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Evaluation of Oral Health and Its Modifying Factors: Oral Microbiome, Genetic Markers, and Patient Knowledge in Phenylketonuria and Type 1 Diabetes

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Annotation

Background: Phenylketonuria (PKU) is an autosomal recessive inherited disorder of phenylalanine metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase. Individuals with PKU must adhere to a lifelong low-protein diet and consume a specialised amino acid mixture daily. However, these amino acid supplements are often acidic, creating an unfavourable oral environment. The resulting low pH promotes plaque accumulation, which can lead to gingivitis and periodontitis, while also increasing the risk of dental caries due to

the persistently acidic conditions in the mouth. Type 1 diabetes (T1D) is a metabolic disorder caused by defective insulin secretion and/or action and diet regimen is often suggested. In T1D chronic hyperglycaemia and impaired immune response increase the risk of periodontal disease, while altered salivary composition contributes to a higher susceptibility to dental caries and oral infections. Advancements in insulin therapy have simplified the disease management, allowing many patients with T1D to maintain a diet that is comparable to that of healthy individuals.

The aim of this study was to evaluate oral health in patients with PKU and T1D compared to individuals in a control group from the Latvian population, and to analyse factors influencing it, such as oral hygiene habits, the oral microbiome, and genetic predisposition, as well as to develop specially tailored oral hygiene recommendations for patients with PKU.

Material and methods: Our study included three groups – individuals with PKU, T1D and a control group without reported chronic diseases and dietary restrictions, all at least 12 years old. Oral health assessment was performed for all participants, followed by collection of saliva for microbial community analysis using *16S rRNA* gene V3-V4 region sequencing and for genotyping of genetic variants in the *IL1, DEFB1, TAS1R2, TAS2R38, CD36* genes.

In this study, two questionnaires on oral hygiene habits and knowledge were prepared and administered by trained medical personnel, with a one-month interval between pre- and post-implementation of oral hygiene recommendations. These recommendations were based on previous oral health examinations and a literature review. The questionnaires were completed by all diagnosed patients with PKU in Latvia who were not lost to follow-up and who were older than two years.

Results: Oral examination data was obtained from 45 patients with PKU (four were excluded due to severe intellectual disability), 31 T1D patients, and 70 control subjects. Clinical evaluation revealed that patients with PKU had the poorest oral hygiene index scores, with statistically significant differences in all variables compared to the T1D and control groups, except for the number of filled teeth. Patients with PKU also reported the lowest consistency in tooth brushing and flossing habits.

Based on the questionnaires among patients with PKU under 18 years of age, 27 % cleaned their teeth once a day, while 6 % did not engage in regular tooth brushing at all. Among those aged 18 years and older, 26 % reported brushing only once a day. Additionally, a mere 39 % of patients with PKU aged ≥ 18 years and 17 % aged < 18 years incorporated flossing in their oral hygiene routine. In total, 45 % of patients with PKU aged ≥ 18 years and 44 % aged < 18 years visited a dentist once a year, but 40 % aged ≥ 18 years and 39 % aged < 18 years reported visiting a dentist less frequently than once per year. After reading the recommendations, a remarkable improvement in daily oral hygiene was observed among patients with PKU under 18 years of age: 25 % improved their toothbrushing habits and 23 % began flossing.

Oral microbiome analysis revealed that the lowest number of shared genera was between the PKU and T1D groups. The highest number of differentially abundant genera was detected in the PKU group (n = 20), with significant enrichment of *Actinomyces* ($p_{adj} = 4.17\times10^{-22}$), *Capnocytophaga* ($p_{adj} = 8.53\times10^{-8}$) and *Porphyromonas* ($p_{adj} = 1.18\times10^{-5}$) compared to the control group. In contrast, the genus *Leptotrichia* ($p_{adj} = 5.79\times10^{-7}$) was enriched in T1D group, whereas the abundance of genera *Fusobacterium* ($p_{adj} = 2.05\times10^{-4}$) and *Alloprevotella* ($p_{adj} = 7.24\times10^{-4}$) was decreased compared to the control group.

Analysis of genetic variant associations with oral health characteristics revealed that the *DEFB1* rs11362 variant was associated with higher Silness-Löe plaque and Greene-Vermillion index scores in patients with PKU ($p = 0.011$ and $p = 0.043$, respectively). The *IL1B* rs1143634 variant was associated with a lower necessity for calculus removal in T1D patients ($p = 0.030$).

Conclusions: PKU and T1D patients have a higher prevalence of carious teeth, and an increased risk of periodontal disease compared to the control group. Differences in the oral microbiome among PKU, T1D patients and controls may contribute to variations in oral health. In addition to metabolic disease and diet, genetic predisposition may also influence oral health outcomes in the PKU, T1D and control groups. Results of the survey on oral hygiene habits before and after receiving targeted recommendations suggest that a tailored approach may be needed to improve oral hygiene practices, particularly among adult patients with PKU.

Keywords: *16S rRNA* sequencing; caries; genetic predisposition; microbiome; oral health; oral hygiene indices; periodontitis; phenylketonuria; recommendations of daily oral hygiene procedures; single nucleotide variants; type 1 diabetes.

Anotācija

Mutes dobuma veselības un tās modificējošo faktoru novērtējums: mutes dobuma mikrobioms, ģenētiskie marķieri un pacientu zināšanas pie fenilketonūrijas un 1. tipa cukura diabēta

Ievads: Fenilketonūrija (FKU) ir autosomāli recesīvi pārmantots fenilalanīna metabolisma traucējums, ko izraisa fenilalanīna hidroksilāzes deficīts. Pacientiem tiek piemērota zema olbaltumvielu diēta mūža garumā. 1. tipa cukura diabēts (1.TCD) ir vielmaiņas saslimšana, ko izraisa traucēta insulīna sekrēcija un/vai darbība. Attīstoties insulīna terapijai, diabēta ārstēšana ir kļuvusi vienkāršāka, ļaujot pacientiem ievērot diētu, kas ir līdzīga veselam cilvēkam.

Šī pētījuma mērķis bija novērtēt mutes veselību FKU un 1.TCD pacientiem, salīdzinot ar kontroles grupu Latvijā, kā arī analizēt tās ietekmējošos faktorus – mutes dobuma higiēnas paradumus, mutes mikrobiomu un ģenētisko predispozīciju.

Materiāli un metodes: Pētījumā tika iekļautas trīs grupas – FKU, 1.TCD un kontroles grupa bez hroniskām slimībām un diētas ierobežojumiem, vecumā virs 12 gadiem. Tika novērtēta mutes veselība, ievāktas siekalas šādām analīzēm: mutes mikrobioma profilēšanai, izmantojot *16S rRNS* V3-V4 gēna sekvencēšanu, kā arī ģenētisko variantu genotipēšanai *IL1, DEFB1, TAS1R2, TAS2R38* un *CD36* gēnos. Katrai no analīzēm grupu lielumi nedaudz atšķīrās.

Pētījumā ar viena mēneša intervālu pirms un pēc mutes higiēnas ieteikumu ieviešanas, balstoties uz iepriekšējo mutes veselības pārbaudi un literatūras apskatu, tika sagatavotas un medicīniskā personāla aizpildīšanai nodrošinātas divas anketas par mutes higiēnas paradumiem un zināšanām visiem Latvijā diagnosticētajiem FKU pacientiem vecumā virs diviem gadiem, kuri ir turpinājuši novēroties.

Rezultāti: Mutes veselības pārbaudi bija iespējams veikt 45 FKU pacientiem (vidējais vecums 24,10 ± 9,04 gadi), no kuriem četri nevarēja piedalīties smagas intelektuālās attīstības traucējumu dēļ, kā arī 31 1.TCD pacientam un 70 kontroles grupas indivīdiem. Visi dati, izņemot plombēto zobu skaitu starp 1.TCD un kontroles grupām, uzrādīja statistiski nozīmīgas atšķirības. Klīniskā izmeklēšanā FKU pacientiem konstatēti vissliktākie mutes higiēnas rādītāji. Viņi arī visretāk tīrīja zobus un izmantoja zobu diegu.

Pamatojoties uz anketu datiem, 27 % no FKU pacientiem vecumā līdz 18 gadiem tīrīja zobus vienu reizi dienā, savukārt 6 % netīrīja zobus regulāri. No pacientiem vecumā virs un 18 gadiem 26 % tīrīja zobus tikai reizi dienā. Tikai 39 % FKU pacientu ≥ 18 gadu vecumā un 17 % < 18 gadu vecumā lietoja zobu diegu. Kopumā 45 % ≥ 18 gadu vecumā un 44 % < 18 gadu vecumā apmeklēja zobārstu reizi gadā, savukārt 40 % ≥ 18 gadu vecumā un 39 % < 18

gadu vecumā – retāk nekā reizi gadā. Pēc ieteikumu izlasīšanas ievērojams skaits FKU pacientu, kas jaunāki par 18 gadu vecumu, uzlaboja savus ikdienas mutes higiēnas paradumus – zobu tīrīšanu (25 %) un zobu diega lietošanu (23 %).

Analizējot mutes mikrobiomu, vismazākais kopīgo ģinšu skaits bija starp FKU un 1.TCD grupām. FKU grupā tika identificēts vislielākais skaits baktēriju ar būtiski atšķirīgu sastopamību (n = 20), no kurām tādas ģintis kā *Actinomyces* ($p_{adj} = 4{,}17\times10^{-22}$), *Capnocytophaga* ($p_{adj} = 8{,}53\times10^{-8}$) un *Porphyromonas* ($p_{adj} = 1{,}18\times10^{-5}$) bija ievērojami biežāk sastopamas salīdzinājumā ar kontroles grupu. Savukārt, 1.TCD grupā tika novērota paaugstināta *Leptotrichia* ģints sastopamība ($p_{adj} = 5{,}79\times10^{-7}$), bet *Fusobacterium* ($p_{adj} = 2{,}05\times10^{-4}$) un *Alloprevotella* ($p_{adj} = 7{,}24\times10^{-4}$) ģinšu sastopamība bija būtiski zemāka nekā kontroles grupā.

Analizējot ģenētisko variantu saistību ar mutes veselības rādītājiem, tika atklāts, ka *DEFB1* rs11362 variants saistīts ar augstākiem *Silness–Löe* aplikuma un *Greene–Vermillion* indeksiem PKU pacientiem ($p = 0{,}011$ un $p = 0{,}043$). *IL1B* rs1143634 variants saistīts ar mazāku nepieciešamību pēc zobakmens noņemšanas 1.TCD pacientiem ($p = 0{,}030$).

Secinājumi: FKU un 1.TCD pacientiem ir augstāka kariesa izplatība un palielināts periodonta slimību risks salīdzinājumā ar kontroles grupu. FKU, 1.TCD un kontroles grupās ir atšķirīgs mutes mikrobioms, kas var ietekmēt mutes veselību. Ģenētiskā predispozīcija papildus vielmaiņas slimībai un diētai var ietekmēt mutes veselību šajās grupās. Anketu rezultāti par mutes higiēnas paradumiem pirms un pēc mērķētu ieteikumu saņemšanas liecina, ka īpaši pielāgotas pieejas ir nepieciešamas, lai uzlabotu mutes higiēnu, īpaši pieaugušiem FKU pacientiem.

Atslēgvārdi: *16S rRNA* sekvencēšana; 1. tipa cukura diabēts; fenilketonūrija; ģenētiskā predispozīcija; kariess; mikrobioms; mutes dobuma veselība; mutes higiēnas indeksi; periodontīts; viena nukleotīda variants.

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Abbreviations used in the Thesis

16S rRNA	16S ribosomal RNA
A	Adenine
BH4	Tetrahydrobiopterin
BRIGHT	Brushing RemInder 4 Good oral HealTh
C	Cytosine
cPKU	Classic phenylketonuria
CI	Confidence interval
CPITN	Community index of periodontal treatment needs
dbSNP	Single nucleotide polymorphism database
DEFB1	Beta-defensin 1
DMFI	Decayed-Missing-Filled Index
DMFT	Index or Decayed-Missing-Filled teeth
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
H	Adenine / Cytosine / Thymine
HbA1c	Glycated haemoglobin
hBD	Human βeta-defensin
HGVS	Human Genome Variation Society
IL-1	Interleukin-1
IL1B	Interleukin-1 beta
IQR	Interquartile range
ISPAD	International Society for Paediatric and Adolescent Diabetes
µmol/l	Micromole per litre
mPKU	Mild phenylketonuria
md/dl	Milligrams per decilitre
min	Minute
ml	Millilitre
mmol	Millimole
MHP	Mild hyperphenylalaninemia
N	Any nucleotide
NBS	Newborn screening
NGS	Next generation sequencing
OR	Odds ratio
ORPHA code	Orphanet nomenclature for orphan diseases

OTUs	Operational taxonomic units
PAH	Phenylalanine hydroxylase
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
Phe	Phenylalanine
PKU	Phenylketonuria
RDA	Redundancy analysis
RFLP	Restriction fragment length polymorphism analysis
RNA	Ribonucleic acid
rpm	Revolutions per minute
QC	Quality control
SNPs	Single-nucleotide polymorphisms
Spp	Species
T	Timine
T1D	Type 1 diabetes
Tyr	Tyrosine
V	Guanine / Cytosine / Adenine
V3-V4	Variable region 3 and variable region 4 of the *16S rRNA*
W	Adenine/ Timine
WHO	World Health Organization

Introduction

Oral health encompasses a range of diseases and conditions, including dental caries, periodontal disease, and more. While hygiene habits are a primary factor affecting oral health (WHO, 2024), the long-term effects of diet, the oral microbiome, and genetic predisposition remains unclear (Ostrowska et al., 2024).

The oral cavity, being open to the environment, serves as a primary entry route for microbes into the human body. Saliva plays a crucial role in maintaining oral homeostasis and contributes significantly to the body's disease-protection mechanisms (Sampaio-Maia et al., 2016). For a considerable time, it has been recognised that diet and oral hygiene strongly affect the composition of oral microorganisms. The majority of reports still associate *Streptococcus mutans* with dental caries (Dianawati et al., 2020; Simon-Soro et al., 2014) and *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* are the primary bacteria identified in periodontitis (Nazir et al., 2020). However, relatively little information exists on the impact of diet on the salivary microbiome, which may in turn affect oral health.

There is evidence that genetic variants in immunity-determining genes (e. g. *IL1B, DEFB1*) and taste receptor genes (e. g. *TAS1R2, TAS2R38*, and *CD36*) predispose individuals to caries and periodontitis (Cogulu & Saglam, 2022; Nibali et al., 2019).

Although the number of patients on medically prescribed diet is increasing, it remains unclear whether these long-term diets affect the human oral microbiome. For patients with certain metabolic conditions, adhering to a strict diet is essential to ensure adequate intake of micro- and macronutrients for normal development. Low-protein diets, for instance, are prescribed for individuals with phenylketonuria (PKU) (van Wegberg et al., 2017). PKU is a metabolic disorder marked by a deficiency or absence of the enzyme phenylalanine hydroxylase (PAH), which is necessary for converting the amino acid phenylalanine (Phe) into tyrosine (Tyr). Without proper management, Phe can accumulate in the blood and brain, potentially leading to cognitive impairments. Consequently, patients with PKU follow a carbohydrate-rich diet supplemented with a low-pH, Phe-free amino acid formula (van Spronsen et al., 2021). This diet increases carbohydrate intake, which heightens the risk of dental caries. The Phe-free amino acid formula is also sweetened and acidic, posing additional risks to oral health (Rocha & MacDonald, 2016).

Type 1 diabetes (T1D) is another heterogeneous disorder characterised by the complete absence of insulin due to the destruction of pancreatic beta cells (Vanderniet et al., 2022). Managing diabetes is a lifelong process that requires constant monitoring, especially as paediatric patients grow. A critical component of treatment is diet therapy, which emphasises

meal regularity and precise carbohydrate counting to optimise glycaemic control (American Diabetes Association Professional Practice, 2021). Both the disease itself and dietary adjustments have been associated with oral health issues, including an increased incidence of dental caries and periodontitis (Ferizi et al., 2022; Gunasekaran et al., 2022).

One of the objectives of this research is to investigate the association between diet and oral health among patients with PKU in Latvia still responsive to follow-up visits. This is the main group of interest, as PKU is a rare disease requiring a low-protein diet. Additionally, T1D patients and a control group (individuals without known chronic diseases or dietary restrictions) will be enrolled to assess oral health status and explore potential influencing factors, such as dietary restrictions, oral microbiome composition, genetic predisposition, and oral hygiene habits. Oral health assessments will be performed for individuals from 12 years of age, as this allows the evaluation of the health of permanent teeth.

Aim of the Thesis

The aim of this study was to assess the oral health parameters associated with caries and periodontal disease risk in patients with PKU and T1D, focusing on dental hygiene status, oral microbiome composition, and the effectiveness of tailored oral hygiene recommendations for patients with PKU in Latvia. Additionally, the study examined whether PKU and T1D patients with the *IL1B* rs1143634, *DEFB1* rs11362, *TAS1R2* rs35874116, *TAS2R38* rs1726866 and rs713598, *CD36* rs1761667 genetic variations have a higher incidence of caries and periodontitis compared to healthy controls.

Objectives of the Thesis

- To evaluate oral health in patients with PKU andT1D
- To develop oral hygiene recommendations for patients with PKU and via survey results evaluate patient / patient caregiver knowledge and daily habits of oral hygiene and recommendation application in everyday practice within one-month period.
- To investigate the effect of oral microbiome characteristics on caries activity and periodontal disease risk in patients with PKU and T1D, compared to healthy individuals in Latvia.
- To analyse genetic predisposition to caries and periodontal disease in PKU, T1D and a control groups, by detecting the genotypes for *IL1B* rs1143634, *DEFB1* rs11362, *TAS1R2* rs35874116, *TAS2R38* rs1726866 and rs713598, *CD36* rs1761667.

Hypotheses of the Thesis

In patients with PKU, daily oral care habits and knowledge regarding oral hygiene are insufficient, which results in poorer oral health outcomes.

Single nucleotide variants *IL1B, DEFB1, TAS1R2, TAS2R38,* and *CD36* influence oral health in patients with PKU and T1D.

In individuals with PKU and T1D, the oral microbiome is altered compared with the control group, and in relation to specific dietary characteristics, this increases the risk of oral diseases.

Novelty of the Thesis

This is the first study to examine the oral microbiome in patients with PKU and T1D and its influence on oral health; previously, only individual microorganisms had been studied. For the first time, oral hygiene guidelines specifically for patients with PKU have been developed.

In Latvia, no prior research has investigated the oral health of patients with rare diseases or endocrine disorders. Until now, neither oral health nor oral health awareness in these patient groups had been studied, as the primary focus was limited to managing the underlying disease. Additionally, this is the first study in Latvia to investigate the association between single nucleotide variants and oral health in context of a specific disease.

Personal contribution

The author's own contribution was to organise, coordinate, and carry out the study's primary components. This includes:

- Designing the research concept together with supervisors.
- Recruiting and enrolling participants in all three groups (PKU, T1D, and control group).
- Performing and/or organising the oral health examinations.
- Preparing and administering the oral hygiene questionnaires for patients with PKU and developing the tailored oral hygiene recommendations.
- Collecting saliva samples for microbiome and genetic analyses.
- Participating in laboratory work, including DNA isolation, genetic analysis.
- Carrying out data analysis (oral health indices, questionnaire results).
- Interpreting results in the context of oral health, diet, microbiome, and genetics.
- Writing the manuscript of the Thesis.
- Author of three first-author publications and co-author of one publication.

1 Literature review

1.1 Oral health

Oral diseases encompass a range of diseases and conditions that include dental caries, periodontal (gum) disease, tooth loss and others. They are among the most common noncommunicable diseases worldwide, affecting an estimated 3.5 billion people (WHO, 2022).

