Doctoral Dissertation

Doctoral Program in Bioengineering and Medical-Surgical Sciences (33rd Cycle)

Candidate Gene Analysis for Orthodontic Tooth Movement. Phase I: Gingival Crevicular Fluid Metabolomics

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To my husband Daniele

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SUMMARY

Aim:

Recent developments in genomic, proteomic and metabolomic approaches have facilitated sensitive analysis of saliva and GCF and are proving increasingly useful for diagnostics.

The aim of this study is to identify a pool of metabolites produced during the first days of Orthodontic Tooth Movement.

Materials and Methods:

A sample of healthy patients undergoing clear aligner orthodontic therapy were selected. The treatment plan provided a single-tooth and single-direction movement, using the contralateral tooth as control. GCF samples were obtained from pressure and tension sites of test tooth and from the mesiobuccal and distobuccal sites of the control tooth at different timings of OTM.

Metabolites concentration was quantified with NMR. Explorative statistical analysis was performed. Normality assumption of the data was evaluated with the Shapiro-Wilk test; homoscedasticity and autocorrelation of the variables were assessed using the Breusch-Pagan and Durbin-Watson tests. Linear regression analysis was performed. The level of significance was set at $p < 0.05$. Post hoc analysis, considering Bonferroni adjustment, was performed.

For each metabolite, unpaired and paired t-tests were carried on. Z-scores were computed, along with their standard errors. The distance among mean values was used to measure the effect size (Cohen's d).

Results

62 metabolites were found in this untargeted NMR analysis. At T1, for mesial sites, several metabolites were significantly down regulated for test teeth: Fucose 2, Lactate, Methionine1, GABALysine1, Putrescine, Taurine1, Taurine2, Uracil2. Glutamine2 was upregulated. For distal sites, some metabolites were significantly down regulated for test teeth: Glucose 2, Sucrose1 and Propionate. Tyrosine1was upregulated.

At T2 metabolites variations between test and control were: alpha-ketoglutarate1=-0,42 (p=0,0276) and tryptophane1=+0,43 (p=0,00835). For distal sites 5Aminovalerate2, Aspartate1, Fucose2 and Glutamate were significantly down regulated. The differences between test and control during follow up (T3-T0) were not evaluated for missing data.

Conclusions

The results obtained in this pilot study showed 62 metabolites found in the first days of OTM; statistical analyses showed variations of several metabolites at different time points; since no significant evidence can be supported with these data, further investigation should be carried on to prove diagnostic or prognostic value of GCF metabolites during OTM.

1. INTRODUCTION

1.1 Metabolomics and personalized medicine

Current clinical practice in the medical field is strongly based on the prognosis, diagnosis and treatment of diseases by methods determined and mediated for the specific sick population. Although this approach leads to a positive outcome in the majority of the population, misdiagnoses, treatment failure and recurrence are common events in many individuals. This phenomenon can be explained by the individual variation found at the level of a patient's genome, transcriptome, proteome and metabolome: the various "omics" approaches aim to investigate the influence of these factors on a molecular scale, with the intention of developing personalized approaches both from the point of view of diagnosis and treatment.

"Omics" is a generic term to describe the study of all genes (genomics), the transcription of these genes (transcriptomics), their translation into their respective proteins (proteomics) and all resulting changes in metabolites (metabolomics) and is aimed at acquiring large-scale data obtained from a single or multiple sample(s)[1].

Because of genetic variables and a variety of other factors including sex and environmental factors and behavior, in fact, not all individuals are equally affected by the same disease nor do all individuals respond to treatment in the same way[2].

Although inter-individual variations may not be so obvious in the initial clinical presentation of the disease, they are most likely to be detectable on a molecular scale.

Traditionally, genomics was considered the most important approach to determining individual characteristics, but there are several intermediate processes between the genotype and phenotype that can influence the development of the disease or the response to treatment: these processes include transcription, translation, and metabolism.

Metabolomics has been the most recently introduced discipline of the domain "omics" and is the closest to the observed phenotype: it reflects the changes that occur at all molecular levels, as well as the influences arising from internal and external factors. By definition, metabolomics is the unbiased identification and characterization of all small molecular compounds (metabolites) in a biological system, through highly sensitive analytical techniques in combination with bioinformatics technologies[3].

Metabolites are products of the physiological metabolism (intermediate reaction) driving significant biological activities in the human body such as signaling, stimulation and inhibition.

Metabolites influence enzymatic activity (sometimes acting as cofactors) and promote intercellular interactions: therefore, their concentration is a complex function of many different processes that take place within the cell.

The metabolome, a collective term for all metabolites of a specific biological system or sample, is the final result of gene expression

(transcription and translation) and therefore reflects changes in the genome, transcriptome and proteome. The identification of the main differences in the metabolome of two different sample groups could be a promising starting point for the discovery of new biomarkers. While metabolomics has been introduced in periodontology in order to identify possible markers of the periodontitis, at the best of our knowledge little is known about the potentials of this approach in orthodontics.

An orthodontic force produces a tissue reaction that involves alveolar bone and periodontal ligament (PDL): in these structures there are cells containing molecules sensitive to mechanical stimuli and structures able to respond to physical changes induced by forces at the level of the extracellular matrix (ECM).

In relation to mineralized tissue, osteocytes play a major role in alveolar bone remodeling: they influence osteoblasts and osteoclasts action, determining bone formation and bone resorption respectively.[4]

At the same time, alterations in the gingival architecture activate purino-receptors that promote the activity of osteoclasts[5].

As already mentioned, non-mineralized tissues undergo changes as a result of the application of orthodontic forces: fibroblasts are mainly involved in events leading to PDL remodeling. OTM alters the neutral state of mechanoreceptors and nociceptors of nerve fibers at PDL level: these somatosensory neurons transmit signals from peripheral tissues to the central nervous system, causing the release of biologically active proteins and thus contributing to the development of local inflammation of neurogenic origin[6].

The physical distortion of nerve endings in periodontal tissues is an important event that occurs early in the chain of events leading to the activation of the remodeling process that is intimately associated with neo-vascularization.

Mechanical stresses applied through orthodontic treatment cause remodeling of the ECM, activating certain signaling pathways mainly through integrins-dependent mechanisms[7].

The entire process is self-limiting when sufficient changes have occurred to support the involved structures.

The above mentioned processes are very complex since each tissue contains its own cellular networks and its own extracellular elements, the remodeling of which, in a coordinated manner, is necessary for the orthodontic movement to occur.

To sum up, when the applied stresses reach certain magnitudes and frequencies, the deformations generated are transducted into biochemical and molecular cellular activities, aimed at achieving a new balance: in particular, in the bone this deformation induces cell proliferation, osteoblastic differentiation and, finally, bone formation[7].

A fundamental condition for remodeling, and ultimately tooth displacement, is the occurrence of an inflammatory process in the periodontal and dental pulp, in response to the mechanical perturbation caused by orthodontic forces.

Recent data suggest that cellular/tissue stress or damage related products can trigger an aseptic inflammatory response.

Their increased levels during OTM led to the hypothesis that interactions between the cells of the nervous, immune and endocrine systems regulate the biological responses occurring after the application of orthodontic forces.

Increased levels of inflammatory mediators of immunological origin , such as prostaglandins (PGs), interleukins (IL-1, IL-6, IL-17) and cytokines of the TNF-α superfamily, which includes the RANK/RANKL/OPG system, have been identified at the PDL and dental pulp levels following the mechanical perturbation induced by an orthodontic force.

Levels of RANKL (RANK-ligand) in the GCF during orthodontic movement (especially in the first 24 hours after force application) increased, while levels of osteoprotegerin (OPG) decreased, suggesting increased bone resorption.[8, 9]

The appearance of osteoclasts is the first step in this remodeling process and bone resorption is a much faster process than bone apposition: it may take three months to replace bone that has been resorbed in just two or three weeks[9].

The biological markers that develop within the oral fluids during orthodontic movement can be classified into three main categories: periodontal tissue remodeling metabolic products, inflammatory mediators, enzymes and enzyme-derived inhibitors from the patient.

In order to develop useful clinical instruments, these markers need to be quantifiable and linked to clinically significant phenomena, such as the type and speed of OTM.

1.2 Gingival Crevicular Fluid (GCF)

Both saliva and GCF are potential economic sources of personal oral information, easy to collect in a non-invasive way.

