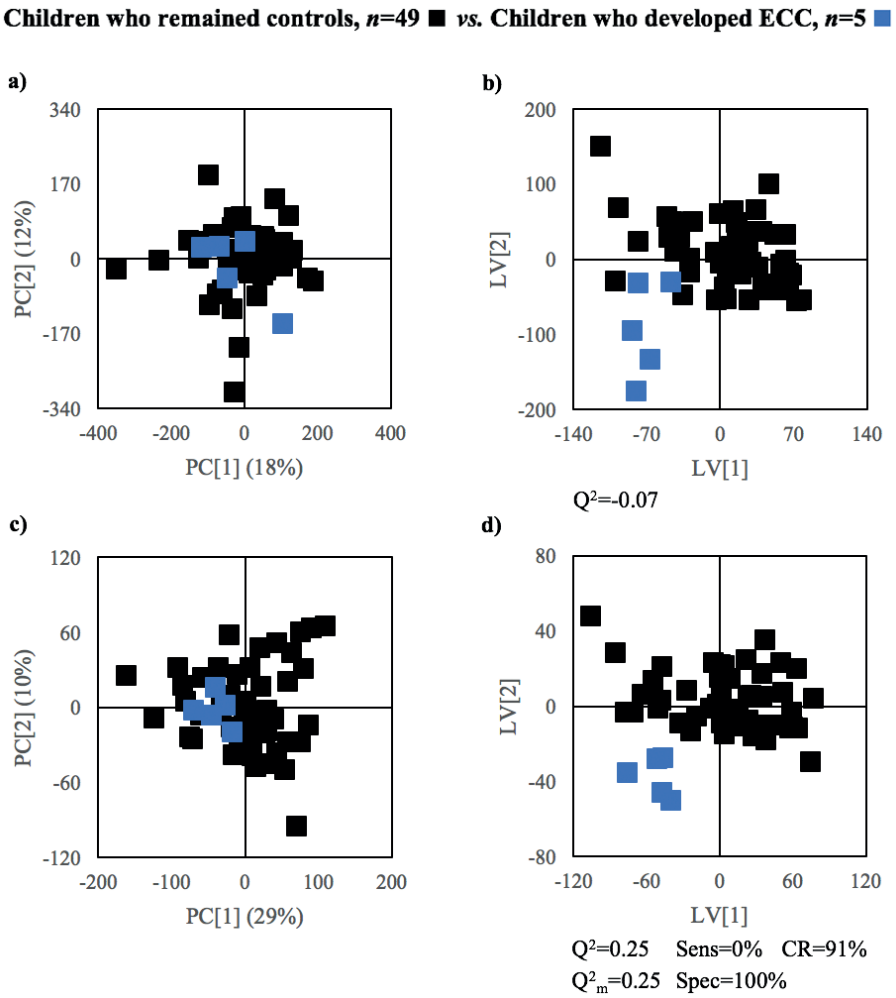

Figure 7.4. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of the disease prediction comparison: children who remained controls throughout the study, *n*=49 (■) *vs.* children who developed ECC between the two evaluations, *n*=5 (■). Q^2 value is indicated for each model and MCCV results are shown for d).

As the present research was designed to test the ability of NMR-based technology to disclose a metabolic signature associated to ECC, we used an untargeted metabolomic profiling approach to study USS samples collected from ECC-afflicted children. In the first set of MVA, baseline USS spectra of ECC-afflicted children were compared to those of controls. Contrary to our expectations the metabolomic profiles observed in ECC-afflicted subjects (*n*=45) and controls (*n*=77) were not significantly distinct, and none of the metabolite variations previously associated to caries in the small cohort of our pilot study were seen.

A probable primary cause for these rather contradictory findings might relate to the fact that this larger cohort, despite being age-matched, may comprise more sources of variability arising from differences in subjects' ethnicity, lifestyle, environment, diet, oral hygiene and oral microflora (Dame et al., 2015). In fact, one of the major challenges encountered in observational studies on human diseases relates to difficulties in adequately controlling such disparities and ascertaining the extent to which these factors contribute to an observed condition in a cohort (L. Zhang, Hatzakis, & Patterson, 2016). It is also plausible that some source of variability has resulted from the wide range of clinical conditions (consistent with the

broad scope of dmft scores) comprised in the ECC-afflicted group, as this subset was defined according to the AAPD definition of ECC and thus included subjects presenting only initial noncavitated carious lesions, children experiencing multiple severe and cavitated lesions, as well as those who had already been subjected to dental treatment(s) and no longer exhibited untreated lesions at all. The high inter-individual variability in the ECC-group may not only reflect metabolic variations related to the presence of carious lesions with distinct extension and severity, but also discrepancies emerging from the different surface roughness conditions found in restored teeth in comparison to sound or untreated teeth, aspects which may influence the bacterial adhesion to hard surfaces, for instance. The controls (n=77) *vs.* ECC-dentin (n=28) models aimed, therefore, at comparing ^{1}H-NMR spectra of healthy controls to those of children who were considered ECC-afflicted owing to the presence of severe untreated caries lesions, excluding subjects with initial lesions or with past history of the disease (*i.e.*, filled or missing teeth), due to the assumption that these conditions could impact differently on the salivary metabolome. A similar approach had already been applied in Fidalgo *et al.* study, in which the ECC-group defined by researchers did not include subjects with previous dental caries history (Fidalgo et al., 2015). However, in the present study no relevant discrimination between samples was still disclosed through these groups comparison. Future research on this topic should be undertaken through longitudinal investigations, as such study design would potentially allow capturing ECC-related phenomena at both the group and the individual level. Longitudinal assessment of ECC impact would, therefore, minimize confounders inherent to inter-subject variability that usually can impact significantly on single time point comparisons.

Several other comparisons were performed to assess the impact of ECC on saliva metabolome as a function of d value, the number of decayed teeth per participant. Though most of these models did not produce noteworthy findings, the comparison between saliva from subjects presenting one to five decayed teeth and samples from those exhibiting six or more decayed teeth (n=33 *vs.* n=6, respectively) was the only yielding moderate predictive power (Q^2=0.33). It can thus be hypothesized that, in our cohort, salivary metabolite variations induced by ECC could be detected from a specific threshold of number of untreated carious lesions.

Regarding comparisons as a function of ECC severity, none of the two performed models ($d_{A\text{-}6}$mft ≤ 5 *vs.* $d_{A\text{-}6}$mft ≥ 6, and 1 ≤ $d_{A\text{-}6}$mft ≤ 5 *vs.* $d_{A\text{-}6}$mft ≥ 6) allowed the detection of meaningful differences between groups. Among five-year old children the disease is classified as S-ECC when a $d_{A\text{-}6}$mft score ≥ 6 is verified or in cases in which one or more smooth surfaces in primary maxillary anterior teeth are cavitated, filled or have been extracted due to caries (American Academy of Pediatric Dentistry (AAPD), 2008, 2017c). The performed models targeted only comparison with S-ECC cases due to $d_{A\text{-}6}$mft ≥ 6, since the inclusion of children presenting only one maxillary anterior affected by dental caries in the same group would substantially increase the subcohort variability and also considering that multiple other models previously conducted in this study had already included such conditions.

Finally, the absence of variations seen in the disease prediction model, carried out based on the ^{1}H-NMR spectra from the baseline evaluation, should be treated with the utmost caution. The model comprised a number of limitations that may have influenced its outcome, namely the considerable disparity between group sizes and the fact that one of the groups was only composed by 5 subjects (n=49 children who remained controls *vs.* n=5 children who developed ECC). Another probable cause for the lack of findings with this regard might result from the inclusion of children with $d_{A\text{-}6}$mft ≠ 0 in the subcohort of subjects who developed ECC, meaning that small sized set of samples was collected from subjects who presented a large scope of clinical conditions, thus introducing more inter-individual variability within the same group.

7.3.2 Cohorts matched for gender and dentition stage

Despite not having found significant metabolomic differences related to gender or dentition stage in neither our pilot study nor in the main cohort, matching approaches considering simultaneously gender and dentition stage were adopted to carry out a final attempt to find ECC salivary metabolic signatures and potential markers. This strategy was chosen on account of the fact that baseline individual characteristics of the participants were somehow imbalanced among the several groups defined for the models previously outlined, perhaps acting as confounding variables and, hence, their possible influence on the research outcomes had to be excluded. Although in these types of observational studies it is not possible to identify and control all extraneous variables, matching was done based on children's gender and dentition stage, in an attempt to make groups more equivalent. The following models will, therefore, include subjects who differ with respect to ECC presence, but are matched on relation to the referred known baseline characteristics.

The first matching approach comprised the comparison between the salivary metabolic profiles of ECC-afflicted males (*n*=23) and matched controls (*n*=17), both at primary dentition stages. Visual inspection of the resulting PCA (Figure 7.5 a)) and PLS-DA (not shown) plots did not indicate any clear group discrimination. In fact, the corresponding PLS-DA not only presented poor predictive power (Q^2=0.10), but also allowed the detection of an outlier control sample. Similarly, upon VS, the recalculated PLS-DA did not show any visible class separation, even though it yielded an increased Q^2 value of 0.75 (plot not shown). This model's MCCV validation parameters and number of selected variables are summarized in Table 7.2, as for all of the PLS-DA models matched for gender and dentition stage performed throughout this chapter. Loadings analysis and peak integration of this model (not shown) demonstrated the only statistically significant variation was a decrease in phenylalanine levels in the ECC group (*p*-value 3.00E-02). This finding was not, however, considered meaningful, as the effect size and standard error values estimated for this variation were similar (-0.64 ± 0.64). In fact, the abovementioned outlier control sample was also found obviously deviated in the PLS-DA scatter plot after VS and visual inspection of its spectrum revealed it presented a high amount of phenylalanine in comparison to the remaining samples, probably accounting for the apparent good Q^2. Accordingly, we chose to recalculate the models after excluding this outlier from the control group. After outlier exclusion, no clustering trends were disclosed in the full dataset PCA (Figure 7.5 b)) nor in the PLS-DA (not shown) plots, and the same was verified after performing VS (plots not shown; Table 7.2) (Q^2=-0.21 and Q^2=0.01 for full dataset and after VS), indicating the initial apparent high prediction power was merely due to the outlier sample. Taken together, these findings indicate the absence of significant differences between ECC-afflicted and controls in male participants in the primary dentition.