Several factors protect/contribute to oral health. These include noncommunicable diseases such as diabetes and phenylketonuria (Botelho et al., 2022), genetic predisposition, saliva production and oral microbiome (WHO, 2024). Saliva is an important factor in a plethora of oral functions, such as mastication, swallowing, antimicrobial activity, cleaning action. Saliva also influences oral health both through its non-specific physio-chemical properties (e.g. buffering capacity, lubrication, mechanical cleansing, and mineral content), as well as through more specific effects, including antimicrobial activity (lysozyme, lactoferrin, peroxidase), immunological defence (secretory IgA), and promotion of tooth remineralisation. (Dodds et al., 2005). Shimazaki and colleagues examined the association of salivary flow rate with dental caries prevalence and periodontal status among 2110 Japanese adults and suggested that individuals with lower salivary flow rates have higher risks for both dental caries and periodontal disease (Shimazaki et al., 2017).

The oral microbiome consists of more than 250 microbial species with varying levels of pathogenicity that could affect oral health. One of the factors affecting oral microbiome is diet. In a study by De Filippis and colleagues on the relationship between restrictive diets (vegan, ovo-lacto-vegetarian, omnivore) and the salivary microbiome, a correlation was observed between a higher variety in diet and increased microbial diversity (De Filippis et al., 2014). Other studies have also shown that even meal frequency affects the composition of the oral microbiome (Viljakainen et al., 2020).

One of the most important risk factors for poor oral health is inadequate oral hygiene habits (WHO, 2024). Oral hygiene can be assessed not only by survey questions but also objectively by using the Plaque index, the Greene-Vermillion Index (measuring the accumulation of dental plaque and calculus) and the Silness-Löe Index (assessing the plaque accumulation on the teeth) (WHO, 2013). Adequate oral hygiene involves daily plaque control consisting of twice-daily toothbrushing with fluoride toothpaste ($\geq$ 1,000 ppm F^-) for ~2 minutes, regular interdental cleaning (using floss or interdental brushes), and is complemented by a sugar-smart diet and periodic professional care (Chapple et al., 2015; Toumba et al., 2019).

1.1.1 Dental caries

Dental caries, commonly known as tooth decay, is a significant healthcare issue due to its global prevalence as the most common illness (WHO, 2022). The term “dental caries” refers to both the disease process and the resulting lesion. It is estimated that nearly 100 % of adults are affected by dental caries (Benzian & Loistl, 2022),

The development of caries is influenced by a combination of genetic and environmental factors as presence of biofilm, dietary choices, and several other factors (Cogulu & Saglam, 2022; Simon-Soro & Mira, 2015).

Dental caries can affect any surface of the tooth. However, due to their complex architecture and fissure system, the occlusal surfaces are the most susceptible and most common sites for caries initiation (Al Saffan, 2023).

Diagnosis and treatment for dental caries

The initial step in the evaluation of caries is always visual examination. It necessitates a dry surface and can be improved using magnifying loupes (Rashid et al., 2022). Traditionally, clinical examination instruments needed for caries diagnosis include mouth mirrors, appropriate lighting, and dental probes. Newer diagnostic instruments, including laser fluorescence detection devices and light-induced fluorescence, provide more precise information regarding the carious lesions (Cho et al., 2021). To evaluate caries the Decayed -Missing-Filled (DMF) Index, also referred to as the Decayed-Missing-Filled Teeth (DMFT) index, was introduced in 1938 (Broadbent & Thomson, 2005). The Index is based on simple formula and its severity categories vary across age group, as defined by the WHO (WHO, 2013).

Treatment strategies for dental caries range from non-invasive preventive measures to more extensive restorative procedures, depending on the severity of the decay. The most effective prevention is appropriate use of fluoride toothpaste in conjunction with good oral hygiene (Toumba et al., 2019). Elimination of plaque is essential in the maintenance of good oral hygiene and hence good oral health. Brushing teeth twice a day with fluoridated toothpaste and flossing in between the teeth can help reduce plaque accumulation (Rimondini et al., 2001). The ability of fluoride to prevent tooth decay is widely recognised. Inadequate fluoride exposure should also be considered as a contributing factor in the development of dental caries (Carey, 2014). Daily oral hygiene procedures should include the use of mouthwash to reduce plaque accumulation (Arduino et al., 2020; Erbe et al., 2019). Regular dental check-up and professional dental hygiene procedures also improve oral health (Zheng et al., 2021).

Oral microbiome and caries

Dental caries develops when the balance of microorganisms in the oral cavity, known as the biofilm microbiota, shifts from a state of equilibrium to a population that can produce acid and cause tooth decay. This shift is typically triggered by the regular ingestion of sugary foods and drinks (Schwendicke et al., 2016). This change can either go unnoticed in a clinical setting or cause a loss of minerals in the hard structures of the tooth, ultimately resulting in a noticeable decayed area (Giacaman et al., 2022). Thus, dental caries is classified as a dietary-microbial condition that necessitates the presence of a cariogenic biofilm and consistent consumption of fermentable carbohydrates from the diet (Pitts et al., 2017). The biofilm bacteria that generate organic acids, particularly lactic acid, metabolise fermentable carbohydrates. These end products of bacterial metabolism accumulate in the fluid phase of the biofilm, resulting in a pH decrease and demineralisation of the tooth's surface layer (Marshall, 2019). The enamel's porosity increases, the spaces between the crystals broaden, and the surface softens, all of which allow the acids to penetrate deeper into the tooth structure and demineralise the surface (Gevkaliuk & Nazarenko, 2023).

Within the biofilm of a caries lesion, the predominant species is *Streptococcus mutans*; various other acid-tolerant and acid-producing bacteria are also found (Simon-Soro et al., 2014). Nevertheless, *Streptococcus mutans* has been extensively studied due to its significant role in producing the extracellular matrix and its ability to rapidly influence the development of a cariogenic biofilm when fermentable carbohydrates are present in the diet. Dental plaque is the environment in which microbial metabolic activity occurs and where both harmful and protective processes take place, influencing the formation of the lesion (Ilie et al., 2014; Nascimento et al., 2019).

While several studies suggest that the *Streptococcus viridans* group is a normal component of the oral microbiome, others propose that it is associated with dental caries and other oral pathologies (Cheon et al., 2013; Yamashita & Takeshita, 2017). The *S. viridans* group plays an important role in the oral ecosystem by contributing to colonisation resistance by preventing the invasion of more pathogenic bacteria. This group includes species such as *Streptococcus mitis, Streptococcus oralis, Streptococcus sobrinus, Streptococcus salivarius,* and *Streptococcus mutans etc*. Dianawati and colleagues examined the distribution of *S. mutans* and *S. sobrinus* in patients with high levels of dental caries and concluded that these two species are the main contributors to caries development (Dianawati et al., 2020). Cheon and colleagues evaluated the association between *S. mutans* genotype diversity, commonality, and stability with dental caries history in a high-caries-risk community. They reported that lower diversity

and higher stability of *S. mutans* genotypes were strongly associated with fewer decayed surfaces (Cheon et al., 2013).

Genetic predisposition to dental caries

When looking for caries heritability there is evidence from twin studies suggesting heritability, although with low certainty (Dos Anjos et al., 2023). Genetic factors implicated in dental caries are involved in enamel formation, salivary composition and flow, immune system function, and carbohydrate metabolism (Vieira et al., 2014). Since there are obvious population differences (Sandhu et al., 2024) and some new loci are still being discovered (Nogawa et al., 2024), several genome-wide association studies have been conducted to analyse the association with caries and have found a strong correlation with loci where the taste receptor genes *TAS2R38, TAS2R3, TAS2R4*, and *TASR25* are located (Alotaibi et al., 2021).

Associations with genetic variants in taste receptor genes (*TAS2R38*, which influences bitter taste, and *TAS1R2*, which influences sweet taste) suggest that differences in taste perception affect daily habits, potentially leading to a cariogenic diet affecting also oral microbial composition increasing the risk for caries. These dietary patterns may also influence oral microbial composition, increasing the risk for dental caries (de Jesus et al., 2022), although having contradictory results – showing that probably diet itself has stronger impact than genetic variants in the taste receptors itself (Sandell & Collado, 2018). Additional effect on caries formation could be that taste receptors have been implicated in innate immune responses against oral bacteria (Gil et al., 2015).

TAS1R2 encodes the taste receptor type 1 member 2, responsible for detecting sweet and umami taste. The most studied genetic variant is rs35874116 (HGVS nomenclature (NC_000001.10(NM_152232.4):c.571A>G p.(Ile191Val)) – valine allele shows partial loss of function effect on receptor (Serrano et al., 2021). A study of 236 children (18 of them with severe caries) showed that individuals with genotype AA(TT) (Isoleucine homozygotes) have an association with higher sweet intake (Liang et al., 2022).

The *TAS2R38* gene encodes the taste receptor type 2 member 38, responsible for detecting bitter compounds such as phenylthiocarbamide and 6-n-propylthiouracil. Three widely analysed variants in this gene – rs713598 (HGVS nomenclature (NC_000007.13(NM_176817.5):c.145G>C p.(Ala49Pro)), rs1726866 (HGVS nomenclature NC_000007.13(NM_176817.5):c.785C>T p.(Ala262Val)), and rs10246939 HGVS nomenclature (NC_000007.13(NM_176817.5):c.886A>G p.(Ile296Val)) – along with their haplotypes, have been the focus of genetic research (Vieira et al., 2014). Based on genotypes there are three categories – those sensitive to bitter taste, called “tasters” (rs713598 proline,

rs1726866 alanine and rs10246939 valine determining allele), those that have little or no sensitivity to bitter taste called “non-tasters” (rs713598 alanine, rs1726866 valine and rs10246939 isoleucine determining allele) and those with intermediate sensitivity called “medium tasters” (rs713598 alanine, rs1726866 alanine and rs10246939 isoleucine determining allele). Functional studies have shown that the greatest impact on bitter taste perception is linked to the first two variants, as amino acid changes at positions 49 and 262 significantly reduce receptor function. In contrast, the variant at position 296 has a minimal effect on protein activity (Bufe et al., 2005).

A systematic review and meta-analysis by Chisini and colleagues on taste receptor gene variants found, that the minor alleles of rs713598 and rs1726866, which are associated with bitter taste, appear to have a protective role against caries, whereas the minor allele of rs35874116 showed inconsistent results, suggesting a possible association with increased caries risk (Chisini et al., 2021).

One of the genetic risk factors is rs11362 in the *DEFB1*, that in meta-analysis is found to be associated with dental caries in permanent dentition (Hatipoglu & Saydam, 2020). The *DEFB1* gene is one of the genes encoding human beta defensins (hBD) – small antimicrobial peptides – secreted also by salivary glands (Sahasrabudhe et al., 2000). rs11362 is located in the 5’UTR region (HGVS nomenclature: NC_000008.10(NM_005218.3): c.-20G>C). The genetic variant is located in a gene promoter region and it may decrease the expression of the gene, resulting in lower concentration of beta defensin-1 (Liu et al., 2024). Also, some previous studies in a smaller number of patients have not found statistically significant association with beta defensin-1 level (Polesello et al., 2017). The *DEFB1* gene is encoding hBD-1 that is a peptide with the ability to kill viruses, fungi, and both types of bacteria (gram negative and gram positive). It is always present in the oral mucosa but may also be produced in response to other triggers, such as microbial infection (Hans & Madaan Hans, 2014). Defensins are essential components of the innate immune system and are prevalent in the oral environment (Morio et al., 2023). The expression of defensins is observed in multiple locations within the oral cavity, such as the periodontal tissues, oral mucosa, and salivary glands (Ślebioda et al., 2013).

1.1.2 Periodontal disease

Periodontal disease refers to pathological conditions affecting the periodontium, which encompasses the gingival tissue, alveolar bone, cementum, and periodontal ligament, together forming the supporting structure around the tooth (Kwon et al., 2021). Chronic inflammatory periodontal disease, including both gingivitis and periodontitis, are among the most prevalent

inflammatory conditions in humans, impacting up to 90 % of the global population (Pihlstrom et al., 2005). Gingivitis is the medical word for inflammation of the gums caused by buildup of bacteria and debris between the gum line and tooth, which is also referred to as dental plaque. Gingivitis is a responsive disorder that can be reversed when oral hygiene improves (Yu & Van Dyke, 2020). Periodontitis refers to the advanced stage of periodontal disease where inflammation becomes chronic, damaging, and permanent, extending beyond gingivitis. Subsequently, the bacteria can infiltrate farther into the tissues and the periodontium that surrounds them (Kinane et al., 2017). Periodontitis results in the periodontium's loss of attachment, which can progress to alveolar bone loss, potentially leading to the loss of the affected tooth (Takedachi et al., 2022). Schematic comparison could be seen in Figure 1.1.

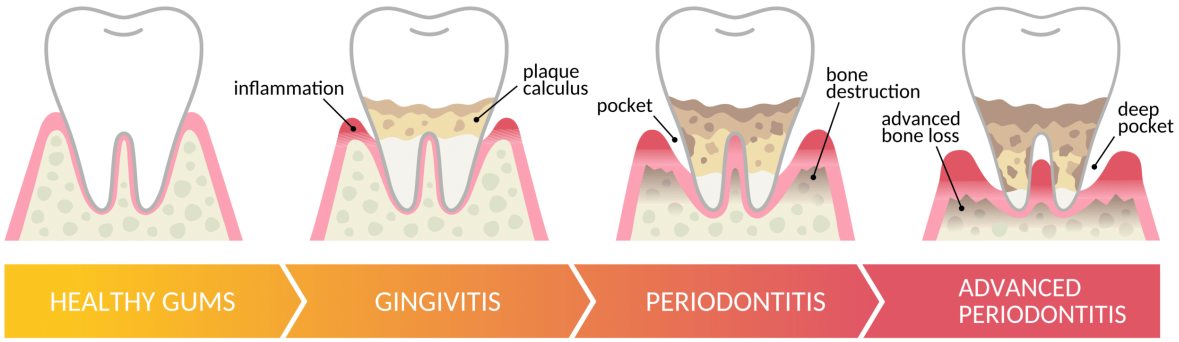

Figure 1.1 **Progression stages for gum inflammation** (Weinstock, 2016)

In 2018, a new classification for defining periodontal disease was introduced. This system aims to provide a more accurate definition based on its stage, which considers the severity and extent of damage to the periodontal tissues as well as the complexity of treatment required. The primary objective of the new classification system is to offer dental specialists a structured approach for personalised diagnosis, treatment, and prevention of periodontal disease (Sorsa et al. 2020). Additionally, the system also considers the grade of periodontal disease, which indicates the risk of future progression (Sorsa et al., 2020; Tonetti et al., 2018).

Diagnostics and management of periodontal disease

Precise and replicable diagnostic methods are essential for identifying persons at risk of illness, detecting disease presence, preferably in its early stages, categorising disease, and assigning diagnoses to individual teeth. The diagnosis of periodontal disease is currently based on clinical and radiographic measures (Tonetti & Sanz, 2019). Clinical examination mostly consists of the use of periodontal probe and visual evaluation of periodontal soft tissues.

A periodontal probe is specifically designed for this purpose, with a 0.5 mm ball tip, to minimise penetration of the probe into soft tissues and also to help in the detection of calculus, a black band between 3.5 and 5.5 mm, and rings at 8.5 and 11.5 mm (Flores-Rodrigo et al., 2022). Clinical probing parameters include bleeding on probing, probing pocket depth and clinical attachment level. Clinical attachment level is measured in relation to the cemento-enamel junction. Periodontal probing allows the clinician to identify subgingival calculus, and irregularities on the root surface. Although plaque and calculus deposits are classified as aetiological factors, they are also assessed as part of the periodontal examination and contribute to the diagnostic process (Lang & Bartold, 2018; Tonetti et al., 2018).

The goals of examining for periodontal disease focus on identifying the presence, severity, and risk factors associated with the condition to guide effective treatment and management (Lang & Bartold, 2018). The Community Periodontal Index of Treatment Need (CPITN) is used to evaluate epidemiological studies, first invented 1982 (Preshaw, 2015). The CPITN is primarily a screening procedure which requires clinical assessment for the presence or absence of periodontal pockets, calculus and gingival bleeding. As part of the evaluation periodontal probing depths and bleeding on probing should be assessed. In addition, recession and clinical attachment loss, tooth mobility, furcation involvement, plaque index, sensibility testing, and occlusion should also be evaluated – see Figure 1.2 (WHO, 2013).

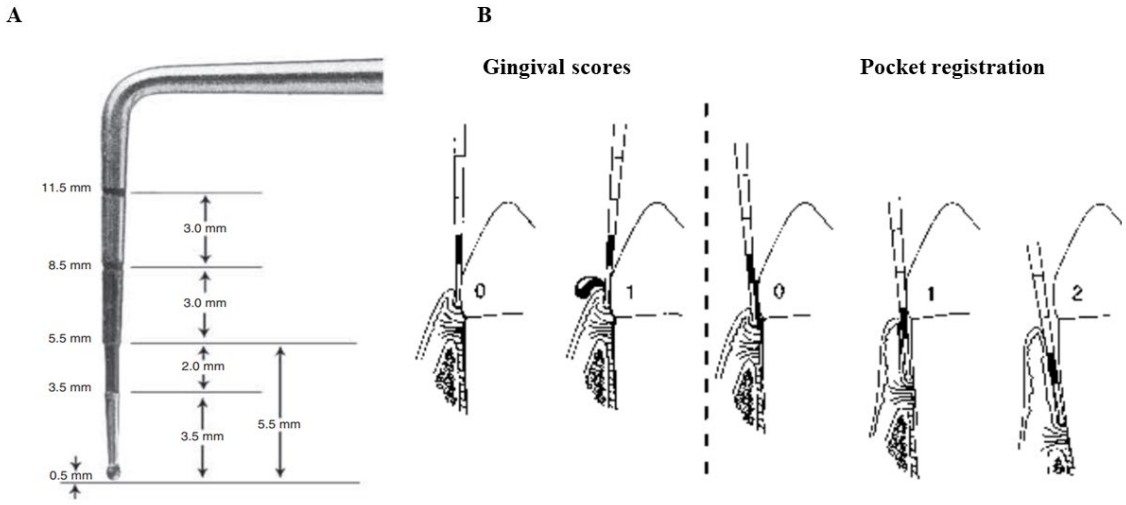

Figure 1.2 **Community Periodontal Index of Treatment Need (CPITN) evaluation (WHO, 2013)**

A – The WHO Community Periodontal Index probe recommended for clinical examination.
B – Coding of periodontal status consistent to the modified Periodontal Index, showing the correct positioning of the WHO Community Periodontal Index probe

Effective management of gingivitis and periodontitis involves a combination of professional dental treatments and diligent oral hygiene practices. The primary goals are to

reduce inflammation, eliminate plaque and calculus, and halt disease progression (Lang & Bartold, 2018).

Oral microbiome and periodontal disease

The initiation and progression of periodontal disease is significantly influenced by inadequate oral hygiene practices. The accumulation of bacteria and plaque on the teeth can result in gingivitis and potentially progress to periodontitis when improper oral hygiene techniques are employed (Ericsson et al., 2009; Music et al., 2021). Anaerobic organisms that are responsible for the progression of periodontal disease can colonise in deeper areas of the periodontium when oral hygiene is inadequate, allowing them to subsequently implement their destructive actions. Several studies have shown that the oral microbiome structure differs in various patient groups with and without periodontitis (Alarcon-Sanchez, 2024; Zhao et al., 2024). *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* are the primary bacteria identified in periodontal disease (Nazir et al., 2020).

Genetic predisposition to periodontal disease

When analysing the heritability of periodontal disease, it was found that one third of phenotypic variability is associated with multiple genetic factors (Nibali et al., 2019) – there are described genetic variants in at least 65 different genes, although results are contradictory (Loos & Van Dyke, 2020) and mainly associated with genetic variants in immune response related genes (Sayad et al., 2020). The immune inflammatory response is primarily responsible for the manifestation of clinical symptoms in pathology. While the immune response is expected to provide protection against periodontal disease, both a weakened response and an overly strong reaction might be harmful to the host (Kwon et al., 2021; Pihlstrom et al., 2005).