The production of gingival crevicular fluid, in the model proposed by Pashley[10] is determined by the fluid passage from the capillaries to the tissues (capillary filtered) and by the removal of this fluid by the lymphatic system. Descriptive and mathematical models of GCF, supported by subsequent experimental results, indicate that the initial fluid accumulated in a healthy gingival sulcus is an interstitial fluid exudate, while in inflammatory or stimulated conditions, GCF is an exudate that reflects the concentrations of metabolites within serum.[11, 12]

As already widely explained, orthodontic forces cause a local inflammatory state and increase capillary permeability locally: since GCF is located closer than saliva to the sites where these processes take place, and is probably also less diluted, it has a greater diagnostic potential than saliva for biological markers of these activities. [13]

The application of orthodontic forces generates changes in the expression of hundreds of genes belonging to cells contained in periodontal tissues. Expression of some genes has been reported, including those that regulate the production of neurotransmitters, signaling molecules, extracellular matrix components, cytokines, chemokines, growth factors, transcription factors, proteases, proteins associated with mineralization and many other molecules that regulate PDL remodeling and bone remodeling to achieve OTM[14]. Understanding these changes and the sequence of events responsible for the

expression of these genes during OTM is critical to fully understand this process, to develop new therapies, to optimize treatment results and to prevent unwanted side effects.

Genetic influences on orthodontic movement have been reported in the existing literature: the presence of allele 2 of IL-1B is associated with a faster orthodontic movement[15, 16] and in the context of the primary failure of eruption a considerable number of cases of parathyroid hormone (PT1HR) receptor 1 mutation has been found. In the literature, gene correlations can also be found with regard to external root resorption (EARR), one of the most severe complications of orthodontic treatment, which is difficult to diagnose at an early stage by radiographic examination alone: in the conclusions of a study by Al-Qawasmi[17] it can be seen that allele 1 of IL-1β predisposes subjects to EARR.

We can therefore conclude that personalized medicine or precision medicine is an emerging approach to the prevention and treatment of diseases that takes into account the individual variety in genes, in each person's environment and lifestyle. Customizing a treatment does not necessarily mean creating a unique drug or medical device for a patient, but it presupposes the classification of patients into subpopulations that differ in their susceptibility to a given pathology/condition or in their response to a specific treatment[18].

In particular, precision orthodontics extends beyond the classic definition of precision medicine by using customized devices and forces.

1.3 Metabolite analysis – H-NMR

The most used techniques for the analysis of metabolites are gas chromatography (GC) and liquid chromatography (LC) coupled with different mass spectrometry detectors (MS) and nuclear magnetic resonance spectroscopy (NMR): these methodologies can be considered complementary, having both their advantages and disadvantages.

Mass spectrometry is more sensitive than nuclear magnetic resonance, but also more selective towards the class of metabolites that can be observed with a given technique and less quantitative.

On the contrary, NMR spectroscopy allows to identify and at the same time to quantify a wide spectrum of organic compounds within the micromolar with a minimum sample preparation, which allows their reuse for further analysis[19].

NMR spectroscopy is therefore an ideal technique for initial metabolomic investigation and its use is now well established for characterize the metabolic profile of a biological fluid and to investigate how it changes the metabolic profile in response to a biological and/or clinical stimulus, although it has a relatively low sensitivity as a limit, such as to make it inappropriate for the analysis of a large number of metabolites present in small quantities

1.4 Anabolism and catabolism

One reason why this analysis of the metabolites present within the GCF can be very useful is to observe whether or not there is a difference

between the metabolites present in the anabolic site and those present in the catabolic site.

Anabolism is one of the two parts of metabolism and includes the set of processes of synthesis of organic molecules more complex than simple ones or nutrients.

Catabolism, on the other hand, represents the totality of metabolic processes whose products are structurally simpler and poorer in energy, releasing the excess in the form of chemical energy (ATP) and thermic energy.

At the level of orthodontic movement, anabolism and catabolism can be synthetically identified in the action of osteoblasts, which cause the apposition of bone (anabolism), and osteoclasts, which cause bone resorption (catabolism).

In the 2009 study by Cattaneo et al. [20] of finite elements generated by micro-CT, it was seen that bone formation is the result of an increase in the loading state in alveolar bone and elongated PDL fibers, while bone resorption is the result of an loaded/unloaded state[21]. Therefore, it can be simplified with a model in which in the compression areas resorption will occur and in the traction areas affixed. However, the same study states that this model is too simplified to reproduce what really happens, because during orthodontic movement it is difficult for an area to be fully compressed or fully tensed.

Moreover, as stated from Alikhani et al. [22] in explaining the Biphasic theory, initial days of OTM are characterized by catabolism all around

tooth perimeter, both in tension and compression areas, while anabolism starts 10-15 days after the orthodontic force application. Based on these observations, it is logical to assume that the biologic response during tooth movement comprises two clearly separated phases that are not strictly site specific.[23]

1.5 Orthodontic appliances

A precise prescription of force application and predictability in prescription expression can be moderately accurate with distalization in Clear Aligner Therapy (CAT)[24]. Castroflorio et al have previously used this technique[25].

Another issue emerging form the reviewed literature is represented by the lack of uniformity of the investigated biomarkers. These inhomogeneous targets do not allow a proper comparison between studies, except for Barbieri et al [26] and Castroflorio et al.[25]. The results of these two studies highlighted the characterization of the anabolic site and of the catabolic site.

Since other orthodontic techniques to distalize upper molars highlighted different factors contributing to intra-arch force dissipation in case of a single movement-single direction prescription or confounding elements, as the plaque that can most easily be deposited around the elastomeric devices resulting in increased inflammation and therefore changing at biomarkers' level, we decided to follow the path opened by these two articles and, thanks to the introduction of new technologies in the analytical field, a new protocol, potentially leading to even more accurate results, was designed.

The innovation is represented by the analysis of metabolites at the periodontium level during orthodontic treatment.

1.6 Aim and Objectives

To date, we cannot accurately predict which individuals will respond in an efficient way to orthodontic treatment from a biological and metabolomic point of view, including response of bone, periodontal and soft tissues.

The aim of this study is to clarify how and how much light forces applied to teeth can affect an ECM response, mineralized and non-mineralized bone components reaction, blood vessels and neuronal tissues responses, This analysis will be carried out by focusing on the metabolites in GCF at different timing of initial application of orthodontic forces. In addition, a protocol to investigate metabolomics in OTM by GCF is presented.

With the present study, we aim to develop a novel, sensitive and reliable method to diagnose and predict endogenous factors influencing biologic response to OTM. We hypothesize that the spectra of metabolites in the inflammatory transudates and exudates around teeth are predictors of future bone reaction to forces stimuli. To test this hypothesis, we have collected gingival fluid exudate samples from subjects undergoing orthodontic treatment and use nuclear magnetic resonance (NMR) spectroscopy to achieve these two specific aims:

The first objective is to use proton nuclear magnetic resonance (H-NMR) spectroscopy in a cross-sectional study to define a unique set of metabolites in the GCF produced during the initial phase of OTM.

The second objective is to profile metabolites that can discriminate between bad responder and good responder patients to OTM by measuring proportional changes of key metabolites relative to orthodontic movement progression over time. This achievement will be performed by stratifying data obtained by sampling site, age, sex, eventually additional devices nowadays used to accelerate tooth movement and age, considering existing literature on different GCF composition in growing and non-growing patients undergoing orthodontic treatment[21].

2. MATERIALS AND METHODS

This randomized prospective clinical pilot study was conducted according to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for prospective case–control studies (Figure 1). This study was performed in accordance with the Declaration of Helsinki; the protocol was approved by Ethics Board of The Health And Science City of Turin (Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino), 15/09/2016, Prot. ref: # 0089263); a signed informed consent was obtained from the patients or patients' parents, before collecting the data, and researchers provided to protect the privacy.

2.1.1 Sample selection

In this prospective-controlled study, a total of 30 healthy Caucasian Stainer Class I, II, III subjects undergoing orthodontic treatment were recruited. Patients were treated by 1 expert orthodontist (TC) in Dental School, Department of Surgical Sciences, Orthodontic Division, University of Turin. The recruiting period lasted from February 2018 to February 2020 (due to Sars-Cov-2 pandemic emergency), patients age ranged from 15 to 32 yo; the average sampling period was 3 weeks.

2.1.2 Inclusion/Exclusion criteria

Participants were recruited from the Department of Orthodontics of CIR Dental School of the University of Torino.

Eligibility criteria for inclusion in the study were:(1) good health status, (2) previous 3rd upper molar extraction if present, (3) molar half or full Class II relationship, (4) mesodivergent craniofacial typology (SpP-GoGn Angle = 25° ± 6°), (5) good control of oral hygiene (value= 0 according to Greene-Vermillion index [27]), (6) no antinflammatory or antibiotics therapy during previous 6 months to T0.

Exclusion criteria were (1) smoking habit and (2) signs of gingivitis or periodontitis, (3) Clinical attachment loss ≥ 2mm at T0 for selected sampling teeth (4) Pocket depth ≥ 4mm for selected sampling teeth, (5) anti-inflammatory or antibiotic therapy in the previous 6 months

2.1.3 Study design

The random split mouth design of the present study allowed to recruit the same patients both for study group and control group. Upper second molars were randomly set as test tooth and control tooth respectively for each patient.

Phase 1 and Phase 2 were set. During phase 1 were set for sampling metabolites from GCF, phase 2 was set as mGwas part of the study.

2.2 PHASE I – Sampling metabolites

Two weeks before beginning the phase 1 of the study, all the subjects underwent a supragingival prophylaxis and were given oral hygiene instructions to follow at home to eliminate inflammation.