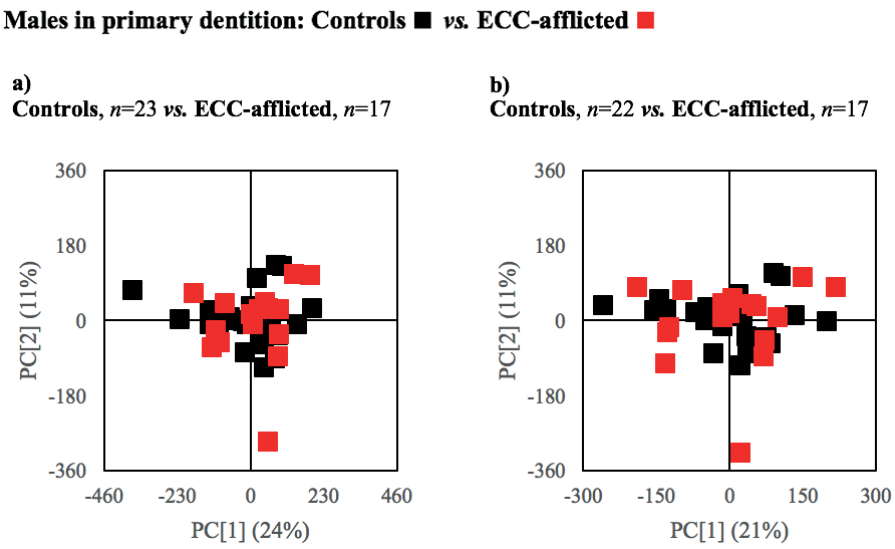

Figure 7.5. PCA scores scatter plots of ^{1}H-NMR spectra of controls (■) *vs.* ECC-afflicted (■) males in primary dentition: a) before (*n*=23 *vs.* *n*=17) and b) after outlier exclusion (*n*=22 *vs.* *n*=17).

Group	Dataset	% full resolution dataset	R^2X	R^2Y	Q^2	Q^2_m	CR (%)	Sens (%)	Spec (%)
Males in primary dentition: Controls, *n*=23 *vs.* ECC-afflicted, *n*=17	Full resolution	–	0.24	0.11	0.10	n.c.	n.c.	n.c.	n.c.
	VS	23	0.32	0.9	0.75	0.34	81	81	81
After outlier exclusion: Controls, *n*=22 *vs.* ECC-afflicted, *n*=17	Full resolution	–	0.20	0.66	-0.21	n.c.	n.c.	n.c.	n.c.
	VS	23	0.18	0.79	0.01	0.45	81	75	85
Females in primary dentition: Controls, *n*=23 *vs.* ECC-afflicted, *n*=7	Full resolution	–	0.19	0.15	0.00	n.c.	n.c.	n.c.	n.c.
	VS	25	0.31	0.74	0.39	0.42	85	54	94
Males in primary dentition: Controls (*n*=23) *vs.* ECC-dentin (*n*=10)	Full resolution	–	0.23	0.69	-0.21	n.c.	n.c.	n.c.	n.c.
	VS	27	0.22	0.75	0.24	n.c.	n.c.	n.c.	n.c.
Females in primary dentition: Controls (*n*=23) *vs.* ECC-dentin (*n*=4)	Full resolution	–	0.26	0.73	0.10	n.c.	n.c.	n.c.	n.c.
	VS	26	0.43	0.69	0.44	-0.26	91	61	96
Males in mixed phase: Controls (*n*=16) *vs.* ECC-afflicted (*n*=6)	Full resolution	–	0.25	0.73	-0.09	n.c.	n.c.	n.c.	n.c.
	VS	25	0.38	0.81	0.56	0.54	89	80	92
Females in mixed phase: Controls (*n*=15) *vs.* ECC-afflicted (*n*=15)	Full resolution	–	0.25	0.64	0.00	n.c.	n.c.	n.c.	n.c.
	VS	19	0.34	0.69	0.33	-0.17	68	73	63
Males in mixed phase: Controls (*n*=16) *vs.* ECC-dentin (*n*=4)	Full resolution	–	0.22	0.81	-0.21	n.c.	n.c.	n.c.	n.c.
	VS	30	0.31	0.85	0.09	n.c.	n.c.	n.c.	n.c.
Females in mixed phase: Controls (*n*=15) *vs.* ECC-dentin (*n*=10)	Full resolution	–	0.27	0.72	0.05	n.c.	n.c.	n.c.	n.c.
	VS	27	0.46	0.68	0.32	0.05	79	65	88

Table 7.2. Q^2 (predictive power) values and MCCV parameters obtained for all the matched models PLS-DA models corresponding to full resolution datasets and datasets obtained after VS. Q^2, cumulative predictive power obtained by cross-validation; Q^2_m, median Q^2 obtained by MCCV; CR: classification rate; Sens, sensitivity; Spec, specificity; n.c., not calculated.

Regarding female subjects at primary dentition, an initial comparison between samples from controls (*n*=23) and ECC-afflicted children (*n*=7) through PCA (Figure 7.6 a)) and PLS-DA (Figure 7.6 b)) methods did not display separation between groups (Q^2 = 0.00) (Table 7.2). Although an apparent improvement in PLS-DA prediction power (Q^2=0.39; Figure 7.6 c)) was achieved when the dataset was reduced by VS, such was a casual result as model validation did not support its robustness (MCCV results on Table 7.2).

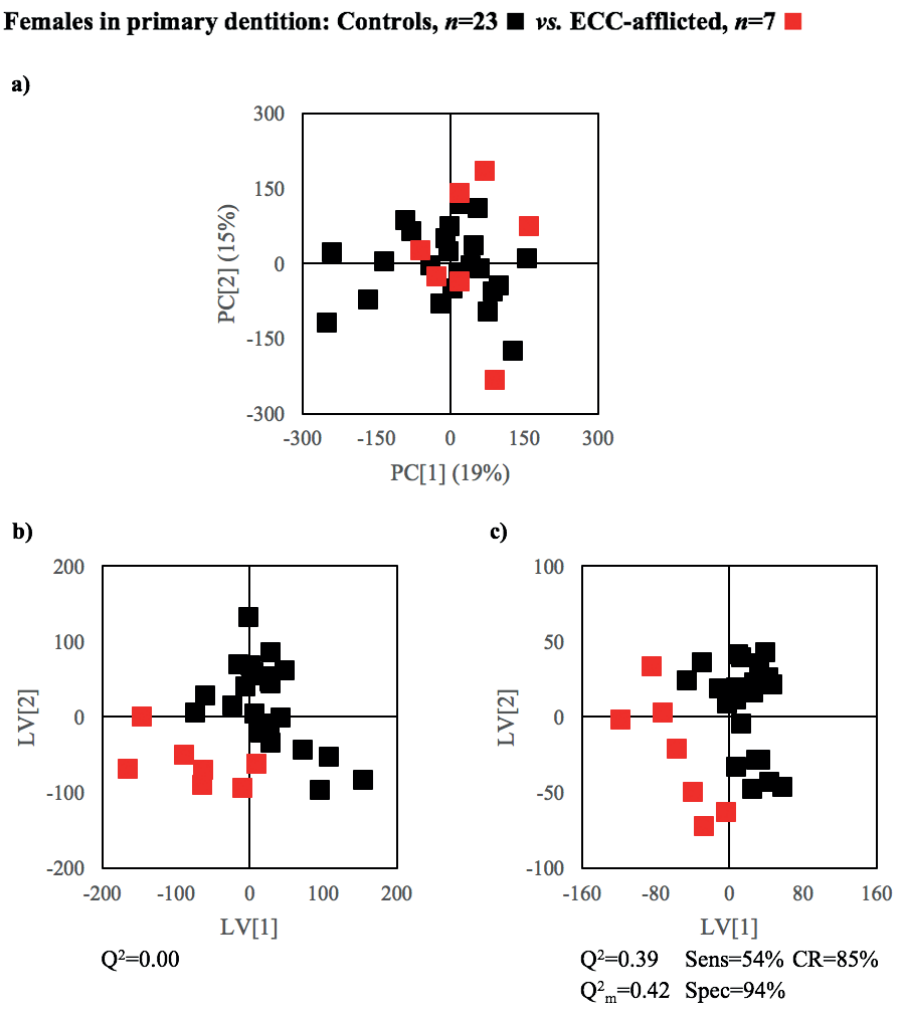

Figure 7.6. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of controls, *n*=23 (■) *vs.* ECC-afflicted, *n*=7 (■) females in primary dentition. Q^2 value and MCCV results are shown for each model.

Apparent variations found in the loading plot (not shown) were still integrated and tested for statistical and biological relevance; however, none of the integrated resonances was found to vary significantly, although a few qualitative trends could be detected by visual inspection of the corresponding spectra. Such qualitative differences comprised increasing tendencies for 5-aminopentanoate (triplet at 2.24 ppm), dimethylsulfone (singlet at 3.15 ppm), formate (singlet at 8.46 ppm) and lower levels of an unassigned compound (doublet at 5.20 ppm, most probably a saccharide) in ECC-afflicted females in primary dentition, as illustrated by Figure 7.7.

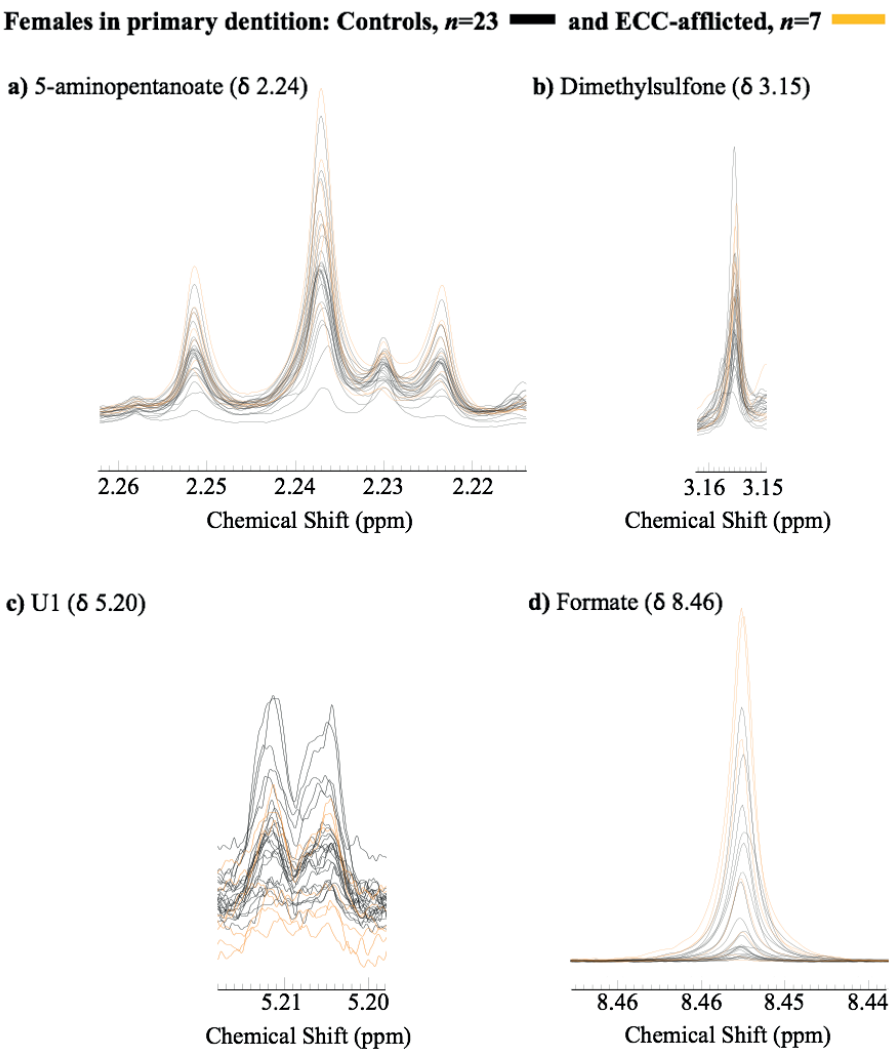

Figure 7.7. Expansions of the a) 5-aminopentanoate (δ 2.24), b) dimethylsulfone (δ 3.15), c) U1 (δ 5.20) and d) formate (δ 8.46) integrated peaks regions in the ^{1}H NMR salivary spectra of controls (black) and ECC-afflicted (orange) females in primary dentition.