The genetic variants of interleukin-1 (*IL-1*) encoding gene are the most widely documented in studies related to the risk of periodontal disease development (Brodzikowska et al., 2019; da Silva et al., 2017; Ding et al., 2012; Ma et al., 2015). The pro-inflammatory cytokine known as interleukin-1 beta (*IL-1B*) plays a significant role in the development of periodontal disease by causing inflammation, bone resorption, and the breakdown of periodontal tissues (Dahash & Kusrat Hussein, 2023). *IL1B* rs1143634 is the most reported genetic variation linked to an elevated susceptibility to the progression of periodontal disease (Abdellatif et al., 2021; Yin et al., 2016). The genetic variant rs1143634 (HGVS nomenclature: NM_000576.2:c.315C>T p.(Phe105 =); legacy name +3954) has been associated in some studies with higher plasma concentration of *IL1B* secretion in patients (López-Anglada et al., 2022).

Defensin, as described above, plays an important role in the innate immune responses and forms the first line of host defines against periodontal pathogens (Gorr & Abdolhosseini, 2011). This protein is found in the oral mucosa and has the ability to combat infections that contribute to the development of periodontal disease (Wang et al., 2015). Between other genetic variants in the *DEFB-1* gene also rs11362 has been analysed in association with periodontal disease, however, a meta-analysis by Shao that summarised seven studies did not identify an association (Shao et al., 2019).

CD36 encodes a multifunctional membrane protein involved in fatty acid metabolism, taste perception – particularly oral fat detection – and overall metabolic regulation. The genetic variant rs1761667 (HGVS: NG_008192.1(NM_001371074.1): c.-180+13244G>A, p.(?), legacy name -31118A-G) has been shown to affect *CD36* expression. Specifically, the A allele is associated with reduced *CD36* expression, which raises the detection threshold for fatty acids; individuals with the AA genotype are therefore less sensitive to fat taste compared to GG carriers (Burgess et al., 2018). Given its wide range of ligands and biological functions, *CD36* has been implicated in several dysmetabolic conditions linked to obesity, including dyslipidaemia, insulin resistance, type 2 diabetes, inflammation, atherosclerosis, and cancer (Hirano et al., 2003; Melis et al., 2017). Although no direct association between rs1761667 and periodontal disease has been reported, *CD36* may be indirectly involved through its role in inflammation – a key pathogenic mechanism in periodontal disease.

1.2 Phenylketonuria

Phenylketonuria (PKU) is the most prevalent inborn error of amino acid metabolism in Europeans caused by the defective hydroxylation of phenylalanine (Phe) to tyrosine (Tyr) due to very low or absent activity of the enzyme phenylalanine hydroxylase (PAH) (Blau et al., 2010). The prevalence of PKU in Europe varies from 1:8500, in Latvia around 1 in 6785 newborns (Hillert et al., 2020; Kreile et al., 2020).

More than 3300 bi-allelic variants in the phenylalanine hydroxylase (PAH) gene, which is located on chromosome 12q22–24.1, have been identified (Blau et al., 2010; van Wegberg et al., 2025). The PAH enzyme, which hydroxylates phenylalanine to tyrosine with the assistance of a cofactor (tetrahydrobiopterin, BH4), molecular oxygen, and non-heme iron, is deficient as a result of these autosomal-recessive inherited variants (van Spronsen et al., 2021).

The metabolic profile is highly heterogeneous, as it is contingent upon the concentration of blood Phe and the extent of residual PAH activity. The daily Phe tolerance is the criterion for determining the severity of PKU. The standard classification is based on the pre-treatment blood Phe concentration and daily dietary Phe tolerance, with the severe classical PKU

characterised by pre-treatment blood Phe concentrations of > 1200 μmol/L, mild PKU characterised by pre-treatment blood Phe concentrations of 600–1200 μmol/L, and mild hyperphenylalaninemia characterised by pre-treatment Phe blood concentrations of 120–600 μmol/L (Blau et al., 2010; Camp et al., 2014).

1.2.1 Diagnosis of phenylketonuria

PKU is diagnosed in a newborn screening, if undiagnosed at birth, symptoms start developing within a few months. In Latvia, PKU has been included in newborn screening since 1985. From 2020 there were 116 individuals diagnosed (Kreile et al., 2020).

In general, untreated PKU leads to global developmental delay or severe irreversible intellectual disability, as well as growth failure, hypopigmentation, motor deficits, ataxia, and convulsions (Jaulent et al., 2020; van Wegberg et al., 2017). Although some late-diagnosed patients with PKU have been reported to escape intellectual disability (van Vliet et al., 2018), they may still face risks such as maternal PKU (Alghamdi et al., 2023).

1.2.2 Treatment of phenylketonuria

Although multiple treatments are under investigation, there is currently no aetiological treatment available for PKU (Smith et al., 2025). Lifelong PKU treatment involves following a strict low-protein diet and consuming a Phe-free amino acid formula approximately three to four times a day, depending on individual requirements (MacDonald et al., 2020). For all patients with PKU, medical nutrition therapy is the primary form of treatment as it is the only way to avoid ingesting the amino acid Phe while meeting their protein intake needs (Hansen et al., 2020). Treatment efficiency is monitored by measuring blood Phe levels, with target concentrations of 120–360 μmol/L in children under 12 years, and 120–600 μmol/L in individuals older than 12 years (MacDonald et al., 2020; van Wegberg et al., 2017).

The PKU diet is primarily composed of low-protein foods, including fruits, vegetables, modified low-protein foods, lipids, and carbohydrates. Unless the dietary restriction is minimal, foods that are high in protein, such as red meat, poultry, fish, milk, eggs, yogurt, legumes, cheese, and beans, are barred from a PKU diet. Foods that are high in fat and contain minimal protein, such as jams, conserves, and desserts that are devoid of gelatine and milk, do not require restriction (Lubina et al., 2023; MacDonald et al., 2020).

The Phe-free amino acid formula contains tyrosine, vitamins, minerals, and trace elements, in addition to the essential amino acids that an individual without PKU would obtain from their daily diet. To accommodate a wide range of lifestyles and preferences, formulas are available in a variety of formats and flavours, including powdered drink mixes, ready-made

drinks, bars, and tablets. These supplements are often sweetened with carbohydrate-containing sweeteners to make them more palatable (Cleary et al., 1997; Daly et al., 2021).

In the diet of a patient with PKU, aspartame is specifically mentioned. Aspartame is an artificial sweetener, consisting of two amino acids Phe and aspartic acid, that is frequently employed in a variety of "sugar-free" products, including foods, beverages, medications, toothpastes, and chewing gums (Newbould et al., 2021). The ingestion of artificial sweetener aspartame and its derivatives is not allowed due to their Phe composition in patients with PKU (Maler et al., 2023).

Sapropterin dihydrochloride (Kuvan®) is a Food and Drug Agency and European Medical agency approved medication for managing PKU – it acts as a synthetic form of BH-4 enhancing its activity and thereby reducing blood Phe concentrations (Feillet et al., 2025; Trefz et al., 2009). A study published by Eshraghi P and colleagues reported that treatment with Kuvan led to a significant reduction in blood Phe levels in paediatric patients, allowing for a more liberal diet (Eshraghi et al., 2019). However, the likelihood of a therapeutic response depends on the specific genetic variants present in the *PAH* gene (Smith et al., 2025). Therefore, a trial period is often recommended to determine individual responsiveness. Additionally, while Kuvan can help manage blood Phe levels, it does not eliminate the need for ongoing dietary management and regular monitoring of Phe concentrations (Smith et al., 2025; Trefz et al., 2009).

1.2.3 Oral microbiome in phenylketonuria

Patients with PKU consume foods that are high in carbohydrates and fat but low in protein, resulting in a less diverse gut microbiome (MacDonald et al., 2020). This likely reduces the number of bacterial species that have co-evolved with traditional diets and rely on nutrient sources that are omitted from PKU dietary regimens (Ostrowska et al., 2023). A study by Bingöl and colleagues showed that, in individuals with PKU, aerobic and facultatively anaerobic *Haemophilus* was considerably enriched, becoming the most prevalent genus. However, broader taxonomic distinctions were less pronounced (Bingol et al., 2024). Furthermore, the dental health indices of patients with PKU were found to correlate with the abundance of *Tannarella*, an anaerobic genus primarily recognised for *Tannarella forsythia*, a species associated with chronic periodontal disease (Griffen et al., 2012).

1.2.4 Caries and periodontal disease in phenylketonuria patients

The specific nutritional needs of patients with PKU and the use of amino acid supplements are significant factors that have the potential to influence their teeth and oral health. For example, during periods of illness, it is recommended that patients with PKU consume supplementary high-energy snacks, for example containing glucose polymers.

Frequent intake of high-glucose and/or sucrose snacks throughout the day results in recurrent drops in salivary pH level, which, combined with insufficient oral hygiene, can result in an increased incidence of caries (van Wegberg et al., 2017).

However, although Phe-free formula is essential for the wellbeing of patients with PKU, it can negatively impact their oral health as it contains a high amount of complex carbohydrates (Yilmaz et al., 2023; MacDonald et al., 2020). There are two types of complex carbohydrates – starch and fibre. Salivary and bacterial amylases in human saliva hydrolyse starch into maltose, maltotriose and low-molecular dextrins (Lingstrom et al., 2000). These by-products in high concentrations are excellent substrates for bacteria to use in acid production resulting in demineralisation of tooth structures causing caries. In a study performed by Bingol and colleagues, which included patients with PKU (both children and adults), a small group with their non-affected siblings and a control group, it was observed that patients with PKU are predisposed to higher levels of caries activity and worse periodontal health due to their dietary needs, and require further periodontal treatment more often (Bingol et al., 2023).

One of the most extensive studies on this subject was conducted by Singh-Hüsgen and colleagues – they examined the oral health of 283 children with PKU and found that they experienced a higher caries level than healthy controls, but not increased risk for the periodontal disease (Singh-Husgen et al., 2016). Ballikaya and colleagues, in their analysis of patients with PKU aged 1 to 22 years (of whom only 16.2 % were older than 11 years), reported a high prevalence of caries among young patients aged 1 to 5 years. In the overall patient group, a moderate gingival index was observed, reflecting periodontal disease status; however, comparisons were made only with literature rather than with a healthy control group (Ballikaya et al., 2020).

1.3 Type 1 diabetes

Diabetes is a chronic, metabolic disease characterised by elevated levels of blood glucose (or blood sugar), which can lead over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves (Abraham et al., 2018; Vanderniet et al., 2022; WHO, 2019). For people living with diabetes, access to affordable treatment, including insulin, is critical to their survival. Diabetes is classified in Type 1 Diabetes (T1D), Type 2 Diabetes, specific types of diabetes due to other causes and gestational diabetes (American Diabetes, 2014; WHO, 2019). Most frequent type that manifests in childhood and adolescence is T1D.

T1D is a disorder that is heterogeneous in nature and is defined by the complete absence of insulin, which is the result of the elimination of pancreatic beta cells. T1D is defined by insulin deficiency and ensuing hyperglycaemia (DiMeglio et al., 2018). T1D is responsible for

5–10 % of the total number of diabetes cases worldwide (Vanderniet et al., 2022). In 2021, there were 8.4 million individuals worldwide with T1D, 1.5 million younger than 20 years, and incidence is increasing especially in Europe (Gong et al., 2024; Gregory et al., 2022). In Latvia in 2022 there were 258 new cases registered (in total 5162 T1D patients) (Health Statistics Database, [https://statistika.spkc.gov.lv/]).

It is now understood that what was once thought to be a single autoimmune disorder caused by a T-cell-mediated attack on insulin-producing cells is actually the result of complex interactions between environmental factors, as well as differences in each patient's microbiome, genome, metabolism, and immune system (Popoviciu et al., 2023).

The complications of diabetes may be categorised into two types: macrovascular and microvascular. Macrovascular complications refer to the development of atherosclerotic cardiovascular illnesses, including coronary heart disease, stroke, and peripheral vascular disease. Hyperglycaemia is the primary cause of each of these problems (Lu et al., 2023). Persistent raising of blood glucose levels causes damage to blood vessels by the excessive accumulation of surface glycoproteins, leading to a weakening of the basement membrane. The endothelium of the retina, kidneys, and peripheral nervous system allows glucose entry even in the absence of insulin, thereby initiating the microvascular complications known as retinopathy, nephropathy, and neuropathy (Tommerdahl et al., 2022).

1.3.1 Diagnosis of type 1 diabetes

Type 1 diabetes can be diagnosed at any age, but the peak is at age 10–14 years (median age for diagnosis is 29 years) (Gregory et al., 2022). A diagnosis of diabetes is based on blood glucose measurements and the presence or absence of symptoms and can be established by the one of the following criteria:

- Classic symptoms of diabetes or hyperglycaemic crisis with random glucose concentration ≥ 11.1 mmol/L (200 mg/dl).
- Fasting plasma glucose ≥ 7.0 mmol/l (≥ 126 mg/dl).
- Two-hour post load glucose ≥ 11.1 mmol/L (200 mg/dl) during oral glucose tolerance test.
- Glycated haemoglobin (HbA1C) level ≥ 6.5 % (Mayer-Davis et al., 2018).

1.3.2 Treatment of type 1 diabetes

Effective management of T1D requires a comprehensive treatment strategy that focuses on insulin therapy, blood glucose monitoring, dietary management, and adjunctive therapies. Advancements in insulin therapy have made it much easier to control the disease, to the point that a diabetic patient can now have a diet somewhat like that of a healthy individual. Treatment

efficiency can be monitored by HbA1 measurement – the target level is ≤ 6.5 %, fasting plasma glucose level of 4–7 mmol/l (72–126 mg/dl) (NICE, 2023). An important part of T1D management is nutritional therapy and its effectiveness are proved in multiple studies. Nutritional therapy recommendations have evolved over time, as insulin substitution enables more precise control of blood glucose levels and helps prevent the complications described above (Evert et al., 2019). In Latvia after receiving a T1D diagnosis patients are educated in nutritional therapy based on bread unit counting. One bread unit corresponds to a quantity of food containing 12–15 g of digestible blood-sugar-effective carbohydrates present in different forms of sugar or starch (Mehnert, 1976; Soczewka et al., 2023).

It is well established that lower HbA1c levels in individuals with type 1 diabetes reduce the risk of both microvascular and macrovascular complications. A meta-analysis by Prigge and colleagues, which included data from 520 392 children and adults, found that glycaemic control was generally better in children; however, it still remained below recommended targets (Prigge et al., 2022).

1.3.3 Oral microbiome in type 1 diabetes

Diabetes mellitus is one of the diseases characterised by distinct alterations in the oral microbiome (Belibasakis et al., 2019; Kunath et al., 2022). It is common for T1D patients to have altered salivary glands, which can influence the composition of saliva and salivary flow rate and ultimately the oral microbiome (Busato et al., 2012). Although recent studies have unequivocally confirmed that the oral microbiome in T1D patients differs from that of control subjects (Kunath et al., 2022; Moskovitz et al., 2021), some studies have not identified such differences (Singh-Husgen et al., 2016). As a result, there is conflicting information regarding the exact nature of these differences and their clinical significance. These discrepancies may stem from differences in sample collection sites within the oral cavity and/or variations in the disease stage of the studied patients (Gregorczyk-Maga et al., 2023). Studies have identified a higher abundance of certain genera such as *Streptococcocus* and *Actinomyces, Prevotella,* and *Veillonella,* along with a reduced abundance of beneficial species like *Streptococcus salivarius* (Kunath et al., 2022; Moskovitz et al., 2021). A study by Ferizi and colleagues in adolescents with T1D identified that the oral microbiome composition was influenced by glycaemic control. Among those with poor metabolic control (n = 46) compared to those with good metabolic control (n = 34), there was a higher presence of cariogenic bacteria such as *S. mutans* and *Lactobacillus* (Ferizi et al., 2022).

Additionally, the subgingival microenvironment in diabetic patients may become altered due to hyperglycaemia, making it more favourable for the growth of pathogenic bacteria (Negrini et al., 2021).

1.3.4 Caries and periodontal disease in type 1 diabetes

It is widely recognised that patients with T1D have a higher risk of developing periodontal disease compared to those without diabetes (Ismail et al., 2015), particularly when glycaemic control is poor (Mealey & Ocampo, 2007), but few examine the link between dental caries and diabetes (Pachoński et al., 2020).

Parameters such as plaque indices, gingival inflammation, and bleeding on probing are commonly used to analyse periodontal disease. Firatli and colleagues and other authors observed significantly elevated plaque, gingival indices, pocket depths, and attachment loss in diabetic children and adolescents, correlating attachment loss with diabetes duration and glycaemic control (Al-Shammari et al., 2006; Firatli, 1997; Lalla et al., 2006; Orbak et al., 2008).

Previous studies focused on the importance of metabolic control for oral health in diabetics. Poor metabolic control has been associated with gingival inflammation and periodontal disease (Ferizi et al., 2022). It has also been suggested that an improvement in the periodontal condition has, in turn, a positive effect on metabolic control (Shinjo & Nishimura, 2024; Simpson et al., 2022). The accumulation of plaque and colonisation of microorganisms in the periodontal pockets of diabetic patients has been found to be swifter and of a more severe nature than in healthy controls. In addition, it has been discovered that patients having T1D tend to develop an earlier and higher inflammatory response to a bacterial challenge than healthy counterparts (Dicembrini et al., 2021; Pachoński et al., 2020).

If children has poorer glycaemic control they are at an increased risk of caries (He et al., 2022). The multi-factored nature of dental caries makes the identification of an exact factor that could be responsible for the association between dental caries and T1D difficult. It has been postulated that slow oral clearance of sugar is caused by impaired salivary secretion resulting in changes in the pH of dental plaque and thereby, increasing the risk of caries developing (Scannapieco et al., 1993). T1D also causes alterations in the salivary glands causing a change in the composition of saliva and in the salivary flow rate, in turn having an effect on oral microflora(Busato et al., 2012; Singh et al., 2021).

2 Materials and methods

2.1 Ethics approval

Approval for this study was obtained from the Central Medical Ethics Committee (No 1/19-03-26, Annex 1) and Genome Research Council of Latvia before the commencement of data collection (A-10/19-05-20, Annex 2). The study was performed according to the principles of the Declaration of Helsinki. Study objectives, possible risks, and participation benefits were explained to all participants or to the parents/legal guardians of underage participants prior to them signing an informed consent (Annex 3).

2.2 Material

The study was performed from 2019 to 2023 at the Children's Clinical University Hospital (Riga, Latvia), Rīga Stradiņš University (Riga, Latvia) and the Latvian Biomedical Research and Study Centre.

2.2.1 Phenylketonuria patients

Data from the Rare disease coordination centre database was used, including all diagnosed patients with PKU in Latvia between 1987–2021 (n = 122). Of these, 101 patients who had not been lost to follow up were invited to participate in the study. For all research components that included oral health examinations, only individuals aged 12 years and older were selected, as by that age primary teeth are replaced by permanent dentition, and the second molars have typically erupted.

The inclusion criteria for patients with PKU were as follows:

- aged ≥ 12 years (for oral health and laboratory parts of the study);
- verified diagnosis of PKU;
- not lost to follow-up;
- ability to participate in the study;
- written informed consent obtained from the patient or legal guardian.

Study participation:

- invited: 101 patients (all diagnosed in Latvia, not lost to follow-up);
- enrolled: 49 patients (13 aged 12–18 years, 36 aged > 18 years).

Oral examination: 45 patients examined (4 excluded due to severe intellectual disability). Oral microbiome & genetic analyses: saliva samples collected from the 49 enrolled patients; however, some samples were excluded at quality control (QC), resulting in slightly lower final numbers.

The survey evaluating the effect of tailored recommendations on daily oral hygiene habits was performed only among patients with PKU in Latvia and was distributed to all patients (and/or their parents) who were not lost to follow-up and were aged ≥ 2 years.

2.2.2 Type 1 diabetes patients

Information about the possibility to participate in the study was distributed to T1D patients and their parents at the Children's Clinical University Endocrinology unit. The target population consisted of patients who fulfilled the following inclusion criteria and were selected based on patient registries, with the majority excluded due to poor glycaemic control.

Inclusion criteria for T1D patients:

- Diagnosed T1D prior to the age of five.
- Well-controlled disease, defined as: average glucose level not exceeding 154 mg/dL (8.5 mmol/L), absence of proliferative diabetic neuropathy, absence of diabetic nephropathy (GFR < 60 mL/min), absence of diabetic autonomic neuropathy.
- Adherence to their recommended diet (18–22 bread units, with one bread unit equivalent to 12 g of carbohydrates).
- Written informed consent for participation in the study.