The study for each patient lasted for 21 days, for 4 sampling timings.

At each visit (T0, T1, T2, T3), pocket depths and clinical attachment levels on teeth (PD,CAL), full mouth Silness and Loe Plaque (PI) Index, Lobene Modified Gingival Index (GI)[28], and Bleeding on Probing (BOP) Index were recorded [29]. The BOP Index was evaluated after GCF sampling to avoid possible variations due to periodontal measurements' compression.

GCF samples were obtained both from pressure and tension sites of test tooth and from the mesiobuccal and distobuccal sites of the control tooth at each timing (4 sample sites per timing):

- before 1st aligner delivery (T0);
- 1 hour after 1st aligner delivery (T1);
- after 7 days from 1st aligner delivery (T2);
- after 21 days from 1st aligner delivery (T3).

These timings were chosen as at day 7th from the beginning of orthodontic forces application, indirect resorption starts and at day 21st metabolites turnover occurs[30].

On the basis of this evidence [30], additional samples at T4, T5, T6, T7 were collected for 8 patients already tested for T0, T1, T2, T3, switching the test 2nd upper molar to control 2nd upper molar and vice versa. A

proper prescription adjustment with no movement prescribed for2nd aligner worn for 21 days and 3rd aligner with single tooth distalization was performed.

GCF samples were obtained both from pressure and tension sites of test tooth and from the mesiobuccal and distobuccal sites of the control tooth at each timing (4 sample sites per timing):

- before 3rd aligner delivery (T4);
- 1 hour after 3rd aligner delivery (T5);
- after 7 days from 3rd aligner delivery (T6);
- after 21 days from 3rd aligner delivery (T7).

For these last 4 timings, patients were asked to use Acceledent Aura (Propel Orthodontics LLC, Milpitas, CA, U.S.A.) for 20 minutes per day.

A total of 588 samples were collected from 30 patients; due to pandemic Sars-Cov 2 emergency, 420 were shipped to University of Minnesota and 56 were analyzed.

After isolating teeth with cotton rolls to avoid any contamination with saliva, removing plaque with curette without touching gingival margins, pushing placque away from subgingival sites and gently drying teeth with a light stream of airflow, GCF was collected with Sterlitech Silver Membrane (Sterlitech Corporation, Kent, USA), 5.0 Micron, 5/32” (3.96 mm diameter).

The silver membrane was pushed inside gingival sulcus and kept for at least 30 seconds or until the complete absorption of GCF was achieved.

Silver membranes (99,97% pure silver) used for sampling have 0,2-0,5 micron pores to collect metabolites and have many advantages: the most important is that silver-nanoparticles coverage can prevent bacterial proliferation and mycobacterial biofilm growth on filtering membrane material[31].

A pool of metabolites were detected and their concentration quantified at different timings with NMR, thus studying low molecular weight compounds, with relatively high sensitivity, a quick analysis and rapid identification with minimal sample preparation and non-biased information on multiple components of biofluids.

2.3 PHASE II – mGWAS

Saliva sample for genomic DNA detection should have been collected in a separate timing with ORAGENE DNA collection kit (DNA Genotek Inc., a subsidiary of OraSure Technologies, Inc., Bethlehem, Pennsylvania, USA).

Levels of the obtained metabolites should have been used to differentiate bone reaction under orthodontic forces phenotypes; then untargeted metabolomics-based GWAS should have been performed for identifying metabolite–gene associations.

This genetic resource would had made possible to regard detected metabolites as phenotypic traits, resulting in high-resolution maps of genomic regions associated with metabolite variation, namely metabolic quantitative trait loci (mQTL).

2.1.4 Intervention

The standardized orthodontic intervention for all the patients involved, was performed with clear aligner therapy (Invisalign® Align Technology, San Jose, CA, USA). The treatment was set to provide a single-tooth and single-direction distalization movement of test tooth. The contralateral tooth was considered as control. All the treatment plans were designed and reviewed by the same operator with ClinCheck® software (Align Technology, San Jose, CA, USA) and staging for test tooth distalization was set at 0.25 mm per aligner. Compliance indicators embedded in each posterior segment of the maxillary aligner were monitored during the study. In accordance with the existing literature, the initial distalizing force was 1 N[32].

2.1.5 NMR spectroscopy metabolites analysis: Analysis of Proton Nuclear Magnetic Resonance Output Data

Samples were shipped to University of Minnesota for NMR analysis. After a simple preparation (elution in 50 µL of 1 mM DSS standard in PBS-Deuterium2O buffer), immediately prior to acquisition, each sample was heated to 25°C. A 700 MHz Bruker Advance III NMR spectrometer with a 1.7mm TCI cryoprobe was used to obtain NMR spectra profiles in PISF, saliva and positive/negative control samples. Two-dimensional (2D) NMR spectroscopy of control buffer (A) and of gingival crevicular fluid extracts from control and distalizing upper 2nd molars, both overlaid on the negative control sample. A gradient-enhanced two-dimensional total correlation spectroscopy (2D 1H-1H TOCSY) pulse sequence with water

suppression by excitation sculpting using 32 transients and 128 increments and a 7,000 Hz spectral width in each dimension was utilized.

The NMR signal acquisition was performed after water suppression pulse, then a “rNMR” software was used to process the 2D NMR data. The patients spectra were overlaid onto buffer control spectra (buffer control eluted from silver membrane).

Regions of interest (ROIs) were defined based on Total Correlation Spectroscopy (TOCSY) data from public databases, Madison-Qingdao Metabolomics Consortium Database (MMCD) and Human Metabolome Database (HMDB), corresponding to 77 potential metabolites that had been identified in previous literature as being present in GCF/PISF and saliva samples. Only ROIs that were calculated based on assumptions of non-overlapping resonances with other compounds or glycerol contaminants and not impacted by artifacts from poor water suppression were used for further analysis. Signal intensities for each ROI in PISF spectra were generated using rNMR software. A total of 35 PISF metabolites were assigned. ROIs were not defined on cross-peaks that appeared in the spectra for buffer control samples. The maximum intensity for each ROI was recorded to a spreadsheet. Normalization and statistical analysis of data was performed with the web based MetaboAnalyst 2.0 software suite (Wishart Research Group, U Alberta, Canada). Binned samples were filtered using interquartile range and normalized to a pooled sample of case subjects receiving orthodontic treatment.

2.1.6 Statistical Analysis

These data were log transformed to consent the FC evaluation. Normality assumption of the data was evaluated with the Shapiro-Wilk test; homoscedasticity and autocorrelation of the variables were assessed using the Breusch-Pagan and Durbin-Watson tests. Linear regression analysis was performed to estimate the differences between groups. LogFC was used as estimate of the linear model. Values were showed as Mean ± SD. The level of significance was set at $p < 0.05$. Post hoc analyses, considering Bonferroni adjustment, was performed on the total biomarker list. Correlation between covariates was showed for the variable selection explanation. Statistical analyses were conducted using the R statistical package (version 3.5.3, R Core Team, Foundation for Statistical Computing, Vienna, Austria).

NMR data were first matched by patient ID computing the concentration variation within the two-time intervals of interest (namely $\Delta_1=T_1-T_0$ and $\Delta_2=T_2-T_0$) in both control and test conditions, and for mesial and distal collection sites separately. Afterwards, for each one of the 62 metabolites taken into consideration, marginal paired *t*-tests were carried on to compare metabolite changes in saliva from control tooth with the corresponding variation observed in the samples from the treated tooth of the same patient (two-tailed, paired *t*-test). Since some patients were only present in one of the two groups, for each metabolite a two-independent sample *t*-test was also carried on as a suitable alternative to detect a possible distalization effect by maximizing sample size. To make concentration changes comparable across the different

metabolites, *z*-scores (i.e. standardized values with mean = 0 and standard deviation = 1) were computed for each metabolite and group means, along with their standard errors, were plotted for control and test groups. Finally, the distance among these mean values was used to measure the effect size (Cohen's *d*) induced by the treatment and determine the most affected metabolites.

3. RESULTS

A total of 30 subjects undergoing clear aligner treatment were recruited. These patients were part of both control and test groups, since a single tooth-single direction movement was prescribed as the study design was set as a split mouth one.

GCF samples collected were 608, including all the timings, test and control groups and different sampling sites.

Since only 56 samples were analyzed, from 6 patients and mainly at 3 timings [T0 6 patients (baseline), T1 6 patients (1 hour after), T2 4 patients (7 days after), T3 2 patients (3 weeks after)], these results helped in building the initial statistical model for further analyses; in this study we will present preliminary data of an explorative statistic dataset based on 56 analyzed samples.