Additional models were developed comparing salivary samples from controls to those from participants presenting at least one carious lesion involving dentin, matched for gender in the primary dentition (plots not shown; all Q^2 and MCCV parameters are listed in Table 7.2). The lack of separation between groups seen in the corresponding scatter plots, together with the unsatisfactory performance/robustness presented by those models, revealed no reliable findings could be obtained through such comparisons and, thus, these results were not further explored in the present thesis.

As for subjects in mixed dentition phase comparison between male controls (n=16) and males afflicted by ECC (n=6) only produced noteworthy results upon application of the VS method (plots not shown), expressed by a PLS-DA model showing separation of controls from ECC subjects with a good predictive power (Q^2=0.56; Table 7.2). The corresponding MCCV parameters also reflected the satisfactory robustness of the VS model (89% classification rate, 80% sensitivity, 92% specificity). Investigation of the corresponding loadings (not shown) and spectral integration allowed peak (and

metabolite) variations assessment and evaluation of their univariate statistical relevance. The qualitative trends detected through visual inspection included apparent higher amounts of urea and lower levels of five amino acids (alanine, glycine, tyrosine, lysine and phenylalanine), ethanol and two unassigned peaks (singlets at 2.79 ppm and 2.80 ppm) in ECC-afflicted males in mixed dentition phase. However, from these nine integrated resonances, only alanine was found significantly decreased (*p*-value 1.18E-3) in the ECC-afflicted group, as fully reported in Table 7.3 and illustrated on Figure 7.8 a) boxplot. The controls *vs.* ECC-afflicted comparison was likewise conducted comprising only female participants in the mixed dentition, however, interestingly, no separation between groups could be detected by MVA methods, as exposed by the PLS-DA Q^2 values before (0.00) and after VS (0.33), as well as by the MCCV quality parameters (Q^2_m=-0.17, 68% classification rate, 71% sensitivity and 92% specificity) reported in Table 7.2.

Model	Family	Compound	δ_H/ppm (multiplicity)	ES	p-value
Males in mixed phase after VS: Controls (*n*=16) *vs.* ECC-afflicted (*n*=6)	*Amino Acids*	**Alanine** [a)]	1.48 (d)	↓ **-0.99 ± 0.98** [b)]	1.18E-03
Females in mixed phase after VS: Controls (*n*=15) *vs.* ECC-dentin (*n*=10)				↑ **0.41 ± 0.81** [c)]	1.63E-02

Table 7.3. List of significant metabolite variations, as viewed by NMR, in the models: 1) controls (*n*=16) *vs.* ECC-afflicted (*n*=6) males in mixed phase after VS and 2) controls (*n*=15) *vs.* ECC-dentin (*n*=10) females in mixed phase after VS, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and *p*-value. d: doublet. [a)] Metabolite variation to our knowledge not reported in previous studies on children; [b)] variation reported in our pilot study, but in opposite direction; [c)] variation detected in our pilot study. Only variations with *p*-value < 0.05 are shown.

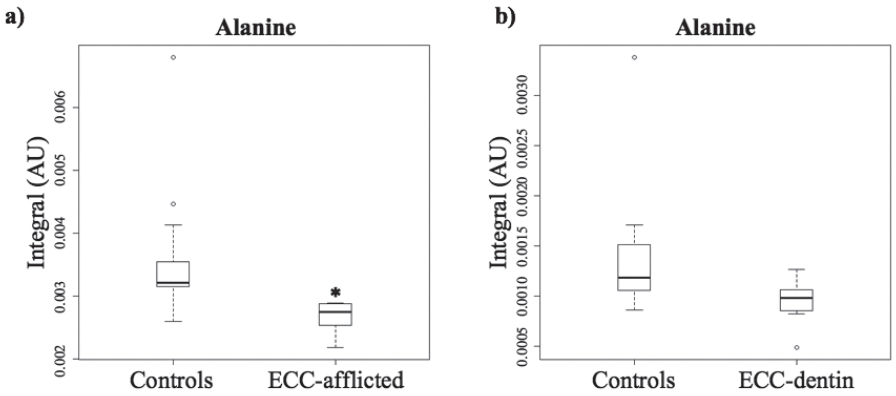

Figure 7.8. Boxplots representations illustrating the statistically significant variations (*p*-value < 0.05) found in a) ECC-afflicted subjects compared to male controls in the mixed phase and b) ECC-dentin subjects compared to female controls in mixed phase. * indicates *p*-value < 0.01.

Lastly, potential differences between the salivary metabolome of controls and ECC-afflicted children due to the presence of carious lesions involving dentin (*ECC dentin*) were explored in children in mixed dentition phase, matched for gender. MVA analysis of spectra from controls (*n*=16) vs. ECC-dentin

(*n*=4) male subjects in mixed phase did not expose any significant variations between sample groups (data now shown) neither considering full dataset nor after application of VS strategy (Q^2=-0.21 and Q^2=0.09, respectively; Table 7.2). Conversely, when the same comparison was performed in female participants (controls, *n*=15 *vs.* ECC-dentin, *n*=10), class separation was observed to some extent in the PLS-DA model upon VS (plot not shown). Though the model only presented moderate predictive capacity and performance (Q^2=0.32; MCCV parameters summarized in Table 7.2), loadings analysis and signal integration revealed that alanine was found to vary significantly among samples (*p*-value 1.63E-02). Interestingly, in contrast to the previously described model concerning controls *vs.* ECC-afflicted males in mixed dentition phase, in this model alanine was found in increased levels in females in the same dentition phase presenting ECC lesions involving dentin (Table 7.3 and Figure 7.8 b)).

Despite having previously rejected the hypothesis of relevant variations related to gender or dentition stage occurring in the salivary metabolome of this cohort, (due to inherent group dispersion), the application of matched strategies yielded models with higher predictive power (listed on Table 7.2) than the unmatched or not perfectly matched approaches (summarized in Table 7.1). A reasonable explanation for such results may relate to a possible variability decrease after performing the best-balanced matching available in diseased and control groups, even though each group sample size was substantially reduced through this process. This improvement was mainly observed in matched models concerning female participants, for reasons not entirely understood, but probably related to specific metabolic characteristics of female saliva composition in terms of hormonal and growth effects. When the salivary metabolome of ECC-afflicted females in the primary dentition was compared to gender and age-matched controls (Figure 7.6), only some qualitative trends towards compositional changes without statistical significance were noticed (Figure 7.7). Higher dimethylsulfone levels had already been seen in our CA pilot cohort with statistical relevance, as it can be noted from Table 6.2 in Chapter 6. All in all, it can thus be conceivably hypothesized that some changes related to caries dynamics might be preferentially noted for female cohorts. Regarding dimethylsulfone specifically, this compound is known to arise from diet or oral bacteria metabolism and had also been detected previously in saliva from adults with poor periodontal status (Figueira et al., 2016). The fact that this salivary compound is here found varying, even despite lacking statistical significance, suggests that its role in the context of oral bacteria-related disease should be further investigated. To the best of our knowledge, alterations in 5-aminopentanoate had never been reported in relation to dental caries, whilst for formate, curiously, weak decreasing tendencies without statistical significance had been noticed in the saliva of our small CA pilot cohort, in contrast to the apparent increase referred here. In fact, literature has demonstrated this short-chain fatty acid is generated from bacterial fermentation occurring in the oral cavity and therefore is tendentiously increased in subjects affected by dental caries (Dame et al., 2015; Fidalgo et al., 2013; Gardner et al., 2019; Rzeznik et al., 2017; C. J. L. Silwood et al., 2002; C. J. Silwood et al., 1999).

As for matched models involving children in the mixed dentition phase, the comparison between controls and ECC-afflicted males evidenced group discrimination, whilst in females in the same dentition phase, separation trends were only observed when comparing controls to subjects exhibiting caries lesions involving dentin (Table 7.2). These results reveal that, unexpectedly, differences between groups in females were only noticed when more severe lesions were exclusively considered. Nevertheless, of all the integrated resonances in both models mentioned, the only metabolite found varying significantly was alanine (Table 7.3; Figure 7.8). Though to our knowledge this variation had not been reported in literature concerning dental caries in children, increased levels of this amino acid had been identified in the saliva of the CA group of our pilot study, consistently with the findings of the comparison between controls and ECC-dentin in females in mixed phase here described. As to the

model controls *vs.* ECC-afflicted males in the mixed dentition phase, alanine was observed varying in the opposite direction. The remaining pilot study observations relative to increased concentrations of several free amino acids, previously attributed to bacterial proteolytic activity and associated to degradation of both soft and hard oral tissues (Aimetti et al., 2012; V M Barnes et al., 2011; Virginia M. Barnes et al., 2010; Liebsch et al., 2019; Mikkonen et al., 2016), were not detected in this main cohort.

Overall, the NMR-based investigation performed using the USS spectra of this large cohort did not confirm the putative dental caries metabolic signature proposed by our previous pilot study findings. In fact, the only significant metabolite variation registered here relative to alanine salivary levels displayed interesting opposite tendencies in the subcohorts affected by ECC. Although not having unequivocally discriminated metabolic profile of ECC from healthy controls, we believe this metabolomic approach has the potential to provide robust ECC biochemical salivary markers and, accordingly, some limitations of the present research should be carefully addressed when extrapolating its findings, as well as when designing further studies using adequate methodology and sample size.

One of the major challenges in the development of this study involved avoiding unintended bias of any sort, from establishing inclusion criteria to compose a large but strictly defined cohort to controlling variables that could possibly influence subjects' salivary flow and composition during collections. Several efforts were made to conduct the research under well-controlled conditions, as described in Chapter 2. However, multiple drawbacks typically inherent to observational studies in humans, and particularly in children, may have occurred, in this case likely aggravated by the fact that sample and data collection were developed in preschools settings.

Firstly, it is possible that legal tutors may have omitted or provided dishonest information on children's metadata, necessary for meeting the inclusion criteria. In an attempt to confirm provided data, the main researcher tried to verify the most relevant data with preschool teachers before each evaluation; still, there is some likelihood that some participants presented systemic diseases which may have impacted to some extent on the salivary metabolome, for instance. Also, following multiple literature recommendations on saliva collection, legal tutors and preschool staff had been previously asked for participants to refrain from eating, drinking and using any oral hygiene product prior to each evaluation. However, as some children arrived at different timings to preschools, it was not possible to fully assure such requests had been rigorously followed and, consequently, such conditions may have accounted for the obtained results. Conversely, in the pilot study, all of the abovementioned set of data and recommendations may have been more accurately provided and followed, respectively, considering saliva collections took place in a pediatric dentistry clinic and each legal tutor verbally provided the requested information directly to the main researcher, allowing the establishment of a more strictly defined cohort. In addition, although the preschool staff was asked for participants to avoid any physical activity (such as running) before evaluations, we are aware that, realistically, this might have been a critical issue for them to control in these young aged cohort. Physical activity has been demonstrated to influence the levels of multiple salivary metabolites (Dame et al., 2015; Gardner et al., 2018, 2019; M. C. Walsh et al., 2006) and thus not controlling for this effect might have led to excessive variation within groups or, eventually, differences between groups.