The study included only well-compensated T1D patients to ensure reliable and comparable results. Poorly controlled diabetes may lead to additional oral health problems (due to chronic hyperglycaemia or diabetes-related complications), making it difficult to determine whether the observed differences stem from diabetes itself or from poor control. Furthermore, we assumed that during tooth development these patients had regular meal patterns due to diabetes management. By selecting stable patients, researchers reduced confounding factors, improved safety, and ensured fair comparison with patients with PKU and healthy controls. The strict criteria also explain the limited number of older T1D patients. Since older patients often have longer disease duration, poorer control, and more complications, they were rarely eligible.

Study participation:

- Interested: 60 patients.
- Excluded: 20 (did not meet inclusion criteria), 9 (approved but did not attend the dental visit).
- Enrolled: 31 patients (22 aged 12–18 years, 9 aged > 18 years).
- Oral examination: all 31 examined.
- Oral microbiome & genetic analyses: saliva samples collected from all 31 patients (the final number of analysed samples slightly reduced after QC).

2.2.3 Control group

Control individuals were recruited primarily at the Stomatology Institute of Rīga Stradiņš University, where dentists invited patients attending regular prophylactic visits who met the following criteria, and additional saliva samples were collected from students at various vocational schools.

Inclusion criteria for control group:

- Sex and age matched the included PKU and T1D patients.
- Absence of known chronic diseases.
- No specific diet (e. g. veganism, vegetarianism).
- Written informed consent for participation in the study.

In addition, individuals for the control group were recruited from other institutions. Besides patients attending prophylactic visits, students were invited to participate as well. Family members of control group participants were also included for oral health examination and oral microbiome analysis, as their inclusion did not affect comparability; however, relatives were not included in the genetic variant analysis.

Study participation:

- Enrolled: 71 individuals (16 aged 12–18 years, 54 aged > 18 years).
- Oral examination: 70 individuals (1 excluded/lost).
- Oral microbiome & genetic analyses: saliva samples collected from all enrolled individuals; relatives were included for oral/microbiome comparisons, but excluded from genetic variant analysis.

Figure 2.1 provides an overview of the three study groups, together with their description and the analysis performed.

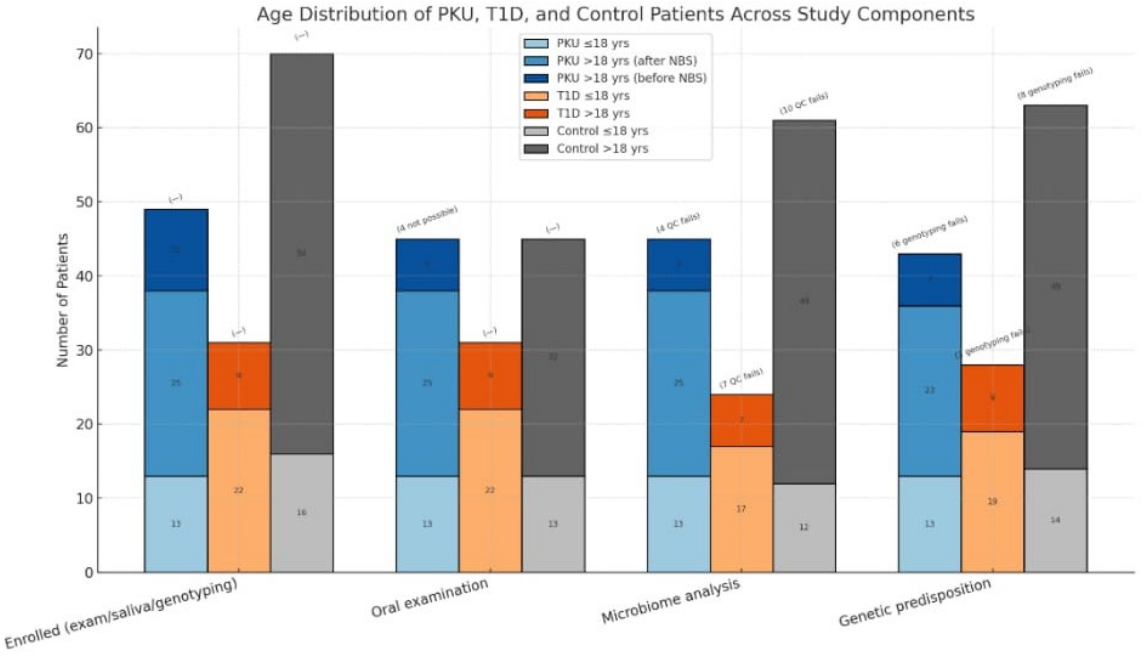

Figure 2.1 **Characterisation of included phenylketonuria, type1 diabetes patients and control group at different stages of the observational study**

PKU – phenylketonuria, T1D – type 1 diabetes.

The number of analysed individuals was affected by their ability to have an oral examination or to collect the saliva. The quality of the isolated DNA had an impact on the size of the research group, which modified the number of persons in the molecular examination section (oral microbiome and genetic variant analysis).

2.3 Clinical examination of teeth and periodontal tissues

Oral examinations were performed for 45 patients with PKU (mean age 24.10 ± 9.04 years), 31 T1D patients (mean age 17.51 ± 6.91 years) and 70 control group individuals (mean age 27.25 ± 1.26 years). A single dentist evaluated each participant, and during the appointment, they completed a clinical card that included questions about their general health and oral hygiene practices (Annex 4). For patients with PKU' additional questions were asked: at what age the diagnosis was established; their adherence to the PKU diet (intake of Phe-free medical formula and other low-Phe foods, as well as how successfully patients are avoiding high-Phe foods); their current and usual plasma Phe concentrations (Annex 4).

Examination was carried out by the same dentist for all patients under appropriate and uniform lighting conditions. Dental status was assessed by identifying decayed teeth, missing teeth, and filled surfaces of teeth using visual–tactile dental examination with a sharp dental probe, mirror, 3–1 syringe, and dental magnifying loupes worn by the dentist. Several indexes were used to assess oral hygiene and gingival health: CPITN index, Silness-Löe plaque index,

and the Greene-Vermillion index, which was calculated upon detection of the presence and abundance of plaque, calculus for specific teeth, the depth of gingival sulci and/or periodontal pockets, and evaluation of gingival bleeding on probing with a periodontal probe.

For the CPITN index, the presence of gum bleeding (score 1), the presence of tooth calculus with the entire black edge of the probe visible (score 2), the presence of 4–5-mm periodontal pockets, the gingival margin positioned against the black probe area (score 3) and the presence of periodontal pockets of 6 mm and above were determined, where the black probe area is not visible (score 4). Patients with CPITN scores of 1 or 2 were classified as having gingivitis, while patients with CPITN scores 3 or 4 were considered at possible risk of periodontitis disease. Ten permanent teeth were examined (d17, 16, 11, 26, 27, 36, 37, 31, 46, 47) with a special graduated CPITN probe with a pod at the end. The black area corresponds to 3.5–5.5 mm. The need for calculus removal and complex treatment should be assessed for each sextant, considering that only 1 type of treatment can be applied to each sextant (Preshaw, 2015; WHO, 2013).

Silness-Löe plaque index belongs to oral hygiene indices and helps to determine the amount of soft and mineralised plaque on d16, 12, 24, 36, 32, 44. The scores from the four areas of the tooth were added together and divided by four to determine the plaque index for the tooth with the following criteria: Grade 0 – no plaque; Grade 1 – a thin plaque layer at the gingival margin, only detectable by scraping with a probe; Grade 2 – a moderate layer of plaque along the gingival margin, plaque fills the gum groove and the adjacent surface of the tooth; Grade 3 – abundant plaque along the gingival margin, interdental spaces filled with plaque. Plaque on the surface of an adjacent tooth (WHO, 2013).

The Greene-Vermillion index is a simplified oral hygiene index with plaque and calculus components (Greene & Vermillion, 1964). Six tooth surfaces were scored, four posterior and two anterior (d16, 26, 11, 46, 36, 31). Plaque was scored on a scale of 0 to 3. Calculus deposits were scored for the same surfaces on a scale of 0 to 3. The index values were calculated from the recordings of the calculus and plaque scores. Debris component scores: 0 – no debris or stain present; 1 – soft debris covering not more than one-third of the exposed tooth surface; 2 – soft debris covering more than one-third but not more than two-thirds of the exposed tooth surface; 3 – soft debris covering more than two-thirds of the exposed tooth surface. Calculus component scores: 0 – no calculus present; 1 – supra-gingival calculus covering not more than one-third of the exposed tooth surface; 2 – supra-gingival calculus covering more than one-third but not more than two-thirds of the exposed tooth surface; 3 – supra-gingival calculus covering more than two-thirds of the exposed tooth surface. By adding together all the codes and dividing by the total number of tooth surfaces inspected,

the average plaque and calculus indices are determined. The two average numbers are then totalled (Greene & Vermillion, 1964).

2.4 Evaluation of daily oral hygiene habits

When inspecting the oral health for all individuals the dentist asked questions about daily oral hygiene (Annex 4):

- Frequency of tooth brushing (answer variants – twice per day, once per day, doesn't brush teeth).
- Usage of fluoride toothpaste.
- Usage of other preventive measures (dental floss, interdental cleaning, mouthwashes).
- Frequency of dental visits.
- Frequency of dental hygienist visits.
- Smoking.

2.4.1 Survey about oral hygiene habits for phenylketonuria patients

As poorer oral health was observed in patients with PKU a more detailed survey about daily oral hygiene habits was prepared. The structured survey based on STROBE guidelines (Ghaferi et al., 2021), composed in Latvian, was finalised and submitted to an expert for requested feedback regarding the content, sensitivity, and standard settings. The questionnaire was validated on 15 non-PKU individuals, including both health care professionals and members of the general population. Modifications were made as necessary to resolve ambiguities. People who participated in the pilot study were not included in the main study. Only one response was available for each query. Parents completed questionnaires for patients under the age of 18.

A survey (Annex 5) was distributed by trained medical personnel, who conducted phone interviews with all patients with PKU who agreed to participate. It was conducted both prior to and after the introduction of the special recommendations to patients with PKU (Annex 6).

2.4.2 Preparation of recommendations for oral health habits in phenylketonuria patients

As the survey results showed poor knowledge of oral hygiene and specific needs for patients with PKU, recommendations for oral health habits targeted to patients with PKU were prepared in Latvian, based on the results of survey and literature search. Recommendations were created by dentists and paediatricians tailored for patients with PKU and distributed to patients and their parents. The purpose of these recommendations was to improve the comprehension of oral health. After a one-month period each participant (family) was

contacted by a phone call and a repeated survey was conducted to evaluate the impact of the provided recommendations. In the group ≥ 18 three patients refused to answer repeatedly.

2.5 Oral microbiome analysis

2.5.1 Saliva sample collection for oral microbiome analysis

All study participants were instructed to have breakfast and brush their teeth after in the morning as usual but no later than 2 hours before saliva sample collection. The salivary sample collection was performed **to determine basal salivary secretion rate.** Participants were provided with a graduated plastic tube for sample collection and a quiet, private space. A 10 ml saliva sample was collected by using the unstimulated drain method; no specific procedures were required other than sitting with the head slightly downward and spitting spontaneously secreted saliva into the collection tube. Afterwards, the 10 ml were divided by number of minutes required for the participant to provide the sample and determined whether their salivary flow is within the normal range of 0.3–0.4 ml/min. The salivary flow rate was expressed as > 60 seconds (it took participants more than 60 seconds to secrete 0.3–0.4 ml of saliva, which indicates decreased salivary flow) and <60 seconds (it took participants less than 60 seconds to secrete 0.3–0.4 ml of saliva, which indicates salivary flow within normal range).

The saliva samples were further prepared as described by Lim and colleagues – samples were centrifuged within an hour after collection at 1000 rpm for 15 minutes to separate epithelial cells and bacteria from the cell-free solution; only the aqueous solution was removed and the 3–5 mL solution was stored at −80ºC prior to DNA extraction (Lim et al., 2017).

2.5.2 Microbial DNA isolation

Thawed saliva samples were transferred to Lysing Matrix E tubes (MP Biomedicals, USA) and homogenised using a MP Biomedicals' FastPrep-24™ bead-beating system (MP Biomedicals, USA) at a speed setting of 6.0 for 40 seconds. The samples were then centrifuged at 14 000× *g* for 10 min and a supernatant was used for DNA extraction using the phenol–chloroform method described by Rovite and colleagues (Rovite et al., 2018).

DNA extraction was performed using the following protocol: the salivary supernatant was transferred to a 15 ml tube and 1 ml Cell Suspension Solution (0.5 M EDTA, 5M potassium chloride) was added. Next, 1.6 mL of the sample mixture was transferred to a Lysing Matrix E tube (MP Biomedicals, USA) and homogenised using the FastPrep-24™ system (MP Biomedicals, USA) at a speed setting of 6.0 for 40 seconds. Samples were then centrifugated for 10 min at 14.500 rpm and the supernatant was transferred to a new 15 ml tube. Then 85 µl of 10 % Sodium Dodecylsulfate solution (Sigma Aldrich, USA) and 5 µl of Proteinase K (Thermo Fischer Scientific, USA) were added to the samples, briefly vortexed,

and incubated at 50°C for 1 hour. To the samples 2 ml of phenol (Sigma Aldrich, USA) was added and mixed by gentle inversion for 15 min. Samples were then centrifuged at 4000 rpm for 10 min, and the supernatant was transferred to a new 15 ml tube. Next, 2 ml of chloroform (Sigma Aldrich, USA) was added and mixed by inverting for 5 min. Samples were centrifuged again at 4000 rpm for 10 min, and the supernatant was transferred to a new sterile tube. Subsequently, 2 ml of isopropyl alcohol (Sigma Aldrich, USA) was added, and the tubes were gently inverted to facilitate DNA precipitation. Once the sample-isopropyl alcohol became homogeneous, the samples were centrifuged at 4000 rpm for 10 minutes. Immediately after centrifugation, the supernatant was removed, and 2ml of 70 % ethanol was added. The pellet was resuspended by vortexing. Samples were then centrifuged at 4000 rpm for 10 min. The supernatant was removed, and the pellet was air-dried at room temperature for at least 15 min, or until the tube appeared completely dry. Subsequently, the pellet was dissolved in 100 µl of Low-TE buffer (1M Tris HCl pH7.6, 0.5 M EDTA). DNA quantity was assessed using the Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA).

2.5.3 *16S rRNA* V3–V4 region amplification and sequencing

A two-stage polymerase chain reaction (PCR) protocol was applied for library preparation. Primers were designed for PCR amplification of the *16S rRNA* gene V3–V4 region specific to the domain bacteria with Illumina overhang adapters as previously described by (Fadrosh et al., 2014) (Table 2.2).

Table 2.2

The primers used for microbiome study with Illumina overhang adapters to amplify the *16S rRNA* V3–V4 region (adapted from (Fadrosh et al., 2014))

Name	Sequence*
16S V3 Fw (341F)	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*NNNNNN* CCTACGGGNGGCWGCAG
16S V4 Rev (805R)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*NNNNNN* GACTACHVGGGTATCTAATCC

* Contains Illumina adapter, heterogeneity spacer, and *16S rRNA* V3-4 region primer. A – Adenine, C – Cytosine, G – Guanine, T – Timine, N – any nucleotide, W – Adenine/ Timine, H – Adenine / Cytosine / Thymine, V – Guanine / Cytosine / Adenine.

Each PCR reaction batch contained negative controls to monitor the purity of the reaction. Microbial DNA (4 ng) was amplified with V3 and V4 primers (Metabion, Germany) using Phusion U Multiplex PCR Master Mix (Thermo Fisher Scientific, USA) under the following reaction conditions: denaturation at 98 °C for 30 sec, 35 cycles of 98 °C for 10 sec, 67 °C for 15 sec, 72 °C for 15 sec, and fragment elongation at 72 °C for 7 min. The yield of the acquired PCR products was assessed using 1.2 % agarose gel electrophoresis and products were purified using a NucleoMag NGS Clean-Up and Size Select kit

(Macherey-Nagel, Germany). The concentration of the PCR products was measured using a Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer and samples were normalised to 4 ng/µL. During the second PCR stage, Illumina MiSeq i7 and i5 (Illumina Inc., USA) indexes were added to the 4 ng of V3 and V4 PCR products using custom-ordered Nextera XT Index Kit (Illumina Inc., USA) and primers (Metabion, Germany). For this reaction, Phusion U Multiplex PCR Master Mix was used under the same thermal cycler reaction conditions specified for the first PCR stage. The *16S rRNA* PCR products were then pooled and purified for the sequencing reaction using NucleoMag magnetic beads. The quality and acquired amount of the *16S rRNA* V3–V4 amplicons were assessed using an Agilent High Sensitivity DNA Chip kit and Agilent 2100 BioAnalyzer (Agilent Technologies, USA) and a Qubit dsDNA HS Assay Kit and Qubit 2.0 Fluorometer, respectively.

Prior to sequencing, all samples were pooled at equal molarities and diluted to 6 pM. They were then paired-end sequenced using a 500-cycle MiSeq Reagent Kit v2 and an Illumina MiSeq (Illumina Inc., USA). Each run was expected to produce at least 10 000 reads per sample. After the sequencing run was completed, the individual sequence reads were filtered using MiSeq software (Illumina Inc., USA) to remove low-quality sequences. All MiSeq quality-approved, trimmed, and filtered data were exported as FASTQ files.

2.5.4 *16S rRNA* sequence analysis

Sequence reads were demultiplexed using Illumina's MiSeq Reporter Software and quality filtered using Trimmomatic v.0.39 (Bolger et al., 2014) with the leading quality of Q20, trailing quality of Q20 and sequences shorter than 36 bp were discarded. All quality-approved sequences were imported into the QIIME 2 v.2021.11 (Bolyen et al., 2019) environment for further analysis. The DADA2 (Callahan et al., 2016) plug-in was used to pair forward and reverse reads, as well as for extra sequence quality control and chimeric sequence removal using a pooled consensus method. The resulting feature table and sequences were used for *de novo* clustering employing VSEARCH plug-in (Rognes et al., 2016) using 97 % identity threshold. Thereafter, *de novo* multiple sequence alignment was performed using the MAFFT method (Katoh & Standley, 2013), while phylogenetic trees were constructed using FastTree 2 (Price et al., 2010). *De novo* clustered sequences were used for taxonomic assignment based on the expanded Human Oral Microbiome Database v.3 (RefSeq v.15.22) identity reference database that was compiled using the RESCRIPT tool (Robeson et al., 2021).

2.6 Genomic DNA analysis

2.6.1 Saliva sample collection for genetic analysis

Genomic DNA sample collection was performed using the unstimulated drain method to collect a salivary sample. The OG-500 (DNA Genotek, Stittsville, ON, Canada) Oragene saliva collection tube was provided to each patient for sample collection. Participants were instructed to assume a seated position with their heads slightly inclined downwards and to spit the saliva that was spontaneously secreted into the designated tube until it reached the indicated mark. The collection tube was then closed, gently mixed, and stored at room temperature until genomic DNA isolation was performed according to manufacturer protocol.

2.6.2 DNA isolation for genomic DNA analysis

In short – firstly the tube with saliva is gently mixed by inversion for a few seconds then the tube is incubated at 50 °C for 1 hour. Further 500 µl of the mixed sample is transferred to a 1.5 ml tube where 20 µl of prepIT-L2P solution (DNA Genotek, Stittsville, ON, Canada) is added and carefully mixed for a few seconds. Afterwards the tube is placed on ice for 10 minutes, and then centrifugated for 5 min at 15 000 × g. The supernatant is transferred to a new microcentrifuge tube and 600 µl of room temperature 95 % ethanol is added and mixed gently by inversions 10 times. The sample is left to precipitate at room temperature, and then is centrifugated for two minutes at 15 000 × g. The supernatant is removed for the DNA platelet to dissolve and 100 µl of TE buffer (1M Tris HCl pH7.6, 0.5 M EDTA) is added.

2.6.3 Genotyping

There were selected single-nucleotide variations that were reported previously with oral health status periodontitis or caries, their nomenclature and allele frequency description is provided in the Table 2.3.