Demographic characteristics of the analyzed subjects and the clinical parameters at T0 are summarized in Table 1. No CAL and PD>4mm were recorded at T0 and consecutive timings. A total of 62 metabolites were found in this untargeted H1-NMR analysis.

	study group/ control group
subjects (n°)	6
age (y,m)	18,1
sex (f/m)	4 f / 2 m
BOP (%)	0 ± 0
Plaque (%)	2,39 ± 0,47
PD (mm)	2,3 ± 0,3
CAL (mm)	0 ± 0

Table 1. Clinical demopragphic characteristics of the study and control group.

3.1 Metabolites variation T1-T0 – test vs control

As described in Table 2, 1 hour after the first sampling, many metabolites showed significant decrease between test and control teeth at T1 with respect to T0.

Differences between test and control during follow-up (T1-T0).					
Biomarker	MD	Std. Error	P value	95%CI L	95%CI U
X1.3.Diaminopropane.1	-1,53	0,61	0,024800	-2,74	-0,33
X1.3.Diaminopropane.2	-1,10	0,52	0,052000	-2,13	-0,07
X5.Aminovalerate.1	-1,32	0,59	0,040000	-2,47	-0,17
X5.Aminovalerate.2	-1,24	0,60	0,056000	-2,41	-0,06
Alanine.Lysine.1	-1,51	0,82	0,080000	-3,11	0,09
Alanine.Lysine.2	-1,49	0,79	0,080000	-3,05	0,06
Alpha.ketoglutarate.1	-1,45	0,58	0,020000	-2,59	-0,32
Alpha.ketoglutarate.2	-1,31	0,55	0,030000	-2,40	-0,23
Arginine.1	-1,56	0,66	0,030000	-2,86	-0,26
Arginine.2	-1,58	0,67	0,032000	-2,88	-0,27
Aspartate.1	-1,45	0,72	0,064000	-2,88	-0,03
Aspartate.2	-1,43	0,64	0,040400	-2,68	-0,18
Betaine.1	-1,47	0,57	0,021400	-2,60	-0,35
Betaine.2	-0,92	0,79	0,264000	-2,48	0,63
Biotin.1	-1,37	0,62	0,044000	-2,60	-0,15
Biotin.2	-1,42	0,59	0,031500	-2,59	-2,24
Cadaverine.Lysine.1	-1,60	0,71	0,038700	-2,99	-0,21
Cadaverine.Lysine.2	-1,48	0,65	0,036900	-2,76	-0,21
Choline	-1,31	0,64	0,059800	-2,57	-0,05
Creatine	-1,38	0,67	0,057300	-2,70	-0,07
Formate	-1,14	0,64	0,095100	-2,39	0,11
Fucose.1	-1,29	0,60	0,047500	-2,47	0,12
Fucose.2	-1,55	0,60	0,022000	-2,73	-0,36

GABA.Lysine.1	-1,44	0,74	0,070800	-2,89	0,01
GABA.Lysine.2	-1,32	0,68	0,071900	-2,66	0,02
Glucose.1	-1,40	0,57	0,026300	-2,51	-0,28
Glucose.2	-1,58	0,56	0,013100	-2,68	-0,48
Glutamate	-1,40	0,69	0,060500	-2,76	-0,05
Glutamine.1	-1,50	0,60	0,025600	-2,68	-0,31
Glutamine.2	-1,04	0,58	0,095900	-2,18	0,11
Isoleucine.1	-1,41	0,69	0,057800	-2,76	-0,06
Isoleucine.2	-1,29	0,59	0,046300	-2,46	-0,13
Lactate	-1,41	0,65	0,047400	-2,70	-0,13
Leucine.1	-1,47	0,68	0,046900	-2,80	-0,14
Leucine.2	-1,38	0,60	0,037400	-2,55	-0,19
Methionine.1	-1,45	0,59	0,028600	-2,62	-0,28
Methionine.2	-1,44	0,58	0,025800	-2,59	-0,30
N.Acetylneuraminate.1	-1,48	0,55	0,017700	-2,58	-0,39
N.Acetylneuraminate.2	-1,10	0,58	0,077000	-2,24	0,03
Proline.1	-1,34	0,61	0,045100	-2,54	0,01
Proline.2	-1,38	0,61	0,038900	-2,57	-0,18
Propionate	-1,44	0,62	0,033700	-2,65	-0,23
Putrescine.Lysine	-1,56	0,81	0,073600	-3,16	0,03
Sucrose.1	-1,54	0,56	0,015000	-2,64	-0,44
Taurine.1	-1,45	0,67	0,046500	-2,77	-0,14
Taurine.2	-1,38	0,68	0,060100	-2,72	-0,05
Threonine.1	-1,56	0,68	0,038100	-2,90	-0,22
Threonine.2	-1,39	0,61	0,039600	-2,60	-0,18

Trehalose.1	-1,03	0,88	0,265000	-2,76	0,71
Trehalose.2	-2,38	0,54	0,000544	-3,45	-1,31
Tryptophan.1	-1,20	0,59	0,059600	-2,36	-0,05
Tryptophan.2	-1,35	0,57	0,031000	-2,47	-0,24
Tyrosine.1	-1,27	0,58	0,047100	-2,41	-0,11
Tyrosine.2	-1,39	0,60	0,035400	-2,57	-0,21
Uracil.1	-1,43	0,61	0,033100	-2,62	-0,23
Uracil.2	-1,47	0,58	0,023800	-2,61	-0,32
Uridine.1	-1,24	0,56	0,043500	-2,34	-0,14
Uridine.2	-1,37	0,57	0,029000	-2,49	-0,26
Valine.1	-1,36	0,69	0,067700	-1,72	-0,01
Valine.2	-1,25	0,78	0,130000	-2,79	0,28
Tyramine.1	-1,53	0,74	0,056200	-2,98	-0,08
Tyramine.2	-1,67	0,76	0,044100	-3,17	-0,18

Control group was used as reference. Log transformed values were considered.

Table 2. Differences between test and control at T1

3.2 Metabolites variation T2-T0 – test vs control

In Table 3, at T2 (1 week after the first sampling), the metabolites that showed variations between test and control were only 2: alpha-ketoglutarate1 -0,42 (p=0,0276) and tryptophane1 +0,43 (p=0,00835).

The differences between test and control during follow up (T3-T0) were not evaluated for lack of data.

Differences between test and control during follow up (T2-T0)					
Biomarker	MD	Std. Error	P value	95%CI L	95%CI U
X1.3.Diaminopropane.1	-0,081	0,155	0,613	-0,39	0,22
X1.3.Diaminopropane.2	0,13	0,21	0,554	-0,28	0,54
X5.Aminovalerate.1	0,1	0,1	0,366	-0,11	0,31
X5.Aminovalerate.2	-0,09	0,12	0,493	-0,33	0,15
Alanine.Lysine.1	-0,16	0,45	0,73	-1,05	0,72
Alanine.Lysine.2	-0,16	0,48	0,748	-1,1	0,78
Alpha.ketoglutarate.1	-0,42	0,15	0,0276	-0,78	-0,11
Alpha.ketoglutarate.2	-0,23	0,16	0,196	-0,54	0,088
Arginine.1	-0,123	0,15	0,426	-0,41	0,16
Arginine.2	-0,19	0,16	0,27	-0,51	0,12
Aspartate.1	-0,3	0,34	0,414	-0,98	0,38
Aspartate.2	-0,34	0,22	0,15	-0,77	0,08
Betaine.1	-0,15	0,27	0,571	-0,68	0,35
Betaine.2	0,34	0,35	0,35	-0,34	1,04
Biotin.1	-0,04	0,12	0,76	-0,28	0,21
Biotin.2	-0,00375	0,004163	0,394	-0,11	0,44
Cadaverine.Lysine.1	-0,246	0,33	0,479	-0,89	0,4
Cadaverine.Lysine.2	-0,246	0,33	0,479	-0,89	0,4
Choline	-0,13	0,24	0,61	-0,6	0,34
Creatine	-0,12	0,27	0,68	-0,65	0,42
Formate	0,22	0,28	0,441	-0,32	0,78
Fucose.1	-0,34	0,26	0,226	-0,85	0,17
Fucose.2	-0,15	0,12	0,236	-0,39	0,08

GABA.Lysine.1	-0,09	0,415	0,83	-0,98	0,78
GABA.Lysine.2	0,005	0,39	0,99	-0,77	0,78
Glucose.1	0,12	0,27	0,65	-0,4	0,65
Glucose.2	0,1	0,2	0,64	-0,3	0,5
Glutamate	-0,19	0,32	0,56	-0,83	0,44
Glutamine.1	-0,18	0,11	0,151	-0,41	0,04
Glutamine.2	-0,44	0,42	0,32	-1,27	0,38
Isoleucine.1	-0,13	0,27	0,64	-0,66	0,4
Isoleucine.2	-0,12	0,2	0,569	-0,51	0,27
Lactate	0,13	0,18	0,48	-0,22	0,49
Leucine.1	-0,44	0,34	0,23	-1,12	0,22
Leucine.2	-0,13	0,15	0,41	-0,43	0,16
Methionine.1	-0,11	0,15	0,46	-0,41	0,17
Methionine.2	0,033	0,12	0,801	-0,21	0,28
N.Acetylneuraminate.1	-0,11	0,16	0,5	-0,43	0,2
N.Acetylneuraminate.2	0,037	0,12	0,78	-0,21	0,29
Proline.1	-0,14	0,16	0,42	-0,45	0,17
Proline.2	-0,12	0,13	0,37	-0,38	0,13
Propionate	0,097	0,23	0,69	-0,36	0,56
Putrescine.Lysine	-0,06	0,48	0,9	-1,009	0,89
Sucrose.1	-0,036	0,07	0,64	-0,18	0,11
Taurine.1					
Taurine.2	0,07	0,32	0,828	-0,559	0,7
Threonine.1	-0,24	0,509	0,64	-1,24	0,76
Threonine.2	0,065	0,36	0,86	-0,64	0,77