During evaluations, multiple difficulties related to the participants' young age were faced, including limited collaboration on oral examinations and saliva collection. Children who were restless or noncompliant during procedures were encouraged to cooperate, but still no excessive efforts were done in order

to obtain salivary samples, since the induced stress could potentially affect the nature of the collected specimen. In addition to children's noncompliance, another source of random missing data emerged from the inability of some participants' to passively drool adequate amounts of saliva, in spite of the small sample volume required for NMR analysis. The high inter-individual variability regarding USS flow rate characteristic of pediatric ages might partially explain this difficulty in providing adequate sample amounts, though we cannot rule out the hypothesis that some children were not at all mature to understand how to passively drool saliva, instead of spiting or swallowing it.

Some reports exploring the human saliva composition have referred subjects should rinse the oral cavity with water minutes before saliva collection, aiming to obtain a more representative USS sample (Dawes & Kubieniec, 2004; Henson & Wong, 2010), whilst others mention measurement of salivary flow rate should precede the actual collection (Fidalgo et al., 2015), possibly either to account for the metabolic output arising from biofilms dependent on salivary flow (and on dental plaque indices) or to exclude children presenting altered saliva flow. Due to the limited physical and logistic conditions encountered in preschools, along with the challenges that additional methodological steps would imply in terms of children's collaboration (often already difficult to manage during evaluations), such procedures were not adopted in this study's protocol. Even though at this point we are unable to speculate on the extent to which this metabolic output arising from dental plaque may have impacted differently in our cohort, we can still state that, as all of the participants whose samples were included in the study were able to expectorate the required volume of saliva within a five minute period (maximum), all of them presented an average individual flow rate of at least 0.3 mL per minute (considered a normal flow rate for children, according to literature).

Although previously addressed in Chapter 3, some limitations inherent to oral health screenings in epidemiological settings might as well have reflected on the research outcome. One of the strengths of this study comprised the adoption of ICDAS-II criteria in oral examination, as such criteria allowed the detection of noncavitated carious lesions and hence enabled ECC diagnosis in early development stages. Notwithstanding, difficulties in oral examination due to more limited visualization in comparison to diagnosis performed in dental clinic settings, along with the lack of radiographic assessment, might have led to underdetection of carious lesions, particularly in proximal surfaces, or of dental surfaces presenting tooth-colored restorations. As a consequence, some participants might have been misclassified as controls or have been wrongly attributed an inferior dmft score and thereupon may have been included in incorrect sample groups.

Lastly, despite having intended to present results from the saliva collection of the six-month evaluation, as previously mentioned, it was not possible to fully analyze such all 1H-NMR data yet (working and writing of manuscript in progress). Indeed, the long periods of time necessary to obtain ethical approval and to compose our main cohort, along with the fact that salivary samples could not be left for fully automated acquisition and processing in the NMR spectrometer, not only hindered us from presenting the results from the six-month evaluation in the present dissertation but also prevented the development of a third assessment in preschools (initially planned to occur twelve months after the baseline evaluation). In light of several observations arising from both our pilot and main studies, undertaking further longitudinal investigations in large cohorts should be a vital issue for future research. Although large-scale investigations are often required to identify and validate biomarkers related to human diseases, as previously mentioned wide cohorts also comprise higher inter-subject variability. Research involving data collection at several time points is therefore advised to null or minimize such source of variation and effectively disclose any potential salivary markers of ECC.

Chapter 8. Conclusions and future directions

Chapter 8. Conclusions and future directions

The present work entailed a pilot cross-sectional research and a large-scale observational study, employing a metabolomics approach to characterize saliva composition of children free from and affected by dental caries or ECC, using ^{1}H-NMR spectroscopy. Within the overall limitations of this work, as stated previously mainly in the discussion of Chapter 7, it was possible to demonstrate in our cohorts that:

1. Through 1D and 2D NMR experiments, a total of 47 metabolites were identified in the USS samples of the pilot cohort, establishing the most comprehensive characterization of children saliva described in literature to date, to the best of our knowledge;
2. The comparison of the metabolic profiles of USS and SS, performed for the first time to our knowledge in a children cohort, did not retrieve any significant differences;
3. In comparison to PD, the use of an absorbing device, SCS (Salimetrics, State College, PA, USA), induced significant salivary compositional variations, thus indicating PD as a preferential method for collecting USS for NMR-based studies;
4. The impact of the physiological variables gender and dentition stage in the metabolome of USS collected by PD was not significant in any of the children cohorts;
5. A putative 21-metabolite/resonance USS signature of caries, involving mostly amino acids and monosaccharides observed for the first time to our knowledge regarding children caries, was noted for the pilot cohort;
6. None of the caries-related variations found in the pilot study could be validated, at this point, through the analysis of baseline samples of the large cohort. Interestingly, a novel significant variation involving alanine was found associated to ECC and the direction of which appeared to be dependent upon gender. The possible reasons for these different findings may be related to the challenges encountered while conducting observational studies in children in preschool settings, as it is conceivable that possible ingestion of food or practice of physical activity prior to saliva collection in this cohort may have accounted to such results. This novel observation was only detected after matching subgroups for gender and dentition stage, a finding that also highlights the need for establishing well-defined large cohorts in future studies. Further analysis of NMR data from the six-month collection, still subject of our ongoing work due to the large number of collected samples and the time constraints of this research, may confirm in the future the changes found in this work;
7. A poor oral health condition was found in our preschoolers' cohort, expressed by high ECC prevalence rates (40.10% and 46.50%) and mean dA-6mft scores (1.62 ± 2.52 and 1.54 ± 2.54) detected in baseline and in the six-month evaluation, respectively, and by an impressive percentage of untreated teeth requiring effective treatment (80.40% and 63.40% at the first and second assessment, correspondingly);
8. Within the main cohort, establishment of toothbrushing habits before the first year of life and twice daily toothbrushing were inversely associated to ECC experience and a statistically significant association was found between daily ingestion of sweets and the disease.

Overall, we believe the findings of our research contributed importantly to optimize saliva collection protocol for future metabolomic studies and provided relevant insights on the features of children's salivary metabolome under physiologic conditions. This work also demonstrated the value of metabolomics to gauge the effects of ECC in saliva, an informative and non-invasive diagnostic biospecimen

particularly advantageous in pediatric populations and unveiled the appealing possibility of establishing an ECC metabolic salivary phenotype potentially useful as a biomarker. The identification of consistent metabolic biomarkers in saliva, either of diagnostic nature or with predictive power, would allow establishment of effective individual preventive strategies before the clinical translation of the disease and improve ECC management, thus benefiting public health. Ongoing work of our team entails the metabolomics analysis of USS samples collected in the six-month evaluation, as further research is warranted to explore the findings advanced in our study. However, the search for potential biomarkers of diagnosis and ECC prediction will necessarily involve the confirmation and biological validation of putative hypotheses through the development of future larger-scale studies with longitudinal design, as implementation of wide follow-up strategies for collection of subjects samples and clinical data would be of great value.

Another interesting approach we are currently exploring in this area is the assessment of saliva stability after collection, particularly the impact of short-term and prolonged storage at -20ºC and -80ºC, respectively, before samples processing. We believe that gaining a comprehensive understanding on the influence of such experimental issues on saliva degradability will assist in the implementation of analytical protocol recommendations and promote the development of further studies under well-controlled conditions.

In the future, our research team believes it would be interesting to develop another study assessing the effect of growth in the salivary metabolome of our main cohort, as almost four years have passed since the first saliva collection, as well as to perform an exploratory approach on the metabolic composition of dental plaque, considering this research field has not been explored in children, to our knowledge.

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List of figures

Figure 1.1. a) and b) Two-year-old boy exhibiting ECC cavitated lesions in the maxillary incisors. c) and d) Intraoral photographs of a three-year-old girl presenting ECC lesions in all primary dentition. Distinct clinical stages of the disease can be observed in the affected teeth. Photographs courtesy of Professor Ana Luísa Costa.

Figure 1.2. Illustrative scheme of a metabolomics study flowchart using NMR spectroscopy.

Figure 2.1. STROBE flow-chart of participants in the pilot study.

Figure 2.2. Diagram flow of participants and samples in the main study.

Figure 2.3. Illustrative scheme of sample handling and preparation.

Figure 3.1. Graphic illustration of the distribution of the $d_{A\text{-}6}$mft score components at baseline and at the six-month evaluation.

Figure 4.1. Average ^{1}H-NMR 500 MHz spectra of USS samples from CF children. Peak numbering identifies the main assignments: (1) butyrate, (2) isoleucine, (3) leucine, (4) valine, (5) propionate, (6) ethanol, (7) fucose, (8) lactate, (9) threonine, (10) alanine, (11) lysine, (12) acetate, (13) proline, (14) *N*-acetyl of glycoproteins, (15) acetone, (16) pyruvate, (17) succinate, (18) glutamine, (19) dimethylamine, (20) aspartate, (21) trimethylamine, (22) dimethylsulfone, (23) arginine, (24) choline, (25) methanol, (26) taurine, (27) glycine, (28) galactose, (29) glucose, (30) sucrose, (31) urea, (32) tyrosine, (33) histidine, (34) phenylalanine, (35) uracil, (36) hypoxanthine, (37) formic acid. *Excluded region (water) prior to multivariate analysis. Remaining assignments are shown in Table 4.1.

Figure 4.2. Expansion of the aliphatic region (0.4-3.1 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1. The molecular structure of propionate with indication and assignment of its different types of protons is also included.

Figure 4.3. Expansion of the sugars' region (3.1-5.8 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1.

Figure 4.4. Expansion of the aromatic region (5.8-8.5 ppm) of the average ^{1}H-NMR 500 MHz spectra shown in Figure 4.1. Peak numbering corresponds to that shown in Figure 4.1.

Figure 4.5. Expansion (0-4.6 ppm) of the 2D NMR TOCSY spectra of control (CF) saliva. Some peak assignments are shown and numbering corresponds to that shown in Figure 4.1. In red: illustration of TOCSY use in the confirmation of the propionate assignment (metabolite number 5).

Figure 4.6. Expansion (0-4.6 ppm) of the 2D NMR HSQC spectra of control (CF) saliva. Some peak assignments are shown and numbering corresponds to that shown in Figure 4.1. In red: illustration of HSQC use in the confirmation of the propionate assignment (metabolite number 5).