Table 2.3

Nomenclature and short characterisations for genotyped single nucleotide variants

Gene	dbSNP #	Variants legacy name	Nomenclature by Human Genome Variation Society	Minor (alternative) allele	Allele frequency in EU non-Finnish (GnomAd)**	Allele frequency in total (GnomAd)**
Il1b	rs1143634	+3954C>T	NM_000576.3: c.315C>T p. (Phe105 =)	**T (A*)**	**0.2503**	**0.2184**
DEFB1	rs11362	-20G>A	NM_005218.4: c.-20G>A	**A (T*)**	**0.4345**	**0.4310**

Table 2.3 continued

Gene	dbSNP #	Variants legacy name	Nomenclature by Human Genome Variation Society	Minor (alternative) allele	Allele frequency in EU non-Finnish (GnomAd)**	Allele frequency in total (GnomAd)**
CD36	rs1761667	-31118A-G	NC_000007.14 (NM_001371081.1): c.-666+13244G>A	**A**	**0.5411**	**0.5506**
TAS2R38	rs1726866	V262A	NM_176817.5: c.785C>T p. Ala262Val	**A**	**0.5526**	**0.6298**
TAS2R38	rs713598***	A49P	NM_176817.5: c.145G>C (p. Ala49Pro)	**C (G*)**	**0.4028**	**0.6487**
TAS1R2	rs35874116	I191V	NM_152232.6: c.571A>G p. Ile191Val	**C**	**0.3313**	**0.3305**

*Allele name according to chromosomal position from the GnomAd database v.4.1.;
** Last Access 01.03.2025
***all four nucleotides are described in the position
dbSNP – single nucleotide polymorphism database; EU – European.

Genotyping of the rs1143634 and rs11362 was conducted through the application of restriction length polymorphism analysis (Hsieh et al., 2016; Meenakshi et al., 2013); the method (primer sequences, used restriction enzyme and formed fragment length) is summarised in Table 2.4.

Table 2.4

Descriptions of the genotyping method for *IL1B* and *DEFB1* genetic variants

Genetic variant	Primer Sequences	Restriction Enzyme
IL1B rs1143634	5′-GTTGTCATCAGACTTTGACC-3′ 5′-TTCAGTTCATATGGACCAGA-3′	*Taq1* (fragments for A allele 250 bp, for G allele 135 and 115 bp) (Meenakshi et al., 2013)
DEFB1 rs11362	5′-CAGGGGTTAGCGATTAG-3′ 5′-GCAGAGAGTAAACAGAAGGTA-3′	*BstnI* (fragments for G allele 167 bp and 60 bp, for A allele 227 bp) (Hsieh et al., 2016)

In detail – for PCR a mixture with a total volume of 20 µl was prepared to carry out the PCR: 2 µl 10xPCR buffer (NH4)2SO4-MgCl2, 2 µl 25 mM MgCl2, 0.25 µl 20 mM dNTP mix, 0.05 µl 100 pmol synthetic oligonucleotides, 0.1 µl Taq polymerase (recombinant 5 U/µl). All reaction reagents were produced by ThermoScientific (Waltham, USA), synthetic oligonucleotides – by Metabion (Martinsried, Germany). 1 µl (concentration of 30 ng/µl) of the DNA sample was added. The prepared mixture was placed in an automatic thermocycler. In order to perform analysis of the length of the restriction fragments (RFLP), a mixture with a total volume of 4 µl was prepared: 3 µl ddH2O, 0.8 µl buffer suitable for restriction endonuclease, 0.2 µl appropriate restrictase, 4 µl PCR product. All restrictases and suitable

buffers are manufactured by Thermo Scientific (Waltham, USA). The prepared mixture is incubated for 1–16 h, at the temperature of 37°C. After restriction, the samples were tested in 6 polyacrylamide gel electrophoresis. The result was visualised with ethidium bromide and documented using photo-documentation equipment OmniProGel, manufacturer UVITEC (Cambridge, United Kingdom). Positive and negative controls were added to each evaluation to determine the quality of performed analysis.

For the rest of variants (***TAS1R2*** **rs35874116,** ***CD36*** **rs1761667,** ***TAS2R38*** **rs713598, and rs1726866)** genotyping with TaqMan hydrolysis probes were used according to manufacturer protocol using Quant Studio Pro6 (Thermo Scientific, Waltham, USA) – assay characterisation represented in the Table 2.5.

Table 2.5

Description of genotyping method for genetic variants in *TAS1R2, CD36, TAS2R38* genes

Genetic variant	Assay ID	Allele 1 [VIC]	Allele 2 [FAM]
TAS1R2 rs35874116	C_____55646_20	C	T
CD36 rs1761667	C___8314999_10	A	G
TAS2R38 rs713598	C___8876467_10	C	G
TAS2R3 rs1726866	C___9506827_10	A	G

2.7 Statistical analysis

All statistical analyses were performed using SPSS for Windows (version R 4.1.2, SPSS Inc., Chicago, IL, USA), Jamovi software (v2.3, Sydney, Australia), or SPSS v29.0 (IBM Inc., New York, NY, USA). A p-value < 0.05 was considered statistically significant.

2.7.1 Clinical and Oral Health Parameters

Descriptive statistics were calculated for all variables. Since several of the clinical indices (number of decayed, missing, and filled teeth, CPITN scores, oral hygiene indices) showed non-normal distributions and the sample sizes within subgroups were relatively small, non-parametric methods were applied instead of parametric tests such as ANOVA or t-tests, which require normally distributed data. Group comparisons were therefore performed using the Kruskal–Walli's test, which is robust to non-normality. Where significant overall group differences were observed, chi-square tests were used for categorical comparisons, and Fisher's exact test was chosen in cases where expected cell counts were low, as it provides more reliable results for small samples.

Risk analyses of caries and periodontal disease were performed using odds ratios (OR) with 95 % confidence intervals (CI), which are considered the most appropriate measure of association in clinical studies because they express both the magnitude and the precision of risk. Ordinal regression models were applied when the outcome variables (e. g. CPITN scores, oral

hygiene indices) were ordinal rather than continuous, since regression models allow for adjustment and provide effect size estimates beyond simple group comparison.

2.7.2 Oral Hygiene Habits

Self-reported oral hygiene behaviours (brushing frequency, interdental cleaning) were compared between groups using the chi-square test, which is suitable for categorical data. To validate whether reported hygiene behaviours corresponded to clinical findings, ordinal regression was used to analyse associations between brushing frequency and observed oral health indices. This approach was chosen because it accounts for ordered outcome categories (e. g. plaque index scores from 0–3) and provides stronger inference than binary tests.

2.7.3 Microbiome Analysis

Microbiome data were analysed using dedicated ecological and statistical methods within the R environment. To control for uneven sequencing depth, all samples were rarefied to 10 820 sequences per sample using the vegan package, ensuring comparability across groups. Measures of alpha diversity (observed OTUs, Chao1, Shannon, Simpson) were calculated using the phyloseq package, as these indices capture both species richness and evenness, giving a comprehensive view within-sample diversity. Differences in alpha diversity were evaluated using the Wilcoxon rank sum test rather than parametric ANOVA, since microbiome data are highly skewed and not normally distributed. To correct for multiple comparisons, the Holm method was applied, thereby reducing the risk of false positives while maintaining statistical power.

Beta diversity was assessed using weighted and unweighted UniFrac distances and Jaccard distances, which are considered best practice in microbial ecology because they account for both phylogenetic relationships and compositional differences between communities. Significance was tested with permutation-based ANOVA-like tests, which do not rely on distributional assumptions and are thus well suited to high-dimensional microbiome data. Venn diagrams were used to visualise shared and unique taxa, offering an intuitive complement to distance-based analyses.

To identify bacterial taxa differing between groups, DESeq2 was applied. This method models microbiome count data with a negative binomial distribution, which is specifically designed to handle overdispersed sequencing data and is more accurate than simple t-tests or rarefaction-based comparisons. Significant taxa were visualised using the ggplot2 package. Correlations between bacterial abundances and clinical parameters were evaluated using Pearson correlation analysis, with the Benjamini-Hochberg adjustment applied to control the false discovery rate, which is essential when testing many taxa simultaneously.

To evaluate how explanatory variables (e. g. group, brushing frequency, gender) shape microbiome composition, redundancy analysis (RDA) was applied. This multivariate method is widely used in ecology because it allows the partitioning of variation explained by both categorical and continuous predictors. To avoid overfitting, model reduction was performed based on the Akaike information criterion (AIC) and significance was confirmed by Monte Carlo permutation testing.

2.7.4 Genetic Association Analysis

Genetic association analyses were conducted for participants with both oral health and genotyping data. Associations between alleles, genotypes, and inheritance models (dominant, recessive) and clinical outcomes (oral hygiene indices, CPITN, caries risk, salivary secretion) were tested. The chi-square test was applied for differences in genotype distribution, while logistic regression and ordinal regression models were used to test associations with binary or ordinal oral health outcomes. These models were chosen because they allow assessment of different inheritance modes and provide adjusted effect estimates, which are more informative than raw comparisons.

3 Results

3.1 Oral health in patients with phenylketonuria and type 1 diabetes in comparison with control population

The dental status and periodontal health of all individuals included in the study were evaluated using clinical dental examination (decayed, missing, and filled teeth assessment), CPITN, the Silness–Löe plaque index, and the Greene-Vermillion oral hygiene index. The comparison between the PKU, T1D and the control groups are presented in Table 3.1. All parameters, except for the number of filled teeth between the T1D and control group, showed statistically significant differences. One possible explanation could be age, however in the PKU versus control group, age did not differ significantly ($p = 0.091$), whereas in the T1D group, age difference was significant ($p < 0.001$), which could partially explain the observed results.

Table 3.1

Number of filled, extracted and carious teeth in phenylketonuria and type 1 diabetes patients versus control group

	PKU cases Median number (IQR)	**T1D cases Median number (IQR)**	**Control group Median number (IQR)**	***p*-value (PKU versus control group) ***	***p*-value (T1D versus control group) ***
Filled teeth	4 (8)	4 (4)	7 (6)	< 0.001	0.082
Extracted teeth	0 (2)	0 (0)	0 (1)	0.002	< 0.01
Abraded	0 (3)	0 (3.5)	4 (6)	< 0.001	0.006
Carious teeth	4 (5)	3 (2.5)	1 (2)	< 0.001	0.030

* Kruskal-Wallis test; IQR – interquartile range, PKU – phenylketonuria, T1D – type 1 diabetes.

In the PKU group, we analysed whether patients' adherence to a low-protein diet correlates with oral health parameters using the Kruskal-Wallis test. However, none of the associations were statistically significant: for filled teeth, $\chi^2 = 0.788$, $p = 0.674$; for extracted teeth, $\chi^2 = 5.292$, $p = 0.071$; for abraded teeth, $\chi^2 = 5.300$, $p = 0.071$; and for carious teeth, $\chi^2 = 0.827$, $p = 0.661$.

The presence of periodontal disease was evaluated using the CPITN index (Table 3.2). Both the PKU and T1D groups showed a statistically significant difference compared to the control group. The PKU group had the worst periodontal status, with a median CPITN score of 2 (IQR 2), while the T1D group had a median score of 1 (IQR 1), and the control group also had a median score of 1 (IQR 1.75). Notably, pathological pocket depths (CPITN code 4) were observed only in the PKU group.

Table 3.2

CPITN index evaluation in the phenylketonuria, type 1 diabetes and control group

	patients with PKU (n = 45)	T1D patients (n = 31)	Control group (n = 45)
0 (No disease present)	1	4	20
	2.2 %	12.9 %	44.4 %
1 (Gingival bleeding on probing)	12	18	14
	26.7 %	58.1 %	31.1 %
2 (Supragingival and/or subgingival calculus)	13	6	9
	28.9 %	19.3 %	20.0 %
3 (Pathological pocket depth 4–5 mm)	12	3	2
	26.7 %	9.7 %	4.4 %
4 (Pathological pocket depth >6mm)	7	0	0
	15.6 %	0	0.0 %
Median (IQR)	2 (2)	1 (1)	1 (1.75)
p value*	< 0.001	0.029	

IQR – interquartile range, PKU – phenylketonuria, T1D – type 1 diabetes.

As poorer periodontal health was observed in the PKU group, we evaluated whether it was associated with the age of diagnosis. We found that 80 % of patients with delayed diagnosis were either already affected by or at risk of developing periodontal disease (CPITN index 3 or 4), compared to only 31.4 % of patients with PKU diagnosed before the age of two months through newborn screening (OR = 8.7, 95 % CI: 1.6–48.1, $p = 0.008$) see Table 3.3.

Ordinal regression was used to analyse whether diet adherence affected the CPITN index, but the results were not statistically significant. When comparing those who fully followed the diet to partial followers, the association was not significant ($p = 0.192$; OR = 0.451, 95 % CI: 0.133–1.480). Similarly, no significant difference was found between full followers and non-followers ($p = 0.261$; OR = 0.377, 95 % CI: 0.066–2.06).

Table 3.3

Time of diagnosis of phenylketonuria patients in association with risk of periodontal disease

		Diagnosis		Diet			
		Timely*	Delayed	Following the diet	Partly following the diet	Not following the diet	Total
Risk of periodontal disease	No (CPITN 0-2)	24	2	16	5	5	26
		68.6 %	20.0 %	66.0 %	62.5 %	38.5 %	57.8 %
	Yes (CPITN 3-4)	11	8	8	3	8	19
		31.4 %	80.0 %	34.0 %	37.5 %	61.5 %	42.2 %
Total		35	10	24	8	13	45
		100.0 %	100.0 %	100.0 %	100.0 %	100.0 %	100.0 %

* Through the Newborn screening program

3.1.1 Oral hygiene habits and its association with oral health parameters in phenylketonuria, type 1 diabetes and control group

Information about daily oral hygiene habits – the frequency of tooth brushing and the use of interdental cleaning products, was collected from all participants. The results are shown in Table 3.4. The control group had the highest proportion of individuals who brushed their teeth twice per day *(p* = 0.000). In contrast, only in the PKU group were there five individuals (11.1 %) who did not brush their teeth at all. When analysing the use of interdental cleaning products, the PKU group had the highest proportion of individuals who did not use any interdental cleaning products (34 individuals, 75.6 %), which was statistically lower compared to the control group (*p* = 0.000).

Table 3.4

Oral hygiene habits in phenylketonuria, type 1 diabetes and control group

		PKU cases (n = 45)	**T1D (n = 31)**	**Controls (n = 70)**	***p* value PKU vs Control group**	***p* value T1D vs Control group**
Frequency of tooth brushing	Do not brush	5	0	0	*p* = 0.000 OR 22.667 (CI 95 % 4.92-104.41)	*p* = 0.000 OR31.87 (CI95 % 6.61-153.63)
		11.1 %	0 %	0 %		
	Once per day	13	15	2		
		28.9 %	48.4 %	2.8 %		
	Twice per day[a]	27	16	68		
		60.0 %	61.6 %	97.1 %		
Interdental cleaning products	Do not use	34	17	23	*p* = 0.000 OR 6.316 (CI 95 % 2.72-14.67)	*p* = 0.048 OR 2.481 (CI95 % 1.04-5.89)
		75.6 %	54.8 %	32.9 %		
	Dental floss[a]	7	12	45		
		15.6 %	38.7 %	64.3 %		
	Mouthwash[a]	4	2	2		
		8.9 %	6.5 %	2.8 %		
		40.0 %	61.2 %	67.1 %		

[a] in comparison accepted as favourable behaviour; PKU – phenylketonuria, T1D – type 1 diabetes; OR – odds ratio; CI95 % - 95 % confidence interval.

Table 3.5 presents the self-reported results. Oral hygiene indices were evaluated during the oral examination, and the results are shown in Table 3.5. Both the PKU and T1D groups had statistically significantly worse indices compared to the control group. For example, the absence of microbial plaque was observed in only one PKU patient (2.2 %), five T1D patients (16.1 %), and 18 individuals in the control group (40.0 %). Regarding the Greene-Vermilion index, the worst score (index 4) was not observed in any of the control individuals but was recorded in 16 patients with PKU (35.5 %) and one T1D patient (3.2 %).

Table 3.5

Oral hygiene indexes in the phenylketonuria, type 1 diabetes and control group

	Patients with PKU (n = 45)	T1D patients (n = 31)	Control group (n = 45)
Silness-Löe index			
0 (Absence of microbial plaque)	1	5	18
	2.2 %	16.1 %	40.0 %
1 (Thin film of microbial plaque along the free gingival margin)	14	13	17
	31.1 %	41.93 %	37.7 %
2 (Moderate accumulation with plaque in the sulcus)	15	10	9
	33.3 %	32.2 %	20.0 %
3 (Large amount of plaque in sulcus or pocket along the free gingival margin)	15	3	1
	33.3 %	9.7 %	2.2 %
Median (IQR)	2 (2)	1 (1)	1 (1.75)
p value*	< 0.001	0.022	
Greene-Vermilion index			
0 (No calculus present)	3	5	19
	6.6 %	16.1 %	42.2 %
1 (Supra-gingival calculus covering not more than one third of the exposed tooth surface)	10	12	16
	22.2 %	38.7 %	35.5 %
2 (Supra-gingival calculus covering more than one third but not more than two thirds of the exposed tooth surface, or the presence of individual flecks of sub-gingival calculus around the cervical portion of the tooth or both)	16	13	10
	35.5 %	41.9	22.2 %
3 (Supra-gingival calculus covering more than two thirds of the exposed tooth surface, or a continuous heavy band of sub-gingival calculus around the cervical portion of the tooth, or both)	16	1	0
	35.5 %	3.2 %	0 %
Median (IQR)	2 (2)	1 (1)	1 (1)
p value*	< 0.001	0.008	

* Kruskal-Wallis test; IQR – interquartile range, PKU – phenylketonuria, T1D – type 1 diabetes patients.

The need for professional oral hygiene to remove calculus was evaluated. This intervention was not necessary in only 3 patients with PKU (6.7 %), 9 T1D patients (29.0 %), and 33 control individuals (47.1 %). A statistically significant difference was observed between the PKU and control group ($p = 0.000$, OR = 0.080, 95 % CI: 0.023–0.283), but not between the T1D and control group ($p = 0.125$, OR = 0.459, 95 % CI: 0.185–1.113).

Another factor that may affect oral health is the basal salivary secretion rate, which was significantly lower (indicating slower salivary flow) in the PKU group compared to the control group ($p = 0.007$, OR = 0.326, 95 % CI: 0.150–0.710). No significant difference was observed between the T1D and control group ($p = 0.652$, OR = 0.775, 95 % CI: 0.322–1.864). The results are presented in Table 3.6.

Table 3.6

Basal salivary secretion rate in phenylketonuria, type 1 diabetes patients and control group

		PKU cases (n = 45)	T1D (n = 31)	Control group (n = 70)	*p* value PKU vs Controls	*p* value T1D vs Controls
Basal salivary secretion rate	> 60 seconds	18	19	47	*p* = 0.007 OR 0.326 (CI 95 % 0.150–0.710)	*p* = 0.652 OR 0.775 (CI95 % 0.322–1.864)
		40.0 %	61.2 %	67.1 %		
	< 60 seconds[a]	27	12	23		
		60.0 %	38.7 %	32.8 %		

* Fisher's exact test, [a] in comparison accepted as favourable behaviour, PKU – phenylketonuria, T1D – type 1 diabetes patients, OR – odds ratio, CI95 % - 95 % confidence intervals.

3.1.2 Correlation between oral hygiene habits and observed oral health

The main indicator of oral hygiene habits was tooth brushing frequency, preferably twice per day. For further analysis, the data were reclassified, and ordinal regression was used to analyse its association with hygiene indexes to test reliability of self-reported oral health habits. Statistically significant associations were observed in the control group with clinically significant plaque and bad oral hygiene and in the phenylketonuria group only with elevated caries risk (Table 3.7).