Trehalose.1	0,28	0,19	0,177	-0,09	0,66
Trehalose.2	-0,212	0,18	0,27	-0,566	0,14
Tryptophan.1	0,43	0,12	0,00835	0,19	0,68
Tryptophan.2	0,048	0,15	0,76	-0,25	0,349
Tyrosine.1	0,12	0,1	0,239	-0,069	0,32
Tyrosine.2	0,018	0,05	0,737	-0,085	0,12
Uracil.1	0,02667	0,13	0,849	-0,238	0,29
Uracil.2	0,25	0,14	0,126	-0,037	0,544
Uridine.1	0,15	0,1	0,167	-0,044	0,35
Uridine.2	-0,045	0,1	0,67	-0,248	0,15
Valine.1	-0,21	0,32	0,531	-0,84	0,42
Valine.2	-0,21	0,43	0,63	-1,057	0,62
Tyramine.1	-0,29	0,37	0,44	-1,02	0,427
Tyramine.2	-0,18	0,39	0,65	-0,9	0,58

Control group was used as reference. Log transformed values were considered.

Table 3 Differences between test and control at T2

3.3 T-test Mesial and Distal site at T1-T0: paired/unpaired

In Table 4 we highlighted the significant results obtained with paired and unpaired t-tests to measure metabolites variations for the mesial and distal sites at T1 with respect with T0.

MESIAL			DISTAL		
Metabolite	p Value	t-test analysis	Metabolite	p Value	t-test analysis
Gaba Lysine1	p=0,042659	paired	Glucose2	p=0,032944	paired
Taurine2	p=0,03866	paired	Propionate	p=0,01799475	paired
Uracil2	p=0,0225	paired	Sucrose1	p=0,034529	paired
Methionine1	p=0,002788504	paired	Tyrosine1	p=0,02475	paired
Putrescine.Lysine	p=0,031695029	paired			
Fucose2	p=0,0226	unpaired			
Glutamine 2	p=0,011577089	unpaired			
Lactate2	p=0,005816732	unpaired			
Taurine1	p=0,019979	unpaired			
Taurine2	p=0,0285	unpaired			

Table 4 Differences between test and control groups at T1 for mesial and distal sites

For mesial sites, several metabolites were significantly down regulated for test teeth at T1 with respect with control teeth: Fucose 2, Lactate, Methionine1, GABALysine1, Putrescine, Taurine 1, Taurine 2, Uracil2. Only 1 metabolite was upregulated: Glutamine2 (Figure 1).

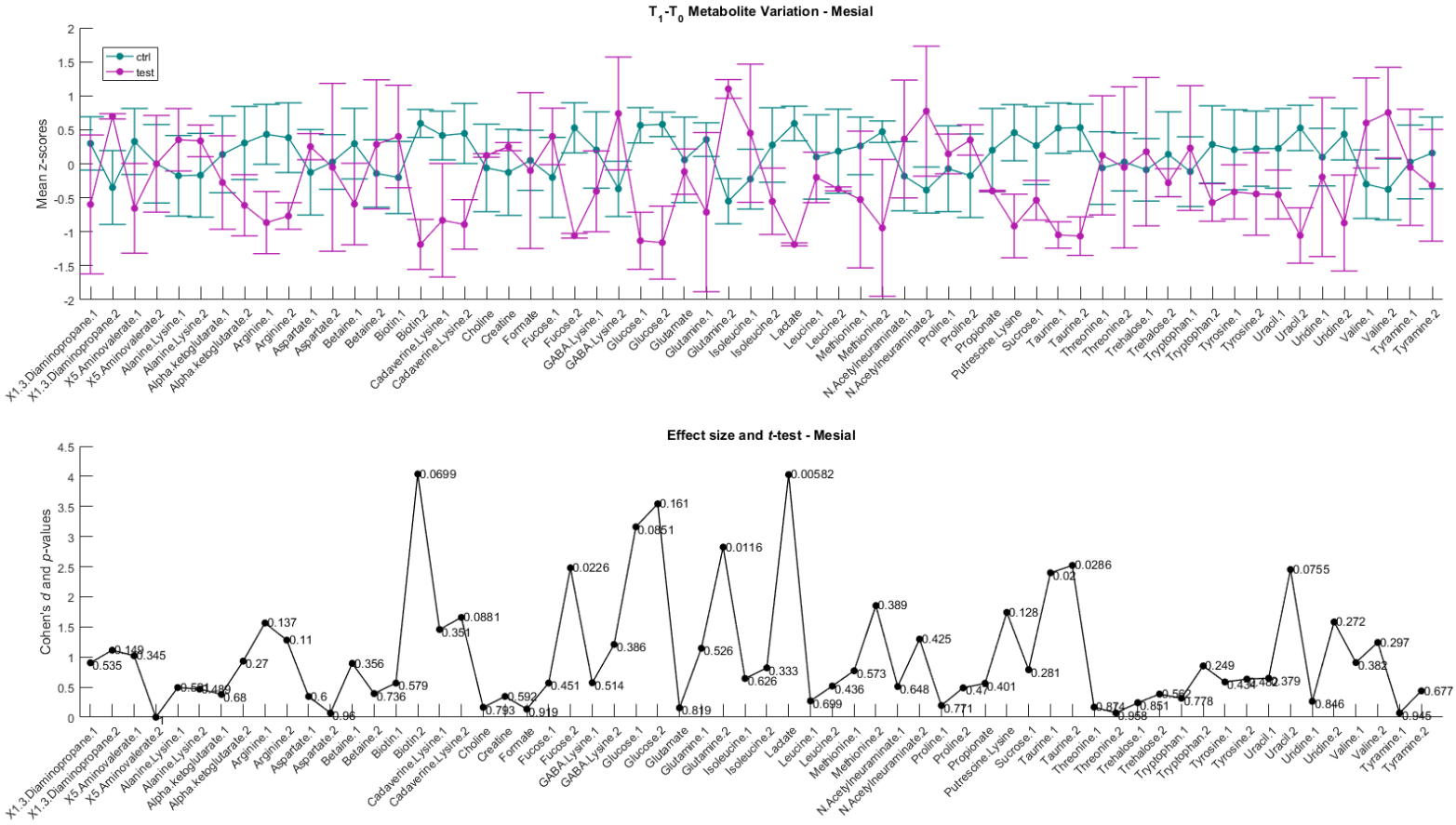

Figure 1. Changes in metabolite composition of saliva samples collected from mesial site were evaluated within the time interval of interest $\Delta_1=T_1-T_0$. Upper Panel: group means of *z*-scored concentration values for both control (teal) and test (magenta) experimental groups, for each one of the 62 detected metabolites taken into consideration. Error bars are the standard error of the mean. Bottom panel: black line and dots represent the Cohen's *d* statistic, which is a measure of the size of the effect induced by the treatment on each metabolite. Numbers above dots are the *p*-values returned by a marginal two-independent sample *t*-test comparing control and treatment groups.

For distal sites, some metabolites were significantly down regulated for test teeth at T1 with respect with control teeth: Glucose 2, Sucrose1 and Propionate. Only 1 metabolite was upregulated: Tyrosine1 (Figure 2).