Figure 5.1. Average 500 MHz ^{1}H-NMR spectra of a) USS and b) SS from the same subset of CF children, matched for gender and dentition stage. Some assignments are noted on a) and major visual changes between spectra are indicated in b) (see arrows). Legend: 1, Butyrate; 2, 0.90(s); 3, Isoleucine; 4, Leucine; 5, Valine; 6, Propionate; 7, Ethanol; 8; Fucose; 9, Threonine; 10, Lactate; 11, Alanine; 12, lysine; 13, acetate; 14, Proline; 15, *N*-acetyl glycoproteins; 16, Pyruvate; 17, Succinate; 18, Dimethylsulfone; 19, Methanol; 20, Glycine; 21, Glucose; 22, Tyrosine; 23, Histidine; 24, Phenylalanine; 25, Formate.

Figure 5.2. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra for saliva stimulation comparison: SS (□, n=30) and USS (■, n=38). Q^2 value and MCCV results are shown for each model.

Figure 5.3. a) PCA and b) PLS-DA scores scatter plots of ^{1}H-NMR spectra of USS from children collected through PD (stripes pattern square, *n*=5) and SCS (Salimetrics, State College, PA, USA) (dots pattern square, *n*=5). In both scatter plots, each participating child is indicated by a number code. Q^2 value and MCCV results are shown for each model. (c) Loadings plot corresponding to the PLS-DA model shown in (b). The loadings plots are colored according to VIP and some assignments are identified. The three-letter code is used for amino acids; *excluded region (water) prior to MVA.

Figure 5.4. Chemical structures and NMR spectra of a) ethylene glycol (1,2-Ethanediol), b) caprate (Decanoate) and c) caprylate (Octanoate). Chemical structures illustrations were adapted from the website and 1D NMR spectra of the compounds are available at http://www.hmdb.ca/.

Figure 5.5. 1D STOCSY obtained using the peak at 3.71 ppm (ethylene glycol) as the root peak (see the arrow), with color scale expressing the correlation (*r*) value and expansions of the 1.16 ppm and 5.20 ppm regions (*r*=1).

Figure 6.1. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR for gender comparison: males, *n*=22 (◇); females, *n*=16 (◆). Q^2 value and MCCV results are shown for each model.

Figure 6.2. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR for dentition stage comparison: primary and mixed phase dentition, *n*=25 (○); permanent dentition, *n*=13 (●). Q^2 value and MCCV results are shown for each model.

Figure 6.3. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of CA, *n*=15 (■) *vs.* CF children, *n*=23 (■). Q^2 value and MCCV results are shown for each model.

Figure 6.4. Loading plot corresponding to PLS-DA after VS scores scatter plot of CA, *n*=15 *vs.* CF children, *n*=23. The loadings plots are colored according to VIP and some assignments are identified. The three-letter code is used for amino acids; *excluded region (water) prior to MVA.

Figure 6.5. Boxplot representations of the main metabolite variations found statistically relevant, with * *p*-value < 0.01, in CA subjects, compared to CF children. Glutamine variation remained significant upon Bonferroni correction.

Figure 6.6. Boxplot representations of the unassigned compound variations found statistically relevant in CA subjects, compared to CF children. * indicates *p*-value < 0.01; ** indicates *p*-value < 0.001. U5 and 2.55-2.60 ppm region remained significant upon Bonferroni correction.

Figure 7.1. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR for gender comparison in controls: males, *n*=39 (◇); females, *n*=38 (◆). Q^2 value is shown for each model.

Figure 7.2. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR for dentition stage comparison in controls: primary, *n*=46 (Δ) *vs.* mixed phase dentition, *n*=31 (▲). Q^2 value is shown for each model.

Figure 7.3. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of controls, *n*=76 (■) *vs.* ECC-afflicted children, *n*=45 (■). Q^2 value is shown for each model.

Figure 7.4. a) PCA, b) PLS-DA, c) PCA after VS and d) PLS-DA after VS scores scatter plots of the disease prediction comparison: children who remained controls throughout the study, *n*=49 (■) *vs.* children who developed ECC between the two evaluations, *n*=5 (■). Q^2 value is indicated for each model and MCCV results are shown for d).

Figure 7.5. PCA scores scatter plots of ^{1}H-NMR spectra of controls (■) *vs.* ECC-afflicted (■) males in primary dentition: a) before (*n*=23 *vs.* *n*=17) and b) after outlier exclusion (*n*=22 *vs.* *n*=17).

Figure 7.6. a) PCA, b) PLS-DA and c) PLS-DA after VS scores scatter plots of ^{1}H-NMR spectra of controls, *n*=23 (■) *vs.* ECC-afflicted, *n*=7 (■) females in primary dentition. Q^2 value and MCCV results are shown for each model.

Figure 7.7. Expansions of the a) 5-aminopentanoate (δ 2.24), b) dimethylsulfone (δ 3.15), c) U1 (δ 5.20) and d) formate (δ 8.46) integrated peaks regions in the ^{1}H NMR salivary spectra of controls (black) and ECC-afflicted (orange) females in primary dentition.

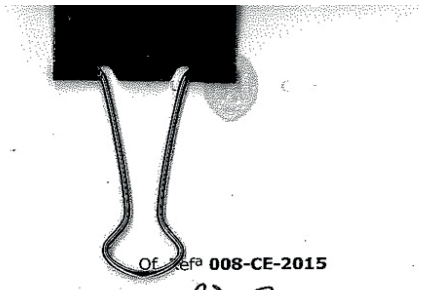
Figure 7.8. Boxplots representations illustrating the statistically significant variations (*p*-value < 0.05) found in a) ECC-afflicted subjects compared to male controls in the mixed phase and b) ECC-dentin subjects compared to female controls in mixed phase.* indicates *p*-value < 0.01.

List of tables

Table 2.1. Characterization of the study population, comprising number of subjects (*n*), average age in years (ranges in brackets), male (M) / female (F), dentition stage and dental caries status, per subgroup.
Table 2.2. Characterization of the main study sample, comprising type, number and location of included preschools (urban (U) / rural (R)), as well as number (*n*) and gender ratio (male (M) / female (F)) of participating children.
Table 2.3. Restoration and sealant coding system of ICDAS-II (adapted from Ismail 2007).
Table 2.4. Dental caries coding system of ICDAS-II (adapted from Ismail 2007).
Table 2.5. Metadata collected through the participation information sheet filled by legal tutors.
Table 2.6. Well-known sociodemographic variables description and operationalization.
Table 2.7. Oral health related behaviors variables description and operationalization.
Table 2.8. Clinical variables description and operationalization.
Table 3.1. Prevalence and severity of ECC in the study sample at baseline evaluation and after six months.
Table 3.2. Descriptive statistics for sociodemographic parameters and bivariate associations with ECC, S-ECC and dentin caries at baseline evaluation.
Table 3.3. Descriptive statistics for sociodemographic parameters and bivariate associations with ECC, S-ECC and dentin caries at the six-month evaluation.
Table 3.4. Descriptive statistics for oral hygiene habits and bivariate associations with ECC, S-ECC, dentin caries, mean d_{A-6}mft and mean d_{4-6}mft.
Table 3.5. Descriptive statistics for dietary habits and bivariate associations with ECC, S-ECC, dentin caries, mean d_{A-6}mft and mean d_{4-6}mft.
Table 4.1. Peak assignments in the ^{1}H NMR spectrum of children saliva. s: singlet, d: doublet, t: triplet, q: quartet, dd: doublet of doublets, dt: doublet of triplets, m: multiplet, br: broad signal.
Table 5.1. Q^2 (predictive power) values and MCCV parameters obtained for the PLS-DA models of saliva collection methods corresponding to full resolution datasets and datasets obtained after variable selection.
Table 5.2. List of metabolite variations in the saliva collected through PD, compared to saliva collected using SCS, as viewed by NMR, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and *p*-value.
Table 6.1. Q^2 (predictive power) values and MCCV parameters obtained for the gender, dentition stage and dental caries PLS-DA models corresponding to full resolution datasets and datasets obtained after VS.
Table 6.2. List of metabolite variations in the saliva of CA children, compared to CF children, as viewed by NMR, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and *p*-value.
Table 7.1. Q^2 (predictive power) values obtained for the gender, dentition stage, ECC impact, ECC severity, number of ECC lesions and disease prediction PLS-DA models corresponding to full resolution datasets and datasets obtained after VS.
Table 7.2. Q^2 (predictive power) values and MCCV parameters obtained for all the matched models PLS-DA models corresponding to full resolution datasets and datasets obtained after VS.
Table 7.3. List of significant metabolite variations, as viewed by NMR, in the models: 1) controls (*n*=16) *vs.* ECC-afflicted (*n*=6) males in mixed phase after VS and 2) controls (*n*=15) *vs.* ECC-dentin (*n*=10) females in mixed phase after VS, along with chemical shift of integrated peak (δ), multiplicity, effect size (ES) and *p*-value.

Annexes

Annex I: Ethical approval from the FMUC ethics committee



FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

COMISSÃO DE ÉTICA DA FMUC

Of. Refª **008-CE-2015**
Data 23/2/**2015**

C/conhecimento ao aluno

Exmo Senhor
Presidente do Conselho Científico da FMUC

Assunto: Projecto de Investigação no âmbito do Programa de Doutoramento em Ciências da Saúde (refª CE-012/2015)

Candidato(a): Joana Leonor de Sousa Almeida Pereira

Título do Projecto: *"Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas".*

A Comissão de Ética da Faculdade de Medicina, após análise do projecto de investigação supra identificado, decidiu emitir o parecer que a seguir se transcreve: ***"Parecer favorável".***

Queira aceitar os meus melhores cumprimentos,

O Presidente,

Prof. Doutor João Manuel Pedroso de Lima

GC

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Annex II: Ethical approval from the ARSC

Ministério da Saúde

ARSC ADMINISTRAÇÃO REGIONAL DE SAÚDE DO CENTRO, I.P.

Exma. Senhora
Dra. Joana Leonor de Sousa e Almeida Pereira

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001799 '15 01-26 15:32

Sua referência	Data	Nossa referência	Data

ASSUNTO: "Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas"

Serve o presente para informar V. Exª., que o Conselho Diretivo da ARS Centro, I.P., deliberou homologar o parecer emitido pela Comissão de Ética para a Saúde (CES), que se anexa.

Com os melhores cumprimentos

O Presidente do Conselho Diretivo da ARSC, I.P.

(Dr. José Manuel Azenha Tereso)

AL

Na resposta indicar a "nossa referência". Em cada ofício tratar só de um assunto.

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COMISSÃO DE ÉTICA PARA A SAÚDE

PARECER FINAL: FAVORÁVEL	DESPACHO: Homologa-se o parecer final da Comissão de Ética para a Saúde 22.1.2015

Conselho Diretivo da A.R.S. do Centro I.P.