Table 3.7

Tooth brushing frequency association with oral health indexes in phenylketonuria, type 1 diabetes and control group

		PKU		T1D		Control group	
		Twice per day	Less than twice per day	Twice per day	Less than twice per day	Twice per day	Less than twice per day
CPITN	No risk for periodontal disease (0-1)	6	7	14	8	51	1
	Risk for periodontal disease (2-3)	12	20	4	5	17	1
		$\chi^2 = 29.8$, $p = 0.591$		$\chi^2 = 0.966$, $p = 0.326$		$\chi^2 = 0.636$, $p = 0.425$	
Silness-Löe index	No clinically significant plaque (0-1)	6	9	10	8	52	0
	Clinically significant plaque (2-3)	12	18	6	7	16	2
		$\chi^2 = 0.000$, $p = 1$		$\chi^2 = 0.267$ $p = 0.605$		**$\chi^2 = 5.948$, $p = 0.015$**	
Greene-Vermilion index	Good oral hygiene (0-1)	5	8	8	9	53	0
	Bad oral hygiene (2-3)	13	19	8	6	15	2
		$\chi^2 = 0.018$, $p = 0.8932$		$\chi^2 = 0.313$ $p = 0.5761$		**$\chi^2 = 6.419$, $p = 0.011$**	

Table 3.7 continued

		PKU		T1D		Control group	
		Twice per day	Less than twice per day	Twice per day	Less than twice per day	Twice per day	Less than twice per day
Caries risk	No risk (1)	9	5	4	4	38	0
	Present caries risk (2-3)	9	22	12	11	30	2
		$\chi^2 = 4.994, p = 0.025$		$\chi^2 = 0.011, p = 0.916$		$\chi^2 = 2.445, p = 0.118$	

PKU – phenylketonuria, T1D – type 1 diabetes patients, χ^2 - Chi Square.

3.1.3 Oral health recommendations for phenylketonuria patients

To educate patients with PKU about oral health habits with specifics related to the phenylalanine restricted diet, oral care recommendations for patients with PKU were created in Latvian. Recommendations were created by rare disease specialists and dentists. Those were distributed to all patients with PKU and their parents, published for health care professionals in national journal "*Latvijas ārsts*". Recommendations are summarised in the Table 3.8.

Table 3.8

Oral health recommendations for phenylketonuria patients

Daily regimen:	1) Teeth brushing twice per day: once after breakfast and once before bedtime. 2) Use fluoride toothpaste (1000-1450ppm) that does not contain aspartame: Ages 0-2: Rice-grain-sized amount (1000 ppm) Ages 2-6: Pea-sized amount (1000 ppm) Ages 6+: Half-length of toothbrush bristle part (1450 ppm). 3) Use a toothbrush with soft bristles (labelled "soft" or "ultra-soft").
Tooth brushing tips	Avoid rinsing after brushing; spit out excess toothpaste. Children under 10 need parental assistance; children aged 10+ can brush independently.
Interdental Cleaning	Use dental floss or an irrigator daily to clean between teeth. Suitable for both baby (deciduous) and adult (permanent) teeth.
Tongue cleaning	For adults (18+), clean the tongue each morning after brushing. Use a toothbrush, tongue scraper, or spoon, moving from back to front of the tongue.
Saliva stimulation	Use aspartame-free chewing gum to stimulate saliva, which helps to remove dental plaque.
After amino acid mixture consumption care	After consuming amino-acid mixtures, rinse the mouth with plain water.
Mouth wash	If recommended, use chlorhexidine mouthwash (0.05 %-0.09 %) twice daily, 30 minutes after brushing. Use 10 mL of liquid and rinse for 30 seconds, avoiding rinsing with water afterward. Alternatives: Rinse with saltwater, coconut oil, or plant-based mouthwashes (Aloe Vera, Echinacea, Chamomile, Grapefruit seed oil) 2-3 times per day. Use for one month, then take a 3-month break. Avoid rinsing with water after use.
Dentist and oral hygienist visits	Schedule dental check-ups every six months as part of preventive care. Visit a dental hygienist every 3-6 months for professional cleaning as part of proactive oral care.

3.1.4 Evaluating the effects of created recommendations and the change of oral hygiene habits in phenylketonuria patients

In a phone survey conducted among patients with PKU, participants were asked detailed questions regarding their oral hygiene habits (Annex 5). The survey was distributed independently of any oral examination, microbiome, or genetic predisposition testing. The results are shown in Table 3.9.

Table 3.9

Daily oral hygiene habits in the phenylketonuria patients before and after received tailored recommendations

		Patients aged 2–18 years						Patients aged 18 and over					
		Before Recommendations		After Recommendations			* *p*-value	Before Recommendations		After Recommendations			* *p*-value
		n = 48	%	n = 48	%	Improvement %		n = 31	%	n = 28	%	Improvement %	
How often do you brush your teeth?	Once a day	13	27	4	8	−19	0.007	8	26	5	18	−8	0.539
	Twice a day	32	66	44	92	25		23	74	23	82	8	
	Less than daily	3	6	0	0	−6		0	0	0	0	0	
Do you use dental floss daily?	Yes	8	17	19	40	23	0.023	12	39	9	32	−7	0.799
	No	40	83	29	60	−23		19	61	19	68	7	

Table 3.9 continued

Do you consistently rinse your mouth with water after each use of an amino acid-based formula?		How often do you go to a dental hygienist?			How often do you visit a dentist?			Patients aged 2–18 years						* *p*-value	Patients aged 18 and over						* *p*-value
								Before Recommendations		After Recommendations					Before Recommendations		After Recommendations				
Drink water or use other methods	No	Yes	Rarely	Once a year	Twice a year	Rarely	Once a year	Twice a year	n = 48	%	n = 48	%	Improvement %		n = 31	%	n = 28	%	Improvement %		
21	18	9	23	17	8	19	21	7													
44	37	19	48	35	17	40	44	15													
23	12	13	6	26	16	1	27	20													
48	25	27	12	54	33	2.	56	42													
4.	−12	8.	−35	19	17	−37	12	27						< 0.001							
0.364			< 0.001																		
3	15	13	17	10	4	12	14	5													
10	48	42	55	32	13	39	45	16													
7	12	9	14	6	8	10	10	8													
25	43	32	50	21	29	36	36	29													
15.3	−5	−10	−5	−11	16	−3	−9	12													
0.284			0.289												0.498						

* Chi-square test

As previously observed, twice-daily tooth brushing was reported by 66 % of patients with PKU under 18 years of age and by 74 % of patients aged 18 and older. Dental flossing was practiced by 17 % of younger patients and 39 % of older patients. Following the consumption of an amino acid mixture, rinsing the mouth with water was reported by 19 % of younger patients and 42 % of older patients.

According to the survey, 87 % of patients with PKU under 18 years and 42 % of those 18 years and older used toothpaste without aspartame. Some patients lacked sufficient knowledge about which toothpaste to use, with many being unaware of aspartame content (48 % under 18 years). However, one month after recommendations and reminders regarding aspartame, survey results showed that approximately 80 % of the under-18 group and 60 % of those 18 and older were using aspartame-free toothpaste. This represents a 20 % improvement in aspartame-free toothpaste use among patients aged 18 and older.

It is notable that 90 % of patients in both groups agreed that the provided recommendations would improve their daily oral care routines. One month after receiving these recommendations, the survey was repeated – results in the Table 3.6. The most significant changes were observed in the group under 18 years of age, suggesting that the parents of younger patients with PKU reviewed the recommendations and are likely to support adherence.

In this follow-up, 52 % of patients under 18 and 43 % of those aged 18 and older reported that the recommendations positively influenced their oral hygiene practices, as confirmed through targeted questions. For example, prior to the recommendations, 66 % of patients with PKU under 18 reported brushing their teeth twice daily, which increased to 92 % after implementation ($p = 0.007$). Additionally, the proportion of patients under 18 who floss daily rose from 17 % to 40 % ($p = 0.023$).

Moreover, 42 % of patients with PKU under 18 indicated plans to schedule biannual dental visits, compared to just 15 % before the recommendations ($p < 0.001$). Similar improvements were noted regarding visits to a dental hygienist, with an increase from 17 % to 33 % ($p < 0.001$).

For the group aged 18 and older, the survey included questions about the educational background. Among adult patients with PKU, 20 % reported having higher education, 23 % required special education, and the remainder held general secondary or vocational secondary education.

3.2 Results of oral microbiome traits in type 1 diabetes and phenylketonuria patients in Latvia

3.2.1 Group information

A total of 130 samples were available for the microbiome analysis based on sequencing quality criteria described in the methods section from the three study groups: T1D patients (24 samples), patients with PKU (45 samples), and generally healthy people as the control group (61 samples).

3.2.2 Taxonomic structure and diversity analysis

All samples were analysed using *16S rRNA* V3–V4 sequencing. After quality-filtering, 7 347 913 sequences were obtained for 130 samples with an average of 56,960 ± 22,037 reads per sample. Subsequently, samples were rarefied to an even sequencing depth, resulting in 10 820 sequences and 78.5 ± 196.0 OTUs per sample.

The most abundant bacterial genera in the control group were *Streptococcus* (55.4 %), *Prevotella* (9.5 %), *Veillonella* (9.1 %), and *Rothia* genera (4.5 %). Similarly, the dominant genera in the T1D and PKU groups were *Streptococcus* (T1D: 53.3 %, PKU: 48.5 %), *Veillonella* (T1D: 10.3 %, PKU: 8.2 %), *Prevotella* (T1D: 9.1 %, PKU: 12.6 %), and *Rothia* (T1D: 4.7 %, PKU: 5.5 %) (Figure 3.1.). Considering the abundance at the species level, the most common bacterial species in the control group were unidentified *Streptococcus* species (54.1 %), *Prevotella melaninogenica* (5.3 %), unidentified *Veillonella* species (4.5 %), and *Rothia mucilaginosa* (4.4 %). The most common bacterial species in the T1D group were unidentified *Streptococcus* species (16.3 %), unidentified *Pseudomonas* species (4.9 %), *Veillonella atypica* (4.5 %), and *Rothia mucilaginosa* (4.5 %), whereas in the PKU group they were unidentified *Streptococcus* species (48.3 %), *Prevotella melaninogenica* (8.0 %), *Rothia mucilaginosa* (5.5 %), and unidentified *Pseudomonas* species (4.6 %).

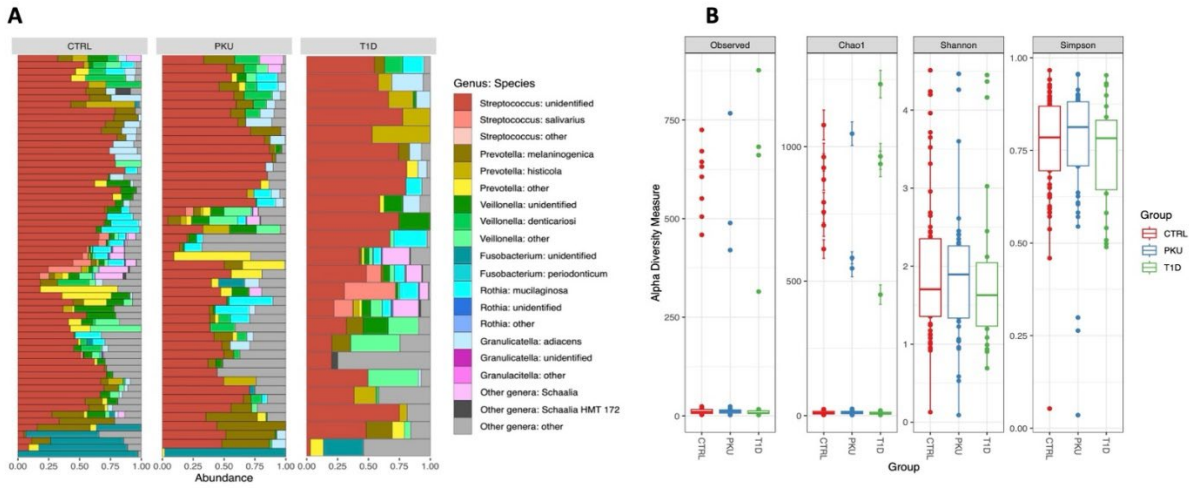

Figure 3.1 **Summary of taxonomic composition and alpha diversity measurements across the three study groups**

(A) Bar plot of taxonomic entities displaying top bacterial genera and their respective species found in CTRL (Control group), PKU (Phenylketonuria group), and T1D (Type 1 Diabetes) patient saliva samples. (B) Alpha diversity metrics were estimated for observed OTUs (operational taxonomic units), Chao1 (total richness), Shannon (mean species diversity), and Simpson indexes (mean species evenness) – coloured according to the study group.

Pairwise comparisons showed that alpha diversity measurements – observed OTUs (number of taxonomic groups observed in the sample), Chao1 (total richness), Shannon index (mean species diversity), and Simpson index (mean species evenness) – did not differ significantly among the three study groups ($p > 0.05$, Figure 3.1.B, Table 3.10), suggesting similar intrasample diversity levels.

Table 3.10

Pairwise comparisons using Wilcoxon rank sum test and Holm p-value adjustment method for alpha diversity metrics between phenylketonuria, type 1 diabetes patients and control group

		Control group	PKU
Observed operational taxonomic units	PKU	0.84	–
	T1D	0.64	0.64
Chao1 index	PKU	0.79	–
	T1D	0.64	0.64
Shannon index	PKU	1	–
	T1D	1	1
Simpson index	PKU	0.94	–
	T1D	0.94	0.94

PKU – Phenylketonuria group, T1D – Type 1 Diabetes group

The PKU group was analysed separately based on adherence to diet (strict, partial, or no diet regime). However, none of the estimated diversity indices showed significant differences between these groups ($p > 0.05$, Table 3.11). The control and T1D groups were not included in this analysis, as the control group subjects had no dietary restrictions and all T1D patients followed a strict diet.

Table 3.11

Pairwise comparisons using Wilcoxon rank sum test and Holm *p*-value adjustment method for alpha diversity metrics between phenylketonuria patients strictly and partly following the diet

		Strict diet	**Partly following the diet**
Observed operational taxonomic units	Strict diet	0.52	–
	Partly following the diet	0.58	0.79
Chao1 index	Strict diet	0.50	–
	Partly following the diet	0.66	0.82
Shannon index	Strict diet	0.63	–
	Partly following the diet	0.63	0.88
Simpson index	Strict diet	0.8	–
	Partly following the diet	0.8	0.8

To examine whether differences existed in intersample variability among the PKU, T1D, and control groups, pairwise comparisons of UniFrac metrics distance were carried out. Weighted and unweighted UniFrac metrics (Figure 3.2) of the salivary microbiome at the genus level by study group presented no significant differences ($p_{adj} > 0.05$, with 999 permutations) in beta diversity among the three groups, suggesting that the global compositions of bacterial communities across our study groups were highly similar. These results were supported by a Venn diagram (Figure 3.2) showing that the intersection (common to all three study groups) had the highest number of species (n = 71, corresponding to 33 %). The lowest number of shared species between two groups was for the PKU and T1D groups (n = 8, corresponding to 4 %), including such entities as *Prevotella* HMT 317, *Eubacterium nodatum, Prevotella intermedia, Alloprevotella tannerae*, and others. The highest number of shared species between two groups was for the T1D and control groups (n = 33, corresponding to 15 %), including such entities as *Eubacterium saphenum, Solobacterium moorei, Filifactor alocis, Oribacterium asaccharolyticum, Capnocytophaga gingivalis*, and others. The highest number of unique species belonged to the PKU group (n = 47, corresponding to 22 %), including such exemplars as *Dialister pneumosintes, Selenomonas noxia, Bifidobacterium breve, Schaalia odontolyticus, Mycoplasma salivary*, and others.

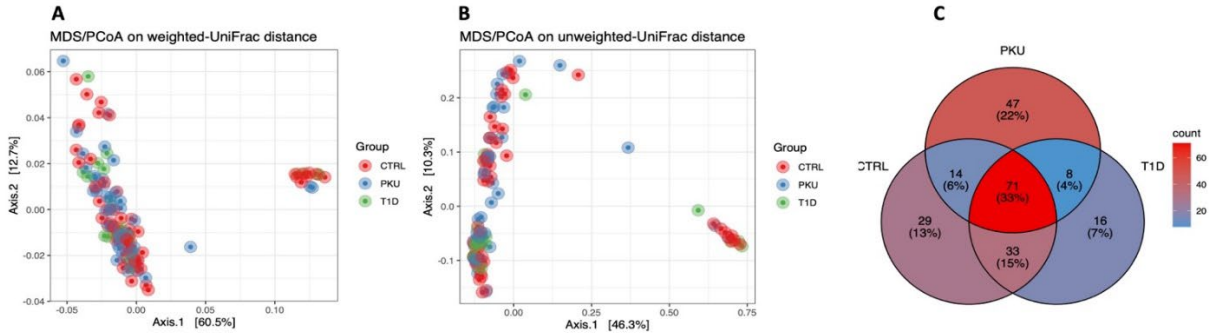

Figure 3.2 **Beta diversity and quantity of bacterial species shared among the phenylketonuria, type 1 diabetes, and control group**

Principal coordinate analysis (PCoA) of the salivary microbiome samples estimated via (A) weighted UniFrac, (B) unweighted UniFrac, and (C) Venn diagram of shared species across the three study groups.

3.2.3 Salivary microbiome differences among the study groups

Differential abundance analysis was used to investigate the bacterial entities that differed significantly between the PKU and T1D groups. Differential abundance testing with DESeq2 ($p_{adj} < 0.001$, $|\log_2(\text{fold change})| > 2$) revealed four species that were differentially abundant across the PKU, T1D, and control group, namely an unidentified *Leptotrichia* species ($p_{adj} = 1.15 \times 10^{-15}$), *Prevotella pallens* ($p_{adj} = 4.88 \times 10^{-12}$), an unidentified *Fusobacterium* species ($p_{adj} = 8.05 \times 10^{-5}$), and an unidentified *Capnocytophaga* species ($p_{adj} = 1.19 \times 10^{-4}$) (Figure 3.3).

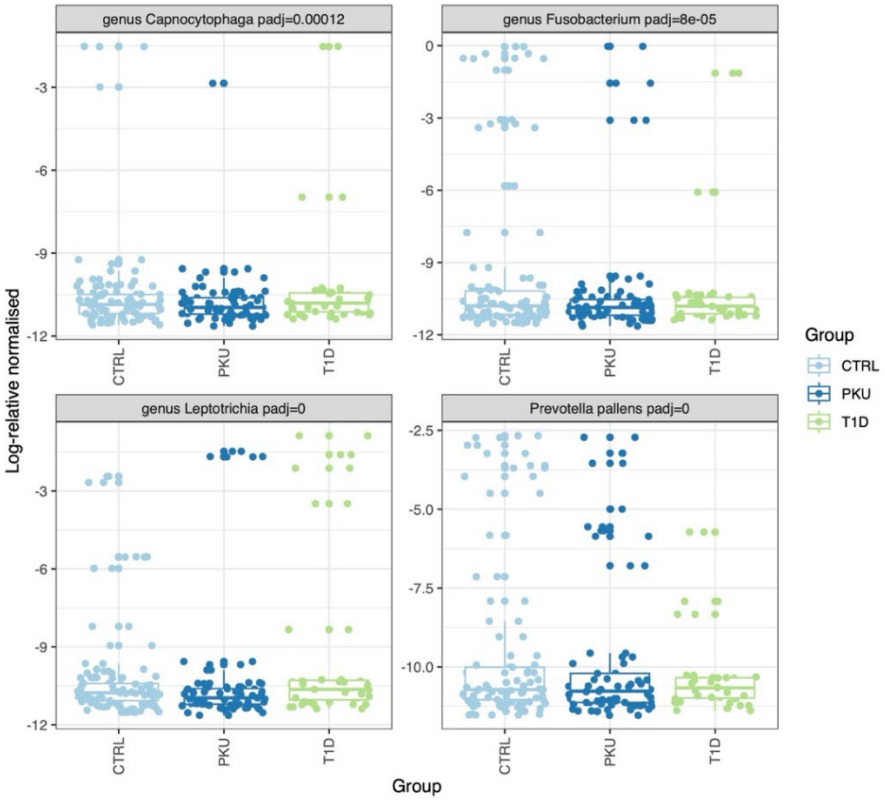

Figure 3.3 **Differentially abundant species-level taxa across all study groups, based on a significance cut-off of $p < 0.001$**

CTRL–control group; PKU–phenylketonuria group; T1D–type 1 diabetes group

Differential analysis was also used to investigate which bacterial species significantly differed between the saliva samples of the T1D and control groups (Figure 3.4). Six species were found to differ significantly between the samples of these two groups, with species enriched in the T1D group including unidentified *Leptotrichia* species ($p_{adj} = 9.65 \times 10^{-9}$) and unidentified *Porphyromonas* species ($p_{adj} = 2.57 \times 10^{-5}$), whereas depleted species within the T1D group included *Prevotella pallens* ($p_{adj} = 2.14 \times 10^{-10}$), an unidentified *Fusobacterium* species ($p_{adj} = 8.04 \times 10^{-5}$), *Veillonella denticariosi* ($p_{adj} = 8.04 \times 10^{-5}$), and *Gemella haemolysans* ($p_{adj} = 1.21 \times 10^{-4}$).