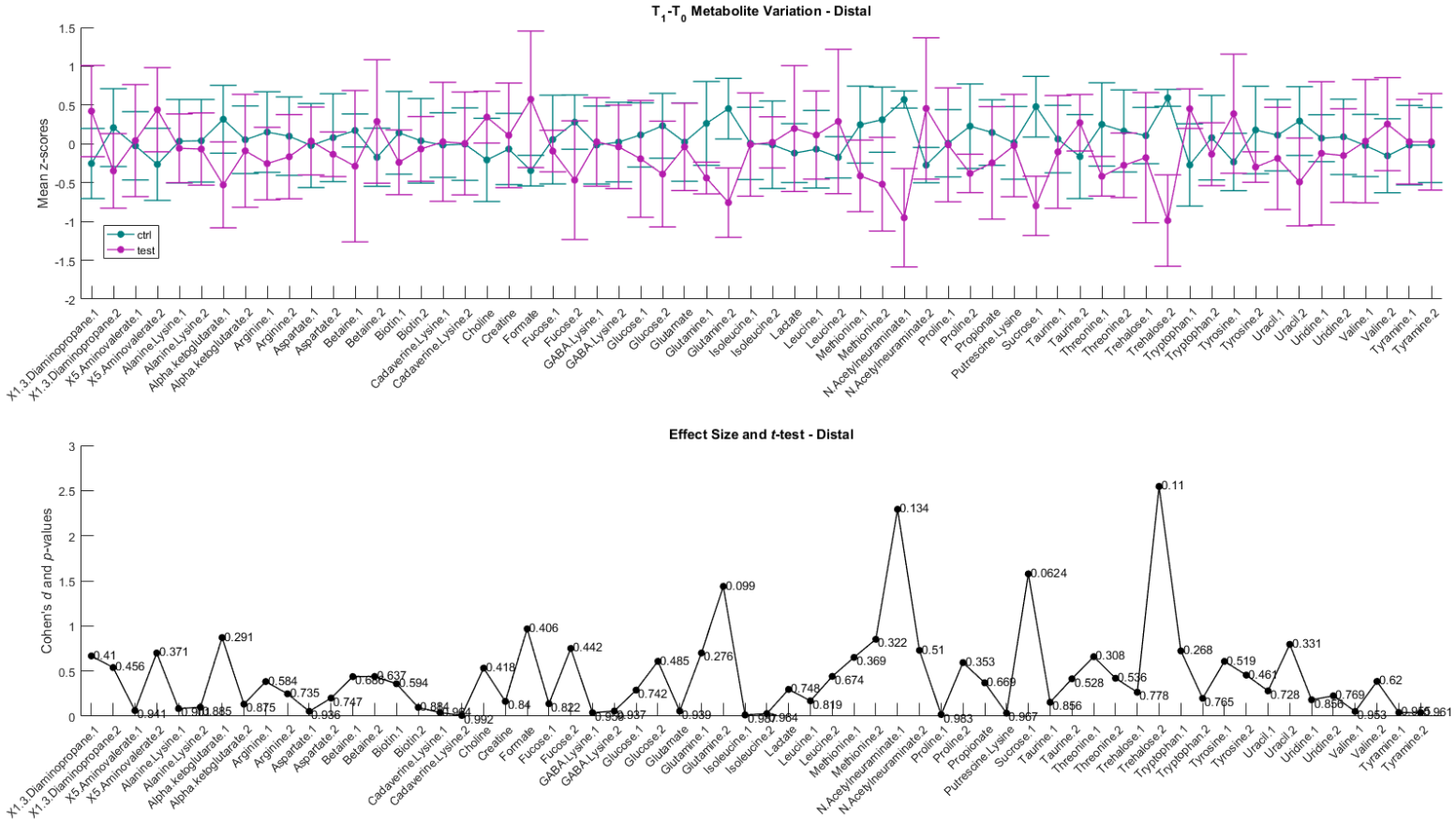

Figure 2. Changes in metabolite composition of saliva samples collected from distal site were evaluated within the time interval of interest $\Delta_1=T_1-T_0$. Upper Panel: group means of *z*-scored concentration values for both control (teal) and test (magenta) experimental groups, for each one of the 62 detected metabolites taken into consideration. Error bars are the standard error of the mean. Bottom panel: black line and dots represent the Cohen's *d* statistic, which is a measure of the size of the effect induced by the treatment on each metabolite. Numbers above dots are the *p*-values returned by a marginal two-independent sample *t*-test comparing control and treatment groups.

3.4 T-test Mesial and Distal site at T2-T0: paired/unpaired

In Table 5 we highlighted the significant results with paired and unpaired t-tests to measure metabolites variations for the mesial and distal sites at T2 with respect with T0. For mesial sites, no significant metabolite variation occurred between test and control teeth.

MESIAL			DISTAL		
Metabolite	p Value	t-test analysis	Metabolite	p Value	t-test analysis
-	-	-	5Aminovalerate2	p=0,0185	paired
			Aspartate1	p=0,021178	paired
			Fucose2	p=0,02635	paired
			Glutamate	p=0,03444	paired

Table 5 Differences between test and control groups at T2 for mesial and distal sites

For distal sites, some metabolites were significantly down regulated for test teeth at T2 with respect with control teeth: 5Aminovalerate 2, Aspartate1, Fucose 2 and Glutamate (Figure 3, Figure 4).

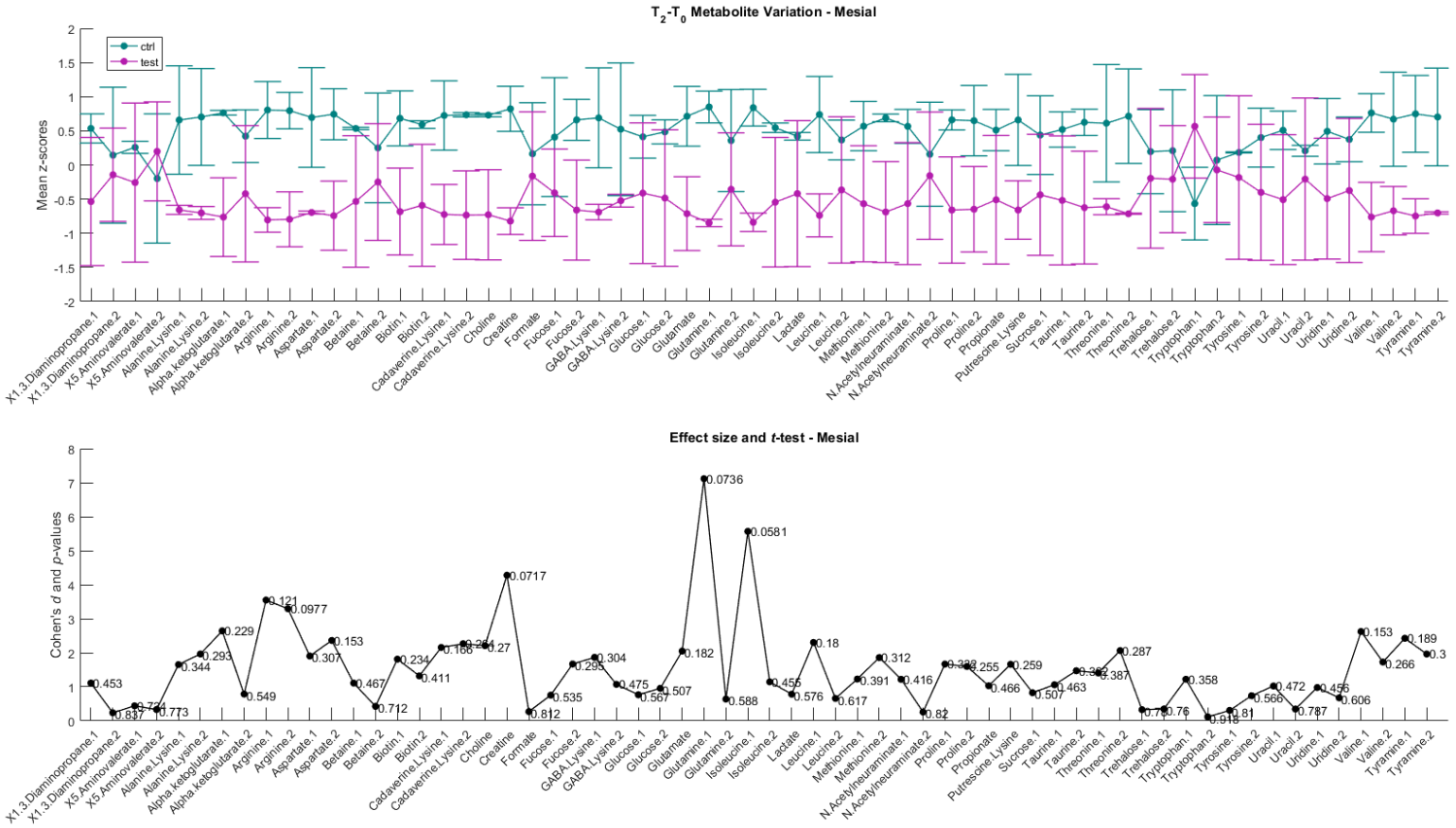

Figure 3. Changes in metabolite composition of saliva samples collected from mesial site were evaluated within the time interval of interest $\Delta_2=T_2-T_0$. Upper Panel: group means of *z*-scored concentration values for both control (teal) and test (magenta) experimental groups, for each one of the 62 detected metabolites taken into consideration. Error bars are the standard error of the mean. Bottom panel: black line and dots represent the Cohen's *d* statistic, which is a measure of the size of the effect induced by the treatment on each metabolite. Numbers above dots are the *p*-values returned by a marginal two-independent sample *t*-test comparing control and treatment groups.