Dr. José Manuel Azenha Tereso
Presidente,

Dr. Luís Manuel Milhão Mendes Cabral
Vogal,

Dr. [illegible] Augusto Mo[illegible]
Vogal,

ASSUNTO:

Estudo 65/2014 – "Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas"

Autores: Joana Leonor de Sousa e Almeida Pereira (IP) (aluna de doutoramento da FMUC), Ana Luísa M. Costa (da FMUC), Ana Maria P Coelho Gil (da Universidade de Aveiro)

Este estudo observacional pretende analisar a composição metabolómica da saliva e da placa bacteriana de uma amostra de 240 crianças de 4 anos (do ensino pré-escolar de Coimbra), em fase de dentição decídua, acompanhadas em três períodos de avaliação (inicialmente, aos 6 e aos 12 meses). Após exame clínico oral, a saliva e a placa bacteriana serão analisadas por RMN. Não qualquer procedimento invasivo.
Os dados dos participantes são anonimizados (os dados da consulta não o serão mas ficam sob o sigilo médico).
O consentimento informado está em "boa e devida forma".

O relator,

Carlos Alberto Fontes Ribeiro

14 de janeiro de 2015

Annex III: Ethical approval from ACeS Baixo Mondego

Andrea Margarida Soares Lopes

De: Joana Leonor de Sousa e Almeida Pereira [joana.leonor.pereira@gmail.com]
Enviado: terça-feira, 26 de Maio de 2015 12:18
Para: Antonio Morais; aces.bm
Cc: aluisacosta Moreira Costa; Maria Alcina Gomes da Silva; jmrelvas@gmail.com
Assunto: Solicitação parecer ACeS Baixo Mondego
Anexos: Anexo 1 -Formulário ética ARS.pdf; Anexo 2 - Parecer etica ARS.pdf; Anexo 3 - Aprovação projeto.pdf; Anexo 4 - Parecer etica FMUC.pdf; Anexo 5 - Consentimento Informado.pdf

ACES BAIXO MONDEGO
ENTRADA
26 MAIO 2015
N.º 11072

Concordo com a candidatura deste projeto de tese em articulação com o plano RISE e SO
27/05/15

Exmo. Sr. Diretor Executivo do Agrupamento de Centros de Saúde (ACeS) Baixo Mondego,

Sr. Dr. António Morais,

Venho por este meio solicitar o parecer do ACeS Baixo Mondego em relação ao desenvolvimento do projeto "*Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas*" em estabelecimentos de ensino pré-escolar do distrito de Coimbra, a iniciar no ano letivo 2015/2016.

O projeto de investigação, a desenvolver no âmbito do Programa de Doutoramento em Ciências da Saúde da FMUC, obteve já parecer favorável das Comissões de Ética da Administração Regional de Saúde do Centro e da FMUC (em anexo).

A cárie precoce da infância (CPI) é uma doença altamente prevalente que, dados os efeitos nefastos que assume nas crianças e respetivas famílias, representa um sério problema de saúde pública. Apesar dos avanços científicos, a sinalização de crianças com suscetibilidade previamente ao surgimento de sinais clínicos da CPI não é ainda possível. A metabolómica salivar constitui uma abordagem analítica promissora que, tendo já permitido estabelecer marcadores de diversas doenças, permanece subexplorada neste âmbito.

Desta forma, este estudo pretende definir os perfis metabólicos salivares e, complementarmente, de placa bacteriana (PB), de uma amostra de 240 crianças de 4 anos ao longo de um ano, em três períodos de avaliação (inicialmente, aos 6 e aos 12 meses), objetivando o estabelecimento de marcadores de caraterização e predição de risco da CPI. O metaboloma salivar e de PB de cada uma das crianças será caracterizado a cada período de avaliação. Uma única examinadora efetuará, nos jardins de infância, o exame intra-oral e as colheitas de saliva não estimulada e PB supragengival presente nas superfícies de dentes anteriores. Estes exames, de caráter não-invasivo, constituem procedimentos simples, rápidos e desprovidos de riscos para os participantes. Previamente ao início do estudo serão entregues aos tutores legais das crianças um consentimento informado duplicado e um documento com a descrição do estudo e requisitos de participação.

Adicionalmente, propomo-nos colaborar/implementar sessões educativas destinadas aos cuidadores das crianças e educadores visando assumir um papel ativo na promoção da saúde oral, especificamente no que diz respeito à prevenção da CPI nesta amostra. Prontificamo-nos igualmente a integrar na consulta de Odontopediatria da FMUC, com cumprimento de determinados critérios e de acordo com a capacidade de resposta, as crianças que participem no estudo e cujos tutores manifestem essa mesma vontade.

Pretendemos desenvolver, em primeira instância, durante o mês de Julho de 2015, um breve estudo piloto de análise comparativa de métodos de colheita de saliva não estimulada para aferir a validade das técnicas analíticas empregues, num número reduzido de crianças.

1

Neste sentido, gostaríamos de solicitar o parecer do ACeS Baixo Mondego no desenvolvimento deste estudo, bem como em relação à sua integração no contexto das atividades já instituídas e em curso no âmbito da saúde oral escolar.

Antecipadamente grata,

Com os melhores cumprimentos,

A Doutoranda

Joana Leonor Pereira

As Orientadoras

Ana Luísa Costa

Ana Maria Gil

Annex IV: Informed consent used in this study

FMUC FACULDADE DE MEDICINA
UNIVERSIDADE DE COIMBRA

FORMULÁRIO DE INFORMAÇÃO E CONSENTIMENTO INFORMADO

TÍTULO DO PROJECTO DE INVESTIGAÇÃO:

CARACTERIZAÇÃO DE PERFIS SALIVARES METABÓLICOS NA CÁRIE PRECOCE DA INFÂNCIA: IMPLICAÇÕES DIAGNÓSTICAS, PREVENTIVAS E TERAPÊUTICAS

PROTOCOLO Nº

INVESTIGADOR COORDENADOR Profª Doutora Ana Luísa Moreira Costa
Profª Doutora Ana Maria Pissarra Coelho Gil

CENTRO DE ESTUDO

INVESTIGADOR PRINCIPAL Joana Leonor de Sousa e Almeida Pereira

MORADA Av. Bissaya Barreto, Blocos de Celas
3000-075 Coimbra

CONTACTO TELEFÓNICO +351 239 484 183

NOME DA CRIANÇA PARTICIPANTE
(LETRA DE IMPRENSA) _____

NOME DO TUTOR LEGAL
(LETRA DE IMPRENSA) _____

É convidado(a) a autorizar o seu educando(a) a participar voluntariamente neste estudo porque na presente investigação se pretendem analisar amostras salivares de crianças nascidas no ano 2011, com e sem Cárie Precoce da Infância (CPI). Este procedimento é chamado consentimento informado e descreve a finalidade do estudo, os procedimentos, os possíveis benefícios e riscos. A participação do seu educando(a) poderá contribuir para melhorar o conhecimento sobre a saúde oral infantil, particularmente no que diz respeito ao diagnóstico e progressão da CPI.
Receberá uma cópia deste Consentimento Informado para rever e solicitar aconselhamento de familiares e amigos. O Investigador ou outro membro da sua equipa irá esclarecer qualquer dúvida que tenha sobre o termo de consentimento e também alguma palavra ou informação que possa não entender.
Depois de compreender o estudo e de não ter qualquer dúvida acerca do mesmo, deverá tomar a decisão de autorizar a participação ou não. Caso autorize que o seu educando(a) participe, ser-lhe-á solicitado que assine e date este formulário. Após a sua assinatura e a do Investigador, ser-lhe-á entregue uma cópia.

1. INFORMAÇÃO GERAL E OBJECTIVOS DO ESTUDO

Este estudo irá decorrer em colaboração com a Faculdade de Medicina da Universidade de Coimbra (FMUC), Centro de Investigação em Materiais Cerâmicos e Compósitos da Universidade de Aveiro (CICECO) e Departamento de Química da Universidade de Aveiro. Pretende-se caracterizar amostras salivares de crianças com e sem CPI através de análise metabolómica possibilitando a identificação de potenciais marcadores de início ou de progressão da doença. Serão incluídas 240 crianças nascidas em 2011 sem patologias sistémicas relevantes.

Trata-se de um estudo observacional, pelo que não será feita nenhuma alteração no estado da cavidade oral ou a nível da saúde geral da criança. Este estudo foi aprovado pela Comissão de Ética da FMUC de modo a garantir a proteção dos direitos, segurança e bem-estar de todos os doentes ou outros participantes incluídos e garantir prova pública dessa proteção.

2. PROCEDIMENTOS E CONDUÇÃO DO ESTUDO

2.1. Procedimentos

Fornecimento de dados – tarefa 1

A participação no estudo inclui o fornecimento de dados sociodemográficos e de comportamentos relativos à saúde oral da criança, solicitados na carta de apresentação do estudo. O preenchimento dos dados deve ser efetuado pelo tutor da criança ou, em caso de ausência, por um indivíduo que o(a) substitua e conheça bem o quotidiano da criança. Solicitamos que responda às questões com sinceridade. Ser-lhe-á ainda solicitado que a escovagem dentária na noite prévia à avaliação não seja efectuada.

Colheita de saliva – tarefa 2

Colher-se-á saliva não estimulada, de forma não-invasiva, durante 5 minutos.

Exame intraoral – tarefa 3

Uma única examinadora, Médica dentista, realizará o exame intraoral da criança e registo dos dados obtidos. Este procedimento será efectuado com recurso a um espelho intraoral e a uma sonda.

Serão, em todos os procedimentos descritos, tomadas as medidas de controlo de contaminação e infeção cruzada preconizadas.

Outros procedimentos

As amostras recolhidas serão devidamente armazenadas até à respetiva análise.

2.2. Calendário das visitas/ Duração

Este estudo envolverá a realização de três avaliações (1º momento da colheita, pós 6 e 12 meses), nas quais se efectuarão todos os procedimentos descritos nas tarefas 2 e 3. Estima-se que a duração total dos procedimentos não exceda os 15 minutos por participante em cada uma das avaliações.

2.3. Tratamento de dados

Os dados fornecidos pelos tutores legais das crianças e os resultados analíticos obtidos serão sujeitos a análise estatística.

3. RISCOS E POTENCIAIS INCONVENIENTES PARA O DOENTE

Não existem quaisquer riscos para o paciente na participação do estudo.

4. POTENCIAIS BENEFÍCIOS

O presente estudo permitirá aprofundar os conhecimentos atuais sobre a CPI, particularmente na caracterização de possíveis marcadores precoces da doença, proporcionando avanços relevantes em termos de prevenção e tratamento, com inegável impacto a nível de saúde pública e na qualidade de vida destas crianças.

5. NOVAS INFORMAÇÕES

Ser-lhe-á dado conhecimento de qualquer nova informação que possa ser relevante para a condição do seu educando(a) ou que possa influenciar a sua vontade de continuar a autorizar a participação no estudo.

6. TRATAMENTOS ALTERNATIVOS

Não se aplica.

7. SEGURANÇA

Os procedimentos a realizar, não sendo em absoluto invasivos, não comprometem a integridade da criança.

8. PARTICIPAÇÃO/ ABANDONO VOLUNTÁRIO

É inteiramente livre de aceitar ou recusar participação do seu educando(a) neste estudo. Pode retirar o seu consentimento em qualquer altura sem qualquer consequência para si ou para a criança, sem precisar de explicar as razões, sem qualquer penalidade ou perda de benefícios e sem comprometer a sua relação com o Investigador que lhe propõe a colaboração neste estudo. Ser-lhe-á pedido para informar o Investigador se decidir retirar o seu consentimento.