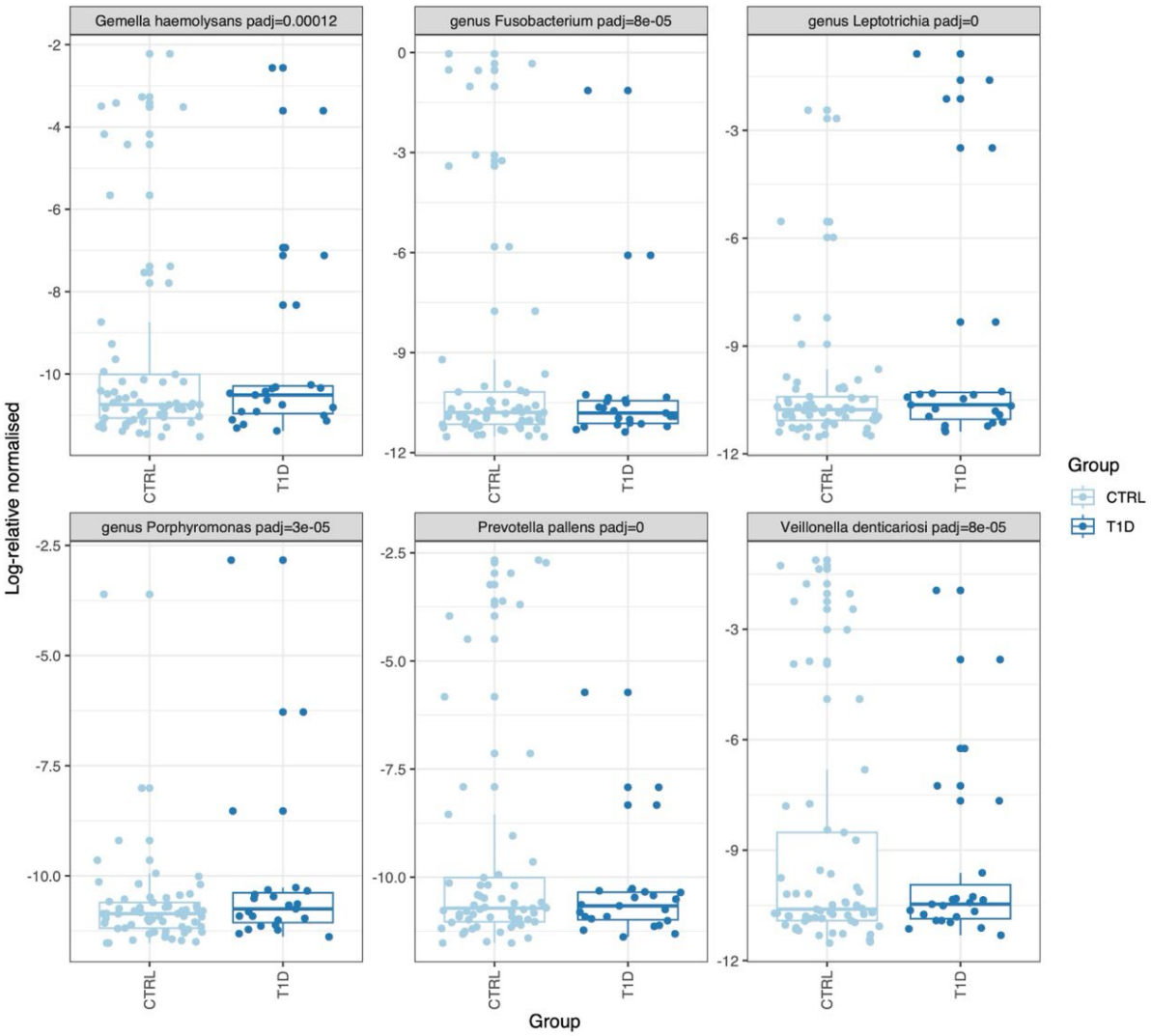

Figure 3.4 **Differentially abundant species-level entities between the control and type 1 diabetes study groups, based on a significance cut-off of $p < 0.001$**

Abbreviations: CTRL–control group; T1D–type 1 diabetes group.

Furthermore, saliva samples from the PKU and control groups underwent a similar analysis. The abundance of 17 bacterial species-level entities was found to differ significantly between the PKU and control groups. Enriched species in the PKU group included such entities as *Veillonella dispar* ($p_{adj} = 9.44 \times 10^{-16}$), *Haemophilus pittmaniae* ($p_{adj} = 2.16 \times 10^{-4}$), and

others, while depleted species included *Streptococcus salivarius* ($p_{adj} = 5.81 \times 10^{-31}$), *Prevotella pallens* ($p_{adj} = 9.53 \times 10^{-4}$), *Prevotella salivae* ($p_{adj} = 2.31 \times 10^{-14}$), *Rothia dentocariosa* ($p_{adj} = 4.08 \times 10^{-9}$), *Megasphaera micronuci formis* ($p_{adj} = 1.08 \times 10^{-8}$), and others (Figure 3.5).

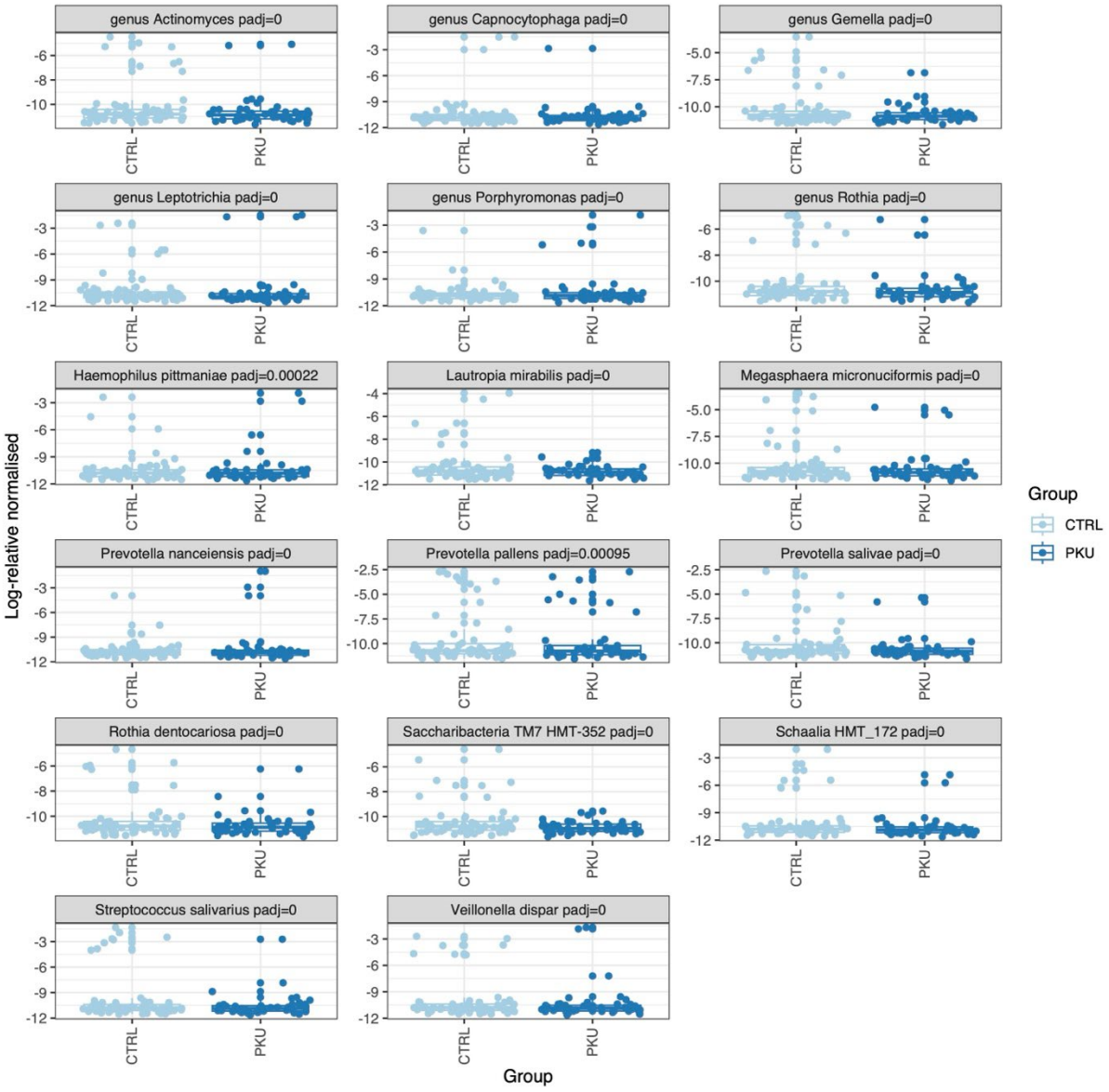

Figure 3.5 **Differentially abundant species-level entities between the control group and phenylketonuria study groups, based on a significance cut-off of $p < 0.001$**

CTRL – control group; PKU – phenylketonuria group.

Furthermore, saliva samples from the PKU group were stratified by dietary regimen (strict, partial, or no diet) and subjected to differential analysis together with control group samples. As a result, the relative abundance of 24 bacterial species differed significantly among the three PKU subgroups and the control group. Notable examples include:

- *Streptococcus salivarius* ($p_{adj} = 1.91 \times 10^{-25}$), which was depleted in all PKU subgroups, though its abundance was slightly higher in individuals adhering to a strict diet.
- *Schaalia* HMT-172 ($p_{adj} = 2.15 \times 10^{-14}$), also depleted across all PKU subgroups, with relatively higher in those with partial adherence to the diet.
- *Veillonella dispar* ($p_{adj} = 2.42 \times 10^{-11}$), enriched in all PKU subgroups, with the highest abundance observed in individuals with dietary adherence.
- *Rothia dentocariosa* ($p_{adj} = 5.08 \times 10^{-10}$), depleted in all subgroups but most abundant in those following a strict diet.
- *Prevotella salivae* ($p_{adj} = 7.44 \times 10^{-10}$), similarly depleted in all PKU subgroups, with relatively higher abundance in the partial adherence subgroup.
- *Porphyromonas gingivalis* ($p_{adj} = 1.64 \times 10^{-7}$), enriched exclusively in individuals following a strict dietary regimen.
- *Porphyromonas pasteri* ($p_{adj} = 2.57 \times 10^{-6}$), depleted across all PKU subgroups.

Additional species showing differential abundance are presented in Figure 3.6.

Figure 3.6 **Differentially abundant species-level entities ($p < 0.001$) between the control group and phenylketonuria subgroups with different levels of dietary adherence**

CTRL – control group; PKU – phenylketonuria group; PKU-No – patients of phenylketonuria group with no diet regime; PKU-Partly – patients of phenylketonuria group with partial diet regime; PKU-Yes – patients of phenylketonuria group with strict adherence to the diet.

3.2.4 Correlations between bacterial communities and clinical parameters

To evaluate the associations between patient-specific characteristics (such as age, carious teeth, extracted teeth, plaque index, and others) and taxonomic entities in the salivary microbiome across study groups, a correlation analysis was carried out (Figure 3.7).

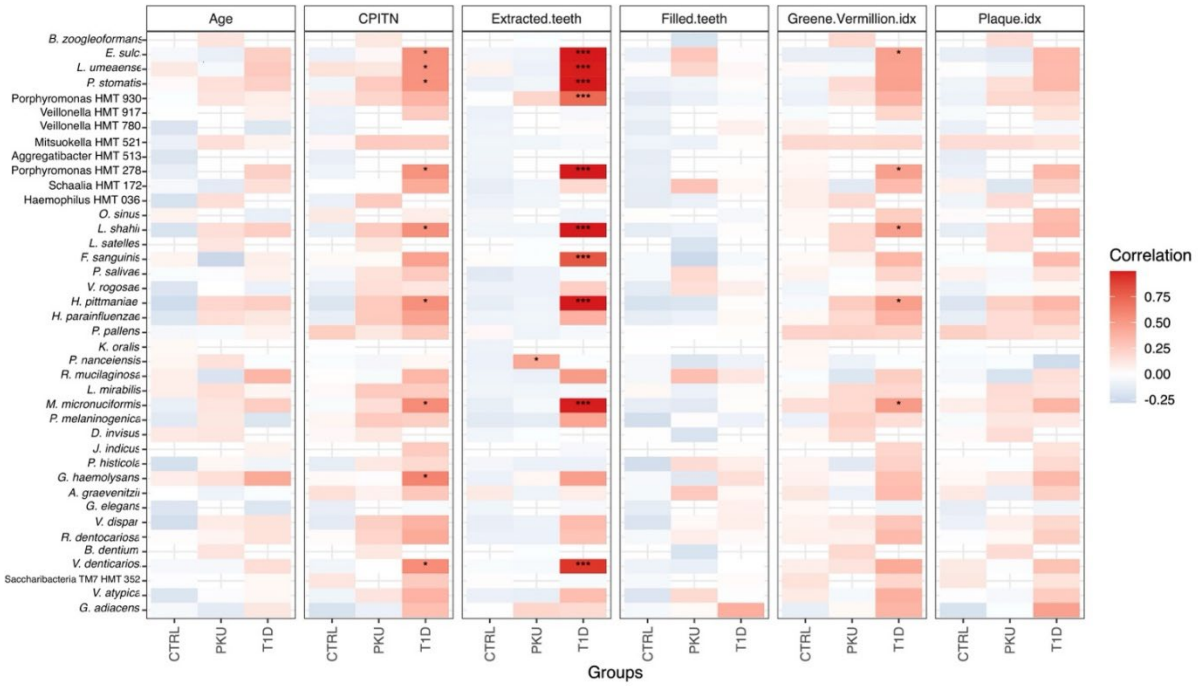

Figure 3.7 **Correlation map of species-level taxonomic entities and patient-specific characteristics across the control, phenylketonuria, and type 1 diabetes groups**

* p = [0.001, 0.05], *** p = [0, 0.001].

Upon testing each variable within the PKU group, we found only one significant correlation – a positive correlation of *Prevotella nanceiensis* with the number of extracted teeth (p_{adj} = 0.038, r = 0.43). On the other hand, many species showed significant positive correlations with patient-specific characteristics in the T1D group; for instance, nine species showed a significant positive correlation with CPITN, including *Peptostreptococcus stomatis* (p_{adj} = 0.029, r = 0.54), *Haemophilus pittmaniae* (p_{adj} = 0.024, r = 0.55), *Megasphaera micronuciformis* (p_{adj} = 0.017, r = 0.57), *Gemella haemolysans* (p_{adj} = 0.018, r = 0.6), and *Veillonella denti cariosi* (p_{adj} = 0.019, r = 0.56); additionally, ten species were found to have a significant positive correlation with the number of extracted teeth in the T1D group, which included such species as *Lachnoanaerobaculum umeaense* ($p_{adj} = 5.32 \times 10^{-17}$, r = 0.99), *Peptostreptococcus stomatis* ($p_{adj} = 1.95 \times 10^{-21}$, r = 0.99), *Fastidiosipila sanguinis* ($p_{adj} = 2.58 \times 10^{-5}$, r = 0.8), *Haemophilus pittmaniae* ($p_{adj} = 3.54 \times 10^{-23}$, r = 0.99), *Porphyromonas* HMT_930 (p_{adj} = 0.00075, r = 0.72), *Megasphaera micronuciformis* ($p_{adj} = 2.56 \times 10^{-18}$, r = 0.99), *Veillonella denticariosi* ($p_{adj} = 3.07 \times 10^{-9}$, r = 0.92), and others. In addition, within the T1D group, the species *Megasphaera micronuciformis* (p_{adj} = 0.041, r = 0.49) was found to have a significant positive correlation with the Greene-Vermillion index. In contrast to the results for the PKU and T1D study groups, no taxonomic entities were found to be significantly associated with any patient-specific characteristics in the control group.

Redundancy analysis (RDA) was conducted to extract and summarise the variation in the dataset that are explained by the explanatory variables. For this purpose, the dataset was divided into two subsets: one comprising control and T1D samples, and the other comprising control and PKU samples.

In the combined dataset of T1D and control groups, ANOVA testing of each variable identified "Group" ($p = 0.002$, 999 perm.) as a significant determinant of microbial composition. To optimise variance explained, the number of explanatory variables was subsequently reduced. However, other patient-specific variables, such as CPITN ($p = 0.175$, 999 perm.), number of extracted teeth ($p = 0.24$, 999 perm.), Greene-Vermillion index ($p = 0.77$, 999 perm.), carious teeth ($p = 0.77$, 999 perm.), plaque index ($p = 0.87$, 999 perm.), age ($p = 0.51$, 999 perm.), and others, were insignificant determinants of the microbial community composition in relation to the study groups (Figure 3.8.). The results were confirmed by testing the parsimonious RDA model with the global model ($p = 0.003$, 999 perm.).

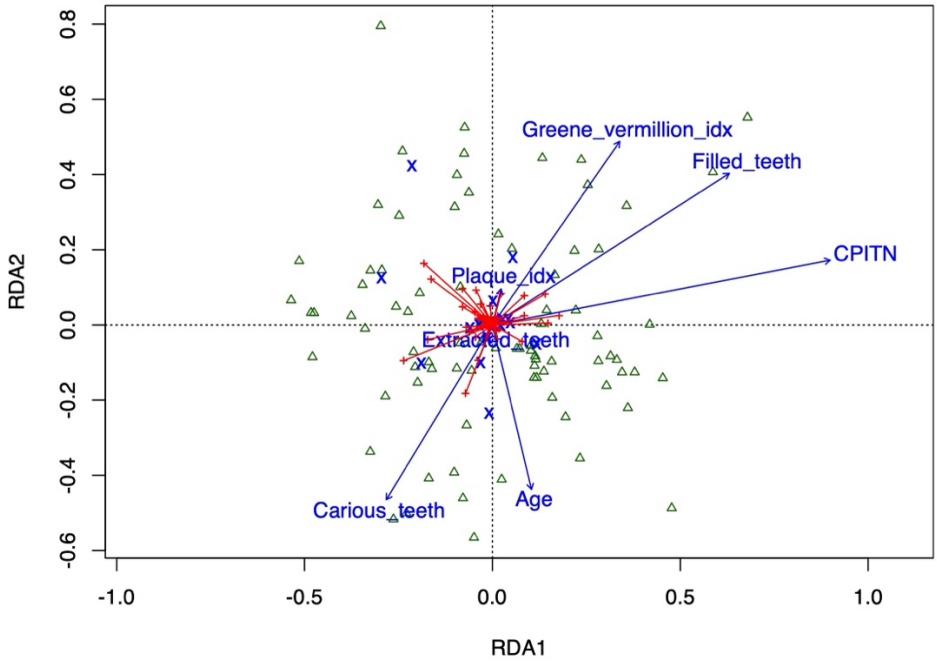

Figure 3.8 **Redundancy analysis (RDA) triplot of salivary microbiome data from the control group and type 1 diabetes patients, based on sensitivity analysis results**

Green hollow triangles represent samples, blue crosses represent the states of a categorical explanatory variables, blue arrows for quantitative explanatory variables with arrowheads indicating their direction of increase, and the taxonomical entities are shown as red plus.

In the combined PKU and control group dataset, upon testing of each variable again identified "Group" ($p = 0.033$, 999 perm.) as a significant determinant of microbial composition. As in the previous analysis, the number of explanatory variables was reduced to optimise the variation explained. In this case, forward selection identified "Frequency of tooth brushing" and "Gender" as significant determinants ($p = 0.005$ and $p = 0.05$, respectively,

999 perm.) in shaping microbial community composition (Figure 3.9). These results were supported by testing the parsimonious RDA model against the global model ($p = 0.009$, 999 perm.).