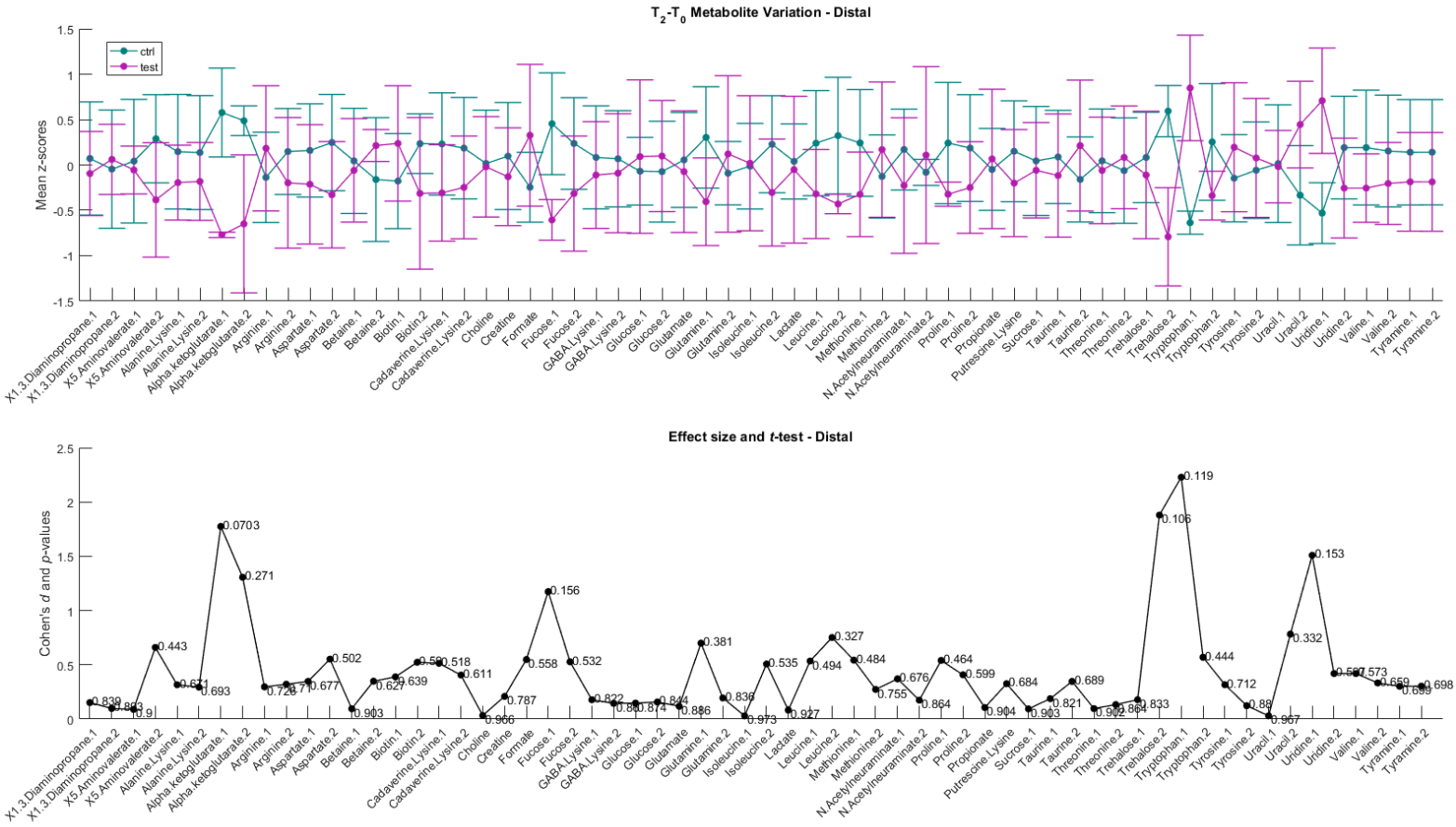

Figure 4. Changes in metabolite composition of saliva samples collected from distal site were evaluated within the time interval of interest $\Delta_2=T_2-T_0$. Upper Panel: group means of *z*-scored concentration values for both control (teal) and test (magenta) experimental groups, for each one of the 62 detected metabolites taken into consideration. Error bars are the standard error of the mean. Bottom panel: black line and dots represent the Cohen's *d* statistic, which is a measure of the size of the effect induced by the treatment on each metabolite. Numbers above dots are the *p*-values returned by a marginal two-independent sample *t*-test comparing control and treatment groups.

4. DISCUSSION

Salivary analysis has become a promising diagnostic mean and has shown great potential to research prognostic factors for several systemic deseases[33].

From a recent study published in 2020[34], there are currently approximately 72 original research articles applying metabolomics to human saliva. According to a specific application field of research, these studies can be categorized into different types: studies investigating disease biomarkers, physiological function and healthy conditions, metabolome association with oral microbiome, oral functions and exercises performances. The majority of these studies are based on prognostic disease metabolites, since the baseline metabolite composition of saliva in healthy individuals is now fairly well characterized, with the majority of NMR spectral assignments "solved"[35], although there remains debate about certain peak assignments.

As seen above, most studies of salivary metabolic composition have focused on salivary metabolite profiling as a means of biomarker discovery. Of these studies, 11 have looked at oral cancer, 8 at periodontal disease and 6 at Alzheimer's disease and the number of these kind of studies is constantly growing.

Nevertheless, saliva can be collected non-invasively and for repetitive sampling[25, 36, 37], so it represents a good biofluid to improve scientific findings.

NMR spectroscopy analysis has started to spread as mean to investigate salivary metabolomics in the last 20 years, but only in the last few years[38] have been set standardized protocols[39] to obtain high quality data from these evaluations[40, 41], even for the most recent pandemic Sars -Cov- 2 emergency[42].

Since a tooth undergoing orthodontic movement, develops an acute inflammatory response characterized by local tissue ischemia, periodontal vasodilatation and migration of leukocytes through the capillaries of the periodontal ligament in the first phase[14], it seems reasonable to think that this first inflammatory reaction can be assimilated to a similar response in case of a patient affected by gingivitis[4, 7].

Nowadays we have data concerning metabolites composition and profiling of saliva by healthy patients and patients with periodontal inflammation of various degrees. So, even if GCF is s an interstitial fluid exudate in healthy patients, while in inflammatory or stimulated conditions, GCF is an exudate that reflects the concentrations of metabolites within serum[11, 12], in this study we tried to compare these preliminary data obtained to existing literature on salivary metabolites in periodontal diseases, in order to explore possibilities of orthodontic metabolites profiling in the first 3 weeks of OTM.

Due to the poor number of GCF samples analyzed, we performed our statistical analyses considering the differences between metabolites concentrations between T0 and T1 or T2 on test teeth compared to control ones (Table 2, Table 3), without distinction between mesial and distal sites, so initially without searching for prognostic metabolites for

compression and tension areas surrounding teeth or predictive factor of biological good respondance to OTM.

However, from a conceptual point of view, this lack of distinction and our consequent findings, could biologically reflect what happens to periodontal tissues involved in OTM, according to the biphasic theory, in the first 7 days[22, 43].

It has been shown in histologic sections at early time points of force application, activation of osteoclasts in both compression and tension sites, which suggests that both compression and tensile forces can traumatize the PDL. In uCT scans of the alveolar bone around moving teeth can clearly be observed radiolucency all around the tooth, so osteoclastogenesis is not limited to the compression side (Fig. 5).