O Investigador do estudo pode decidir terminar a participação do seu educando(a) se não estiver a seguir o plano do estudo, por decisão administrativa ou decisão da Comissão de Ética.

O corresponsável do estudo notificá-lo-á se surgir uma dessas circunstâncias e falará consigo a respeito da mesma.

9. CONFIDENCIALIDADE

Os registos do seu educando(a) manter-se-ão confidenciais e anonimizados de acordo com os regulamentos e leis aplicáveis. Se os resultados deste estudo forem publicados a identidade do seu educando(a) manter-se-á confidencial. Ao assinar este Consentimento Informado autoriza este acesso condicionado e restrito. Pode ainda, em qualquer altura, exercer o seu direito de acesso à informação. Pode ter também acesso à informação médica e dentária através da

Médica dentista neste estudo. Tem também o direito de se opor à transmissão de dados que sejam cobertos pela confidencialidade profissional.

Os registos médicos e dentários que identificam o seu educando(a) e o formulário de consentimento informado que assinar serão verificados para fins do estudo pelo Investigador e/ou por colaboradores do Investigador, e para fins regulamentares pelo Investigador e/ou pelos colaboradores do Investigador e agências reguladoras noutros países. A Comissão de Ética responsável pelo estudo pode solicitar o acesso aos registos médicos e dentários para assegurar-se que o estudo está a ser realizado de acordo com o protocolo. Não pode ser garantida confidencialidade absoluta devido à necessidade de passar a informação a essas partes.

Ao assinar este termo de consentimento informado permite que as informações médicas e dentárias neste estudo sejam verificadas, processadas e relatadas conforme necessário para finalidades científicas legítimas.

Confidencialidade e tratamento de dados pessoais

Os dados pessoais dos participantes no estudo, incluindo a informação médica recolhida ou criada como parte do estudo, tais como registos da observação oral ou resultados de análises, serão utilizados para condução do estudo, designadamente para fins de investigação científica relacionados com a patologia em estudo.

Ao consentir a participação do seu educando(a) neste estudo a informação a ele respeitante, designadamente a informação clínica, será utilizada da seguinte forma:

1. O promotor, os investigadores e as outras pessoas envolvidas no estudo recolherão e utilizarão os dados pessoais do seu educando(a) para as finalidades acima descritas.
2. Os dados do estudo, associados às iniciais ou a outro código que não identifique diretamente o seu educando(a) (e não o nome) serão comunicados pelos Investigadores e outras pessoas envolvidas no estudo ao promotor do estudo, que os utilizará para as finalidades acima descritas.
3. Os dados do estudo, associados às iniciais ou a outro código que não identifique diretamente o seu educando(a), poderão ser comunicados a autoridades de saúde nacionais e internacionais.
4. A identidade do seu educando(a) não será revelada em quaisquer relatórios ou publicações resultantes deste estudo.
5. Todas as pessoas ou entidades com acesso aos dados pessoais do seu educando(a) estão sujeitas a sigilo profissional.
6. Ao dar o seu consentimento para a participação do seu educando(a) no estudo autoriza o promotor ou empresas de monitorização de estudos especificamente contratadas para o efeito e seus colaboradores e/ou autoridades de saúde, a aceder aos dados constantes do seu processo clínico, para conferir a informação recolhida e registada pelos investigadores, designadamente para assegurar o rigor dos dados que lhe dizem respeito e para garantir que o estudo se encontra a ser desenvolvido corretamente e que os dados obtidos são fiáveis.

7. Nos termos da lei, tem o direito de, através de um dos médicos envolvidos no estudo, solicitar o acesso aos dados que digam respeito ao seu educando(a), bem como de solicitar a rectificação dos dados de identificação.
8. Tem ainda o direito de retirar este consentimento em qualquer altura através da notificação ao investigador, o que implicará que o seu educando(a) deixe de participar no estudo. No entanto, os dados recolhidos ou criados como parte do estudo até essa altura que não identifiquem o seu educando(a) poderão continuar a ser utilizados para o propósito de estudo, nomeadamente para manter a integridade científica do estudo, e a informação médica do seu educando(a) não será removida do arquivo do estudo.
9. Se não der o seu consentimento, assinando este documento, o seu educando(a) não poderá participar neste estudo. Se o consentimento agora prestado não for retirado e até que o faça, este será válido e manter-se-á em vigor.

10. COMPENSAÇÃO

Este estudo é da iniciativa do investigador e, por isso, solicita-se a participação do seu educando(a) sem uma compensação financeira para a sua execução, tal como também acontece com os investigadores e o centro de estudo. Não haverá qualquer custo para o participante pela sua inclusão no estudo.

11. CONTACTOS

Se tiver perguntas relativas aos seus direitos como participante deste estudo, deve contactar:
Presidente da Comissão de Ética da FMUC
Azinhaga de Santa Comba, Celas – 3000-548 Coimbra
Telefone: 239 857 707 e-mail: comissaoetica@fmed.uc.pt

Se tiver questões sobre este estudo deve contactar:
Ana Luísa Moreira Costa, Joana Leonor Sousa Almeida Pereira
Tel: +351 239 484 183 Morada: Av. Bissaya Barreto, Bloco de Celas, 3000-075 Coimbra

NÃO ASSINE ESTE FORMULÁRIO DE CONSENTIMENTO INFORMADO A MENOS QUE TENHA TIDO A OPORTUNIDADE DE PERGUNTAR E TER RECEBIDO RESPOSTAS SATISFATÓRIAS A TODAS AS SUAS PERGUNTAS.

CONSENTIMENTO INFORMADO

De acordo com a Declaração de Helsínquia da Associação Médica Mundial e suas atualizações:

1. Declaro ter lido este formulário e aceito de forma voluntária que o meu educando(a) participe neste estudo.
2. Fui devidamente informado(a) da natureza, objetivos, riscos e duração provável do estudo, bem como do que é esperado da parte do meu educando(a).
3. Tive a oportunidade de fazer perguntas sobre o estudo e percebi as respostas e as informações que me foram dadas. A qualquer momento posso fazer mais perguntas ao médico responsável do estudo. Durante o estudo e sempre que quiser, posso receber informação sobre o seu desenvolvimento. O médico responsável dará toda a informação importante que surja durante o estudo que possa alterar a minha vontade ou do meu educando(a) continuar a participar.
4. Aceito que utilizem a informação relativa à história clínica e registos clínicos do meu educando(a) no estrito respeito do segredo médico e anonimato. Os dados do meu educando(a) serão mantidos estritamente confidenciais. Autorizo a consulta dos dados do meu educando(a) apenas por pessoas designadas pelo promotor e por representantes das autoridades reguladoras.
5. Aceito que o meu educando(a) siga todas as instruções que lhe forem dadas durante o estudo. Aceito que o meu educando(a) colabore com o médico e informá-lo-ei imediatamente das alterações não usuais do estado de saúde e bem-estar do meu educando(a) que ocorram.
6. Autorizo o uso dos resultados do estudo para fins exclusivamente científicos e, em particular, aceito que esses resultados sejam divulgados às autoridades sanitárias competentes.
7. Aceito que os dados gerados durante o estudo sejam informatizados pelo promotor ou outrem por si designado. Eu posso exercer o meu direito de rectificação e/ ou oposição.
8. Tenho conhecimento que sou livre de desistir que o meu educando(a) participe no estudo a qualquer momento, sem ter de justificar a minha decisão e sem comprometer a qualidade dos seus cuidados médicos. Eu tenho conhecimento que o médico tem o direito de decidir sobre a saída prematura do estudo e que me informará da causa da mesma.
9. Fui informado que o estudo pode ser interrompido por decisão do investigador, do promotor ou das autoridades reguladoras.

Nome da criança Participante_____ ***Data nascimento:*** _____/_____/____
Assinatura: _____ ***Data:***_____/_____/____

Nome de Testemunha / Tutor Legal:_____
Assinatura: _____ ***Data:***_____/_____/____

Confirmo que expliquei ao participante acima mencionado a natureza, os objetivos e os potenciais riscos do estudo acima mencionado.

Nome do Investigador:_____
Assinatura: _____ ***Data:***_____/_____/____

Annex V: Assent for children's participation in research

CARACTERIZAÇÃO DE PERFIS SALIVARES METABÓLICOS NA CÁRIE PRECOCE DA INFÂNCIA: IMPLICAÇÕES DIAGNÓSTICAS, PREVENTIVAS E TERAPÊUTICAS

ASSENTIMENTO INFORMADO PARA AS CRIANÇAS PARTICIPANTES

Previamente à realização de cada observação oral e de cada colheita salivar, dever-se-á apresentar aos participantes a equipa de investigação, bem como todos os procedimentos do estudo, visando:

- Estabelecer uma relação empática com as crianças participantes, permitindo criar um ambiente calmo e minimizando a possibilidade de ocorrência de falta de cooperação durante o processo;

- Descrever os procedimentos do estudo recorrendo a uma linguagem não técnica, perceptível pelas crianças:
    - *"vou ver os vossos dentes com este espelho pequenino, para saber se eles têm bichinhos"*
    - *"vou pedir a todos os meninos que estejam sentados e bem sossegadinhos, para fazermos o jogo da saliva num instantinho!"*

- Pedir autorização a cada participante para realizar a observação oral e a(s) colheita(s) de saliva:
    - *"deixas-me contar os teus dentinhos?"*
    - *"podes babar para este tubinho, para eu estudar um pouco da tua saliva?"*

Annex VI: Communication letter to inform parents on child's oral health status

(Data)

Caro Encarregado de Educação,

Após a observação da cavidade oral do(a) menino(a) _____ no âmbito do estudo "*Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas*", constatou-se a suspeita da presença de _____ lesões de cárie dentária.

Atendendo a que as condições de exame oral no Jardim de Infância não são as ideais, sugere-se que a criança seja reavaliada no contexto de uma consulta de Medicina Dentária, para confirmação do diagnóstico e instituição de eventual tratamento.

Caso esteja interessado/a que o seu educando possa usufruir de acompanhamento médico-dentário na consulta da clínica de Odontopediatria, a decorrer nos Blocos de Celas, Coimbra, preencha por favor o destacável abaixo e faça-o chegar à Educadora responsável deste Jardim de Infância para posterior contacto para marcação.

Com os melhores cumprimentos,

P'la equipa de investigação,

Joana Leonor Pereira

(Contactos da investigadora)

NOME COMPLETO da CRIANÇA

MORADA

DATA de NASCIMENTO

SUBSISTEMA de SAÚDE / Nº

CONTACTO(s) TELEFÓNICO(s)

Annex VII: Study presentation letter

Ex.mo Sr. Encarregado de educação,

É convidado a autorizar o seu educando a participar voluntariamente no estudo "***Caracterização de perfis salivares metabólicos na cárie precoce da infância: implicações diagnósticas, preventivas e terapêuticas***" que decorrerá em estabelecimentos de ensino pré-escolar do distrito de Coimbra.

A cárie precoce da infância (CPI) é uma doença altamente prevalente que, dados os efeitos nefastos que assume nas crianças e respetivas famílias, representa um sério problema de saúde pública. Este estudo pretende **estudar saliva de crianças com e sem CPI ao longo de um ano**, em três períodos (inicialmente, aos 6 e aos 12 meses), com o objectivo de caracterizar e prever o aparecimento da doença.

Uma única examinadora, médica dentista, efetuará, no jardim de infância, a observação da boca das crianças e as colheitas de saliva de forma **não invasiva, rápida, totalmente indolor** e **sem risco** para a criança. Este estudo foi aprovado pelas Comissões de Ética da Faculdade de Medicina da Universidade de Coimbra (FMUC) e da Administração Regional de Saúde do Centro garantindo-se a proteção dos direitos e bem-estar das crianças.

Enviamos em anexo um documento intitulado consentimento informado que descreve a finalidade e os procedimentos do estudo. **É inteiramente livre de aceitar ou recusar participação do seu educando neste estudo.**

A participação do seu educando contribuirá para melhorar o conhecimento sobre a saúde oral infantil, particularmente no que diz respeito ao diagnóstico e progressão da CPI.

Após cada observação oral, informá-lo-emos acerca do **estado de saúde oral do seu educando**. Atendendo à necessidade de instituir, o mais cedo possível, tratamentos preventivos e/ou curativos evitando complicações mais severas, o Mestrado Integrado de Medicina Dentária da FMUC faculta, no âmbito da consulta da clínica de Odontopediatria, e de acordo com a disponibilidade de marcação, a realização deste tipo de tratamentos. **Caso esteja interessado/a que o seu educando possa usufruir de acompanhamento médico-dentário na consulta da clínica de Odontopediatria**, a decorrer nos Blocos de Celas, Coimbra, preencha por favor o destacável abaixo e faça-o chegar à Educadora responsável deste Jardim de Infância para posterior contacto para marcação.

Adicionalmente, propomo-nos implementar **sessões educativas destinadas aos cuidadores das crianças e educadores**, visando assumir um papel ativo na promoção da saúde oral, especificamente no que diz respeito à prevenção da CPI.

Caso esteja interessado em **autorizar a participação do seu educando**, deverá **assinar e devolver** no Jardim de Infância **uma cópia do consentimento informado dentro de um envelope fechado,** juntamente com os dados da criança participante **devidamente preenchidos** (documentos e envelope em anexo),

Estamos inteiramente disponíveis para quaisquer esclarecimentos adicionais.
Antecipadamente gratas,
P´la equipa de investigação

Joana Leonor Pereira, Ana Luísa Costa, Ana Gil
joana.leonor.pereira@gmail.com

NOME COMPLETO da CRIANÇA

MORADA

DATA de NASCIMENTO

SUBSISTEMA de SAÚDE / Nº

CONTACTO(s) TELEFÓNICO(s)

Annex VIII: Participant information sheet regarding medical, sociodemographic and oral health related behavior metadata

CARACTERIZAÇÃO DE PERFIS SALIVARES METABÓLICOS NA CÁRIE PRECOCE DA INFÂNCIA: IMPLICAÇÕES DIAGNÓSTICAS, PREVENTIVAS E TERAPÊUTICAS

Ex.mo(a) Sr.(a) Encarregado(a) de educação,

Muito obrigada por concordar em participar neste estudo, o sua participação é inteiramente **voluntária** e o **confidencialidade será sempre mantida**. Mesmo que os resultados do estudo sejam publicados, a **identificação dos participantes nunca será divulgada**.
Seria muito útil se pudesse responder a algumas perguntas sobre **o(a) seu(sua) educando(a)**; note que não existem respostas cenas ou erradas.

ESTADO DE SAÚDE DA CRIANÇA

1. O(a) seu(sua) educando(a) tem alguma(s) **doença(s) sistémica(s)**?
☐ Sim ☐ Não Em caso afirmativo, por favor indique qual(quais):

2. O(a) seu (sua) educando(a) tem alguma(s) **alteração do crescimento ou desenvolvimento**?
☐ Sim ☐ Não Em caso afirmativo, por favor indique qual(quais):

3. O(a) seu(sua) educando(a) faz alguma(s) **medicação atualmente**?
☐ Sim ☐ Não Em caso afirmativo, por favor indique qual(quais):

4. O(a) seu(sua) educando(a) tomou alguma(s) **medicação nas últimas duas semanas**?
☐ Sim ☐ Não Em caso afirmativo, por favor indique qual(quais):

5. O(a) seu(sua) educando(a) usou **dentífricos ou elixires antibacterianos nas últimas duas semanas**?
☐ Sim ☐ Não Em caso afirmativo, por favor indique qual(quais):

1

DADOS SOCIODEMOGRÁFICOS DA CRIANÇA

1. Quantas pessoas vivem com o(a) seu(sua) educando(a)? R: _____

2. O(a) seu(sua) educando(a) tem irmãos?
☐ Sim ☐ Não ☐ Não sabe / não responde

3. O(a) seu(sua) educando(a) é:
☐ o primeiro filho ☐ o segundo filho ☐ o terceiro filho ☐ o quarto filho ou mais ☐ Não sabe / não responde

4. Qual o país de origem do(a) seu(sua) educando(a)?
☐ Portugal ☐ País africano de Língua Portuguesa ☐ Brasil ☐ País de Leste da Europa ☐ Não sabe / não responde ☐ Outro Qual?
R: _____

5. Qual o pais de origem da mãe da criança?
☐ Portugal ☐ País africano de Língua Portuguesa ☐ Brasil ☐ País de Leste da Europa ☐ Não sabe / não responde ☐ Outro Qual? R:_____

6. Qual o pais de origem do pai da criança?
☐ Portugal ☐ País africano de Língua Portuguesa ☐ Brasil ☐ País de Leste da Europa ☐ Não sabe / não responde ☐ Outro Qual? R:_____

7. Indique a área de residência do(a) seu(sua) educando(a):
☐ Área rural ☐ Área urbana ☐ Não sabe / não responde

8. Refira qual o nível de instrução do pai e da mãe da criança.

	Pai	Mãe
Licenciatura. Mestrado ou Doutoramento		
Bacharelato		
I 2º ano, 9º ano ou entre 9 e 12 anos de escolaridade		
Entre o 5º e o 9º ano		
Menos de 4 anos de escolaridade ou sem instrução		

9. Dentro dos seguintes grupos de profissões, escolha o que melhor se aplica à situação profissional do pai e da mãe do criança.

	Pai	Mãe
Grandes empresários. Gestores de topo do sector público ou privado. Professores Universitários. Altos potentes militares. Profissões liberais com curso superior. Altos dirigentes políticos.		
Médios empresários. Dirigentes de empresas. Agricultores proprietários. Dirigentes intermédios e quadros técnicos do sector público ou privado. Oficiais dos Forças armados. Professores do Ensino Básico ou Secundário.		
Pequenos empresários. Quadros médios. Médios agricultores. Sargentos ou equiparados.		
Pequenos Agricultores e rendeiros. Técnicos Administrativos. Operários semiqualificados. Funcionários Públicos e membros dos Forças Armadas ou militarizados ainda não referidos.		
Assalariados agrícolas. Trabalhadores indiferenciados ou profissões ainda não referidas.		

HÁBITOS DE HIGIENE ORAL DA CRIANÇA

1. Quando foram iniciados os hábitos de escovagem dentária da criança?
   ☐ Antes do 1º ano de vida ☐ Entre o 1º e o 2º ano de vida ☐ Depois do 3º ano de vida ☐ Não escova ☐ Não sabe / não responde

2. Com que frequência é realizada a escovagem dentária na criança?
   ☐ Não escova ☐ Uma vez por dia ☐ Duas vezes por dia ☐ Mais de duas vezes por dia ☐ Não sabe / não responde

3. A criança tem auxílio durante a escovagem dentária?
   ☐ Não ☐ Sim ☐ Não sabe / não responde

4. Qual o tipo de dentífrico habitualmente utilizado?
   ☐ Dentífrico infantil ☐ Dentífrico de adulto ☐ Não sabe / não responde

3

5. A criança utiliza fio dentário?
   ☐ Não ☐ Sim ☐ Não sabe / não responde

6. Com que idade é que a criança teve a sua primeira consulta de Medicina Dentária?
   ☐ Antes do 1º ano de vida ☐ Entre o 1º e o 2º ano de vida ☐ Entre o 2º e o 3º ano de vida ☐ Entre o 3º e o 4º ano de vida ☐ Depois do 4º ano de vida ☐ Nunca foi ☐ Não sabe / não responde

7. Qual a regularidade habitual de ida ao Médico Dentista?
   ☐ Nunca foi ☐ Só quando tem queixas ☐ Regularmente, mesmo sem queixas ☐ Não sabe / não responde

HÁBITOS DIETÉTICOS DA CRIANÇA

1. A criança foi amamentada pela mãe após o 1º ano de vida?
   ☐ Nunca foi amamentada ☐ Não ☐ Sim ☐ Não sabe / não responde

2. Habitualmente, qual o número de refeições diárias da criança?
   ☐ Menor ou igual a 5 ☐ Mais do que 5 ☐ Não sabe / não responde

3. Qual a frequência habitual de ingestão de doces ou alimentos/bebidas açucaradas, por semana?
   ☐ Menor ou igual a 1 vez por semana ☐ Até 3 vezes por semana ☐ Diariamente ☐ Não sabe / não responde

Por favor **coloque este documento no envelope juntamente com o consentimento informado**, feche-o para garantir a confidencialidade dos dados e **entregue o envelope à educadora** do(a) seu(sua) educando(a).

Estamos inteiramente disponíveis para quaisquer esclarecimentos adicionais.

Antecipadamente gratas,
Pela equipa de investigação,
Joana Leonor Pereira, Ana Luísa Costa, Ana Maria Gil
joana.leonor.pereira@gmail.com

Annex IX: Oral examination assessment form

Metabolómica da saliva para o estudo da Cárie Precoce da Infância na criança

Código participante ____/____/_____ Jardim de Infância: _____

Data ____/____/_____

Número da observação: _____

Ficha de registo clínico

Diagnóstico de cárie atendendo aos critérios ICDAS-II
(Registo por dente: pior resultado das várias superfícies do mesmo)

	16	55	54	53	52	51	12	11	21	22	61	62	63	64	65	26
Restauração e selantes																
Cárie dentária																
Nº superfícies cariadas																
Presença de defeitos de esmalte																
Traumatismo dento-alveolar																

	46	85	84	83	82	81	42	41	31	32	71	72	73	74	75	36
Restauração e selantes																
Cárie dentária																
Nº superfícies cariadas																
Presença de defeitos de esmalte																
Traumatismo dento-alveolar																

Outras lesões

Pigmentação negra extrínseca: _____

Lesão aftosa / ulcerativa: _____

Fístula: _____ Abcesso: _____

Língua geográfica: _____ Glossite rombóide mediana: _____

Outras lesões - especificar: _____