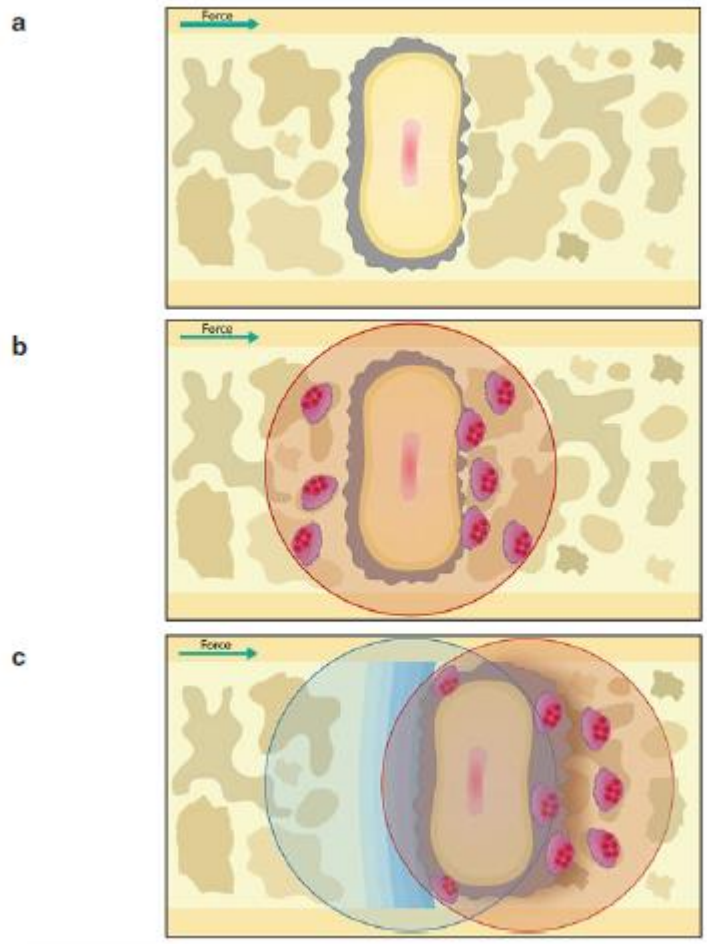

Figure 5. Biphasic Theory of Tooth Movement explained by the coupling of osteoclast activity with osteoblast activity. The biologic response during tooth movement comprises two clearly separated phases. After application of an orthodontic force (a), both the compression and tensile stresses generated by displacement of the tooth cause damage to PDL stimulating a perimeter of osteoclastogenesis (red circle) (b). Once the tooth moves in the direction of the orthodontic force into the space created by osteoclast activity, a perimeter of osteogenesis and bone formation (blue circle) is created roughly in the same area of the alveolar bone where the catabolic response took place (c). The coupling of the catabolic response (osteoclast activity) with the anabolic response (osteoblast activity) during orthodontic tooth movement can occur through different pathways: osteoclast-derived signals working a paracrine fashion, direct cell-cell interaction, and growth factor release from the matrix during bone resorption. (*Biphasic Theory of Tooth Movement: Chapter 3; Cytokine Expression and Rate of Tooth Movement,Cytokines and Rate of Tooth Movement*. M.Alikhani, S. Alansari, C. Sangsuwon, J. Nervina, C. Teixeira. Biology of Orthodontic Tooth Movement 2016, Springer.)

Both catabolic and anabolic responses occur in the alveolus around the entire tooth – regardless of the type of force that is actually experienced at a specific site – ensuring that the alveolus remains intact throughout orthodontic treatment.

The data on osteoclast and osteogenic markers clearly support the temporal relationship. in the Biphasic Theory of Tooth Movement, osteoclasts play an important role in the activation of osteoblasts. This is in agreement with numerous studies that suggest osteoclasts are principle regulator of osteoblast activity[44, 45].

To test the site effect on metabolic GCF profiling, we carried on some measurements on available samples stratifying per mesial and distal subgingival area of the control and test teeth, which resulted as preliminary evidence, even if weak, of different metabolites in tension (mesial) and compression (distal) sites of orthodontic force application at different timings.

We expected partly overlapping results to those found in the GCF of healthy teeth, with some variation in concentration after orthodontic force application; we found out almost equatable metabolites from a 500 MHz 1H-NMR spectrum of human saliva in this previous review[35]: propionic acid; lactic acid; 2-hydroxy valeric acid; putrescine; succinic acid; choline; D-glucose; fucose; L-valine; L-proline; L-tyrosine.

Tsuchida et al.[46], in a 2019 review concerning the state of proteomic technologies to discover and identify biomarkers of gingival crevicular fluids for periodontal disease, compared the results obtained in 8 different articles. Of the 8 articles analyzed, 7 examined proteins within the GCF

and only 1 examined metabolites. In this article by Ozeki et al.[47], the aim was to identify the metabolites present in pockets of different depths: 19 metabolites have been identified by GC/MS with different peaks depending on the depth of the pocket: Propyl amine, Lactic acid, Benzoic acid, Phosphate, Glycine, Succinic acid, Alanine, Hydrocinnamate, Malic acid, Glutamic acid, 5-Aminovaleric acid, Phenylalanine, Ribose, Taurine, Putrescine, Galactose, Lysine, Inositol, Octadecanoate. All of these metabolites' concentrations tended to increase, even if not in a linear association, with increasing pocket depth. Many of those were found in our GCF samples, with some significant variations in down regulation after 1 hour since orthodontic force application (Table 2).

In a study from Rzezink et al.[48], an interesting disease-specific increases of butyrate concentrations were associated with reduced levels of other short chain acids (SCAs), such as lactic acid, acetic acid, formic acid, or γ-aminobutyric acid. The marked reduction of lactate in patients could reflect a shift in the microbial composition of commensal bacteria in the healthy oral cavity as well as in other mucosal ecological niches. This may include lactic acid bacteria. In our explorative statistics, lactate was found to be present and to decrease at T1 in the mesial test site, accordingly.

A more recent systematic review by Baima et al.[49] investigated salivary metabolomics for the diagnosis of periodontal diseases with methodological quality assessment.

This systematic review provided a qualitative synthesis of the literature dealing with untargeted metabolomic analysis of saliva for the diagnosis of periodontal diseases.

Compared to periodontally healthy subjects, patients with periodontitis presented statistically significant differences in 114 metabolites of different classes; for periodontitis patients, pathway enrichment analysis revealed significant activity in the phenylalanine, tyrosine and tryptophan pathway, together within the phenylalanine and the pyruvate metabolism. The increased glycosidase, lipase, and protease activities associated with periodontal inflammation supplied a more favorable energetic environment for oral bacteria[50, 51].

Due to the high complexity of systems targeted by omic research, multivariate analysis is often required in order to minimize random noise and to find latent trends within the data sets. In our study, preliminary assessment of the ROIs reveals the presence in GCF of several amino acids from collagen and metabolites always present in previous SALIVA metabolites sets for healthy and diseased patients.

We did not identified 3-hydrobutyrate or butyrate, that have been previously described in GCF and strongly associated with increased severity of periodontitis[52, 53]. We also identified taurine down regulation at T1, an amino-sulfonic acid that is used by sulfate reducing anaerobic bacteria to sequester hydrogen for the sulfur and pyruvate metabolism pathways[54].

Finally, since this research turned into exploratory, unpaired T-tests and Cohen's D were reported (Figure 1 and Figure 2, Fig. 3, Fig. 4), together

with Z-score plots, respectively for mesial and distal sites, to report the effect size of this small test and control groups. Even though the few statistical relevant significance p-values of metabolites variations, effect size helped us to interpret probabilities in light of sample size and could suggest a clinical relevance of some metabolites after future analyses. Moreover, mean Z-scores gave us an idea of how many standard deviations -below or above the population mean- our raw data were.

In Figure 1 and 2 we explored trends in variations of metabolites in mesial and distal test site compared to control teeth at T1. In Figure 1 – mesial - , it is interesting to see how, despite significant p-values, peaks showed on Cohen's D scheme correlated to important down regulation of Arginine1 and 2, Biotin, Cadaverine-Lysine 1 and 2, Fucose 2, Glucose 1 and 2, Lactate, Methionine 1 and 2, Putrescine, Sucrose, Taurine 1 and 2, Uracil and Uridine 2 and upregulation of GABA-Lysine, Glutamine, Valine 1 and 2. In Figure 2 – distal – down regulation was revealed for Glutamine2, N-Acetylneuraminate1, Sucrose and Trehalose 2.

In Figure 3 and 4 the same trends were examined: at T2, in the mesial site, Glutamine1 was down regulated (opposite trend with respect with T1) as well as Creatine and Isoleucine1. In Figure 4, at distal site, Trehalose confirmed its down regulation as well as at T1 for the distal site and interestingly tryptophane turned upregulated, consistently with the tryptophane pathway of metabolites degradation in perio-patients.

A generalized estimating equation (GEE) approach will be used to account for correlation among sites from the same subject. Bonferroni correction will be used to control multiple testing. Significant ROIs will be

further evaluated in a multivariate model to develop a prediction model for bad and good responders to OTM.

Alternatively, we will use a principal component analysis (PCA) approach to summarize data from the ROIs, and test for association between disease status (PD, CAL/BL) and the top principal components (PCs) using GEE.

5. CONCLUSIONS

5.1 Strengths and limitations of this study

Untargeted metabolite analysis is preferred for providing global profiling and for discovering new pathways.

To the best of our knowledge, this is the first pilot randomized controlled trial in orthodontics, focusing on the application of untargeted GCF metabolomics, aiming to find a set of metabolites expressed in OTM.

Previous studies and reviews, indicated that some metabolites can consistently discriminate between healthy and periodontal inflammatory conditions and may be candidates for future steps in the diagnostic and prognostic validation process.

5.2 Implications for future research

Developing diagnostic tests with high sensitivity and specificity requires to better characterize the metabolic events occurring in the mouth in health and disease.

Reproducibility of salivary and GCF tests, as well as standardized protocols and a huge amount of patients involved in these studies are the key features.

At present, it is not clear whether the state of health, reaction to orthodontic forces application or disease activity have a higher influence on the salivary metabolome or, alternatively, these ones are more dependent upon the genetic or epigenetic susceptibility factors.

In the future, integrating metabolomics with other omics platforms (e.g., genomics, transcriptomics and proteomics) will empower the knowledge on the biological responsiveness, leading to more targeted analyses.

5.3 Concluding remarks

The results obtained from this explorative study, of course, are not specifically diagnostic or prognostic, neighter can be associated to a single biological response condition. More samples are needed to improve diagnostic accuracy, but this panel of metabolites that was found may be the initial step to a pathway to orthodontic metabolomics.

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