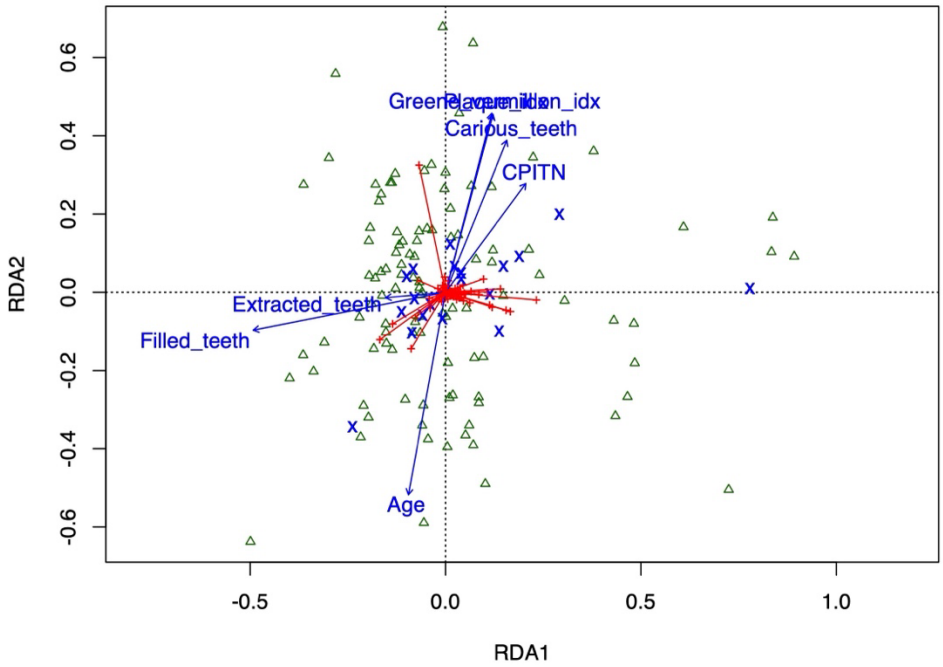

Figure 3.9 **Redundancy analysis (RDA) triplot of salivary microbiome data from the control group and phenylketonuria patients, based on sensitivity analysis results**

Green hollow triangles represent samples, blue crosses represent the states of a categorical explanatory variables, blue arrows for quantitative explanatory variables with arrowheads indicating their direction of increase, and the taxonomical entities are shown as red plus.

3.3 The association analysis of genotyped variants with oral health in phenylketonuria and type 1 diabetes patients

Firstly, the distributions of genotyped variants among patients diagnosed with PKU, those diagnosed with T1D, and individuals from a healthy control group were compared. As anticipated, there was no observed correlation between any of the investigated variants and overall systemic health. Genotype distribution across all three study groups is presented in Table 3.12 The following section presents the findings relating to the correlations between the allelic variants and oral hygiene indices across all three study groups.

Table 3.12

Genotype frequencies in the phenylketonuria, type 1 diabetes and control group

Gene, SNP	Group	Minor allele homozygotes	Heterozygotes	Major allele homozygotes	Minor allele frequency
IL1B rs1143634	PKU	5	10	26	0.2439
	T1D	6	11	10	0.4259
	Control group	7	18	35	0.2666
DEFB1, rs11362	PKU	8	26	9	0.4883
	T1D	9	9	10	0.4821
	Control group	11	30	21	0.4193
CD36, rs1761667	PKU	6	23	14	0.4070
	T1D	6	14	6	0.5000
	Control group	10	38	15	0.4603
TAS2R38, rs1726866	PKU	4	17	22	0.2907
	T1D	8	8	12	0.4285
	Control group	8	30	23	0.3770
TAS2R38, rs713598	PKU	2	18	23	0.2558
	T1D	8	8	12	0.4286
	Control group	8	27	27	0.3469
TAS1R2, rs35874116	PKU	3	14	26	0.2325
	T1D	1	5	21	0.1296
	Control group	3	20	40	0.2063

SNP – single nucleotide polymorphism, PKU – phenylketonuria, T1D – Type 1 Diabetes.

3.3.1 *DEFB1* rs11362

In the PKU group, a statistically significant association with a higher Silness-Löe index score ($p = 0.011$) was observed when the minor allele (A) was suspected to be dominant. Homozygote or heterozygote genotypes were found in 93 % and 92 % of cases with scores of two or three, respectively. In contrast, such genotypes were present in 57 % of cases with a score of one, while the presence of a score of zero was not observed in the PKU group. The Greene-Vermillion index is also linked to the minor allele (A) in a dominant manner ($p = 0.043$). The minor allele was observed in either homozygous or heterozygous state at frequencies of 100 %, 75 %, 70 %, and 33 % among those with index scores of three, two, one, and zero, respectively.

No associations between variant and oral health status were observed in the T1D and control group.

3.3.2 *IL1B* rs1143634

In the T1D group, in cases where the minor allele T was inherited in a recessive way, an association with a requirement for calculus removal was identified ($p = 0.030$). The results revealed that 50 % (4 out of 8 individuals) of individuals with the minor allele in a homozygote state did not require calculus removal, whereas, in the group that did require calculus removal, the corresponding proportion was 10 % (2 out of 19 individuals).

No statistically significant associations were found in the PKU and control groups.

3.3.3 *CD36* rs1761667

A statistically significant difference was identified only in the T1D group for rs1761667 if minor allele is recessive, it is associated with caries risk ($p = 0.033$). The rs1761667 variant genotype presence between different caries risk groups is minor allele homozygotes in group with low risk 1 of 6 (16 %), medium risk 2 of 15 (13 %) and high-risk group 3 of 5 (60 %) individuals.

3.3.4 *TAS2R38* rs1726866

No statistically significant associations were identified with any of the clinical parameters in any of the groups. Only in the control group an association with CPITN index if major allele is dominant ($p = 0.054$). The variant rs1726866 genotype presence (major allele homozygotes / heterozygotes / minor allele homozygotes) between the group with different CPITN index score are as follows – with index 0 – 8/16/1 with 1 – 5/8/6; 2 – 8/6/1; 3-2/0/0.

3.3.5 *TAS2R38* rs713598

Variant rs713598 found to be associated with Green Vermillion index in PKU group ($p = 0.049$). The variant rs713598 genotypes presence in individuals with different index score (major allele homozygotes/ heterozygotes/ minor allele homozygotes) between the group with different index scores - with index 0 – is 1/1/1; with 1 – 8/1/1; 2 – 7/9/0; 3-7/7/0 – major allele homozygotes are more in groups with higher index scores.

It was also associated with viscosity of basal saliva ($p = 0.033$), rs713598 genotype if at least one minor allele is present in famous group was in 14 out of 22 individuals, and in watery group in 6 out of 21 individuals.

3.3.6 *TAS1R2* rs35874116

Variant rs35874116 was not found significantly associated with any oral health parameters in the groups.

4 Discussion

In our study, we investigated three distinct groups – PKU and T1D patients, and a healthy control group – to assess the potential influence of a specific diet on oral health. We examined their oral health status and analysed its association with daily oral hygiene habits, the oral microbiome, and genetic predisposition. Additionally, we developed unique dental care guidelines specifically tailored for patients with PKU as a practical outcome of the research.

4.1 Oral health in phenylketonuria and type 1 diabetes patients

The prevention of dental caries and periodontal disease depends not only on dietary habits but also on patients' home care practices and regular dental visits. In all three study groups (participants older than 12 years), daily oral hygiene habits were assessed. Notably, only individuals in the PKU group reported not brushing their teeth at all (11 %). The proportion of participants brushing twice daily was 60 % in the PKU group, 61 % in the T1D group, and 97 % in the control group. Additionally, when specifically targeting patients with PKU aged 2–18 years, 6 % reported that they do not brush their teeth at all. Tooth brushing is considered a fundamental self-care behaviour essential for maintaining oral health, and brushing twice daily has become a widely accepted social norm (Kumar et al., 2016). However, insufficient oral hygiene habits are prevalent not only among patients with PKU but also globally. This has led the WHO (WHO, 2022) to emphasise the importance of oral hygiene [https://www.who.int/health-topics/oral-health]. National data further illustrates these trends. The most recent report from Latvia (2023) revealed that 45 % of 12-year-olds and 51 % of 15-year-olds brush their teeth two or more times per day, while 5 % and 3 % of these respective age groups do not brush at all (Maldupa et al., 2023). In comparison, data from the UK's BRIGHT trial showed that 69.8 % of secondary school students brush their teeth twice daily, with only 0.3 % never brushing (Marshman et al., 2023). In France, 78.8 % of adolescents were reported to brush their teeth twice daily as of 2014 (de Grado et al., 2021). Similarly, a Finnish study involving individuals aged 15–64 years found that 66 % brushed twice daily and 95 % at least once per day (Raittio et al., 2021). These data indicate that oral hygiene behaviours among the PKU group in Latvia are inferior to those of the T1D and control groups.

The high proportion of twice-daily brushers in the control group may be attributed to the fact that these individuals were recruited during routine dental visits, indicating potentially higher health awareness and literacy compared to the general population.

In the literature, there are many studies on type 2 diabetes, even suggesting that good oral hygiene is associated with lower diabetes risk (Chang et al., 2020). One of studies performed in Hungary about T1D included a self-reported oral health evaluation and

information about oral hygiene in well controlled T1D patients older than 18 years. It identified tooth brushing twice per day in 45.5 % (15/33) and additional dental flossing in 18.2 % (6/33) that was worse than in our group (Liptak et al., 2025). More concerning data were presented form Kosovo, where T1D children were separated and compared into two groups, defined by good glycaemic control (n = 18) and bad glycaemic control (n = 31). In this study the good glycaemic control group reported tooth brushing twice per day in 29.4 %, compared to 17,4 % in the group with bad glycaemic control (Ferizi et al., 2022). In the performed study no additional tool usage were analysed. This could be explained by the fact that countries are often classified based on Gross Domestic Product (GDP) to analyse their economic development and living conditions.

Using floss or interdental brushes in addition to brushing is also very important to remove plaque between two adjacent teeth. It may reduce gingivitis more than toothbrushing alone (Fleming et al., 2018). It is strongly recommended to use daily interdental cleaning devices and mouthwash, in addition to brushing, to prevent and control periodontal diseases and dental caries (Worthington et al., 2019). In our groups the highest proportion using interdental cleaning products was in the control group (67.1 %) and lowest in the PKU group (24.5 %). There are not many data on interdental cleaning product usage at all, e.g. information from the UK data reports that 37 % of the general population use mouthwash and 30 % use dental floss. Unfortunately, no individual was able to mark multiple answers, resulting in missing information about the proportion that are not using any of the additional interdental cleaning methods [https://www.gov.uk/government/statistics/adult-oral-health-survey-2021].

Patients with diabetes, including the T1D group, are reported to be predisposed to periodontal disease. There are other reports about oral hygiene habits in this population, a study by Zgang and colleagues evaluated 892 self-reported diabetic patients older than 30 years of age. In this group 69.5 % reported flossing (Zhang et al., 2023), which is higher than we observed in our T1D group, where only 38.7 % reported using dental floss. The fact that our study patients were younger may have contributed to their lack of motivation to floss their teeth. Conversely, the periodontal condition of our study patients was superior, as only patients with well-controlled diabetes were included, and their relatives and patients themselves maintained records of their health status.

4.2 Caries activity in phenylketonuria, type 1 diabetes patients and control group

Caries prevention and treatment are of major importance in the dental care of patients with PKU. Ballikaya and colleagues reported higher caries activity, moderate plaque accumulation, and gingival inflammation in patients with PKU, with nearly all of them

requiring professional oral hygiene procedures due to excessive plaque and calculus (Ballikaya et al. 2020).

The available studies report contradictory results, which are consistent with our finding that the PKU patient group exhibited the highest caries activity (44.44 %).

For example, Kilpatrick and colleagues examined 40 children with PKU and found no difference in the amount of dental caries compared with age/sex-matched healthy controls. However, significantly more children with PKU exhibited signs of tooth wear compared to their healthy counterparts (33 % vs. 24 %). The Kilpatrick and colleagues research focused on children, thereby precluding a comprehensive comparison of the outcomes (Kilpatrick et al., 1999). The caries activity in our observed T1D group (data available for 24 persons) was lower than that of the control group; however, high and medium activity levels did not exhibit statistically significant differences.

The association between poorer glycaemic control in T1D and increased caries risk is marked in a previous study by Ferizi and colleagues and others. The significant association between poor glycaemic control and caries risk is linked to decreased salivary flow. Saliva neutralises acids and removes food particles. If saliva becomes more enriched with glucose, it facilitates bacterial proliferation (Ferizi et al., 2022; Triebl et al., 2024).

More recent research has reported no difference in the caries prevalence in children with T1D (Al-Badr et al., 2021; Wang et al., 2019). This could be explained by the fact that, over time, advancements in insulin therapy regimes have led to a better control of the disease, reducing the difference in diet regime between T1D and control group children (Aronson et al., 2020). Patients included in our study with T1D had good glucose level control, permanent teeth, and most of them ate regular meals not enriched with carbohydrates; we found this last aspect very important for comparing the influence of dietary habits on the oral microbiome. It should be noted that only well-controlled T1D patients were included in this investigation. Since poorly controlled diabetes is more common during adolescence, this strict inclusion criterion also explains why the number of patients differed between study groups. Singh-Hüsgen and colleagues found that T1D patients displayed slightly higher Silness-Löe index values compared to patients with PKU and healthy controls, but it must be noted that this research only included children between the ages of 3 and 18 years (Singh-Husgen et al., 2016). In our study, in addition to adults, individuals from the age of 12 years with all secondary teeth present were included.

One of the risk factors stated is a diminished salivary flow rate. Our study showed that individuals with PKU have a reduced salivary secretion rate compared to healthy controls and the T1D group, with 60 % of patients with PKU having a flow rate exceeding 60 seconds.

Higher Phe levels in blood and saliva increase the pH of saliva, hence influencing mineral balance and affecting tooth health. Numerous PKU-specific amino acid formulae exhibit acidity, with a pH about 4.1 for flavoured variants. These formulas possess considerably elevated titratable acidity, allowing them to sustain an acidic environment in the oral cavity for an extended duration (Ghasemi et al., 2023). Shimazaki and colleagues examined the association of salivary flow rate with dental caries prevalence and periodontal status among 2110 Japanese adults and suggested that individuals with a lower salivary flow rates have higher risks for both dental caries and periodontal disease (Shimazaki et al., 2017). Saliva is an important factor in a plethora of oral functions, such as mastication, swallowing, antimicrobial activity, cleaning action. Saliva also influences oral health both through its non-specific physio-chemical properties, as well as through more specific effects (Dodds et al., 2005).

4.3 Periodontal disease risk in phenylketonuria, type 1 diabetes patients and control group

Our results revealed that patients with PKU exhibited the highest scores in oral hygiene indices as Silness-Löe and Greene-Vermillion that are useful periodontal disease risk indicators (de Vasconcelos Calixto et al., 2024; Machado et al., 2020) and CPITN index, that determines periodontal problems (Benigeri et al., 2000). All three indices were considerably higher than those in the control group.

Besides diet, our PKU group reported the worst tooth brushing and flossing habits out of all three study groups, subsequently had the worst gingival health clinically. Although self-reported tooth brushing frequency is higher than that reported by Ballikaya and colleagues in their study on patients with PKU where 85.3 % did not brush their teeth regularly and 90.4 % had never visited a dentist (Ballikaya et al., 2020) – in our group, 27 % reported brushing their teeth once per day, 66 % twice per day, and only 6 % brushed less frequently. Consequently, this is likely to lead to plaque accumulation and, over time, periodontal disease. (Kuzucu et al., 2025) It is crucial to note that Mediterranean countries have a higher prevalence of moderate PKU in contrast to the severe classic PKU seen in Eastern Europe. Mild hyperphenylalaninemia is the predominant phenotype in Turkey, as confirmed by numerous national studies (Kahraman et al., 2025). Previous studies have shown that although children with T1D possess a lower caries risk, they have an increased risk of developing periodontal disease (Saxena et al., 2020). Numerous clinical studies indicate that diabetes mellitus serves as a risk factor influencing the prevalence, progression, and severity of periodontal disease. Periodontal disease is regarded as the sixth most prevalent complication associated with diabetes (Costa et al., 2023; Dakovic et al., 2015). Those with poor metabolic control seemed to have a higher inclination toward periodontal disease risk (Bui et al., 2019). Our study showed that T1D patients had a higher

risk of gingivitis, which is a reversible stage of periodontal disease, provided that the patient follows an oral hygiene routine.

These findings underscore the importance of regular dental check-ups, oral hygiene instructions, and preventive dental care for PKU and T1D patients especially to minimise oral health complications.

4.4 Oral microbiome in phenylketonuria, type 1 diabetes and control group patients

With the importance of human and microbiome interactions becoming increasingly evident, greater attention is now being paid to the elucidation of the healthy states of microbiomes and to the identification of the microbiome markers that might contribute to disease development in the human host. In the case of the oral microbiome, acquisition of such knowledge could benefit the development of treatment options or probiotic guidelines for patients with unhealthy oral cavities. Therefore, this study aimed to evaluate the oral status and microbiome alterations that influence caries activity and periodontal disease risk in PKU and T1D patients compared to healthy individuals.

Our results revealed that the most dominant bacterial genus in all collected saliva samples, regardless of study group, was *Streptococcus viridans* group*,* a genus frequently referenced in oral microbiome studies. For example, Dianawati and colleagues examined the distribution of *Streptococcus mutans* and *Streptococcus sobrinus* in patients with high levels of dental caries and concluded that these two bacterial species are the main factors that cause dental caries (Dianawati et al., 2020). Cheon and colleagues evaluated the association of *Streptococcus mutans* genotype diversity, commonality, and stability with dental caries history in a high-caries-risk community. They reported that lower diversity and higher stability of *Streptococcus mutans* genotypes were notably associated with fewer decayed surfaces (Cheon et al., 2013). Thus, it is well established that oral bacteria within the dental plaque biofilm play a central role in the development and progression of dental caries – one of the most prevalent chronic diseases worldwide (Chen & Jiang, 2014). However, in our study, no association between bacterial abundance and oral health status was observed, possibly due to the limited sample size.

In our research of children with T1D, an increased prevalence of *Streptococcus mutans* and *Lactobacillus casei* was observed in their oral microbiome. These species are considered to be cariogenic by some researchers (Bimstein et al., 2019). However, neither our study nor several other authors reported increased caries activity in the T1D group. A study by Singh-Hüsgen and colleagues investigated the clinical oral cavity condition and bacterial composition in children with PKU and T1D. They found that children with PKU exhibited

lower levels of *Lactobacillus* species, whereas children with T1D had higher levels of *Lactobacillus* and *Streptococcus mutans*, but reduced levels of *Porphyromonas gingivalis* (Singh-Husgen et al., 2016).

We found that there were intersample dissimilarities between the PKU and T1D groups, as indicated by Jaccard distance measurements. While only a limited amount of research has been conducted comparing the oral microbiomes of PKU and T1D patients with healthy subjects, some studies suggest that poorer glycaemic control correlates with a higher bacterial load and increased alpha diversity in children with T1D (de Groot et al., 2017). There are also differences in microbiome beta diversity depending on the site of sample collection for example, between the *gingival sulcus* and buccal mucosa. Jensen and colleagues proposed that changes in alpha diversity in subgingival plaque may be a marker of higher periodontitis risk in children with T1D (Jensen et al., 2021). They found that children with more plaque and poorer oral hygiene habits exhibited more pronounced alpha diversity. Regarding adults with T1D, it has been reported that there are marked differences in the oral microbiome, especially in beta diversity. Several studies have concluded that T1D is characterised by a higher microbial load in dental plaque (Jensen et al., 2021). In our study, microbiome analysis was performed on saliva rather than plaque, which may have influenced the results. Additionally, T1D patients were not stratified by age (e. g. children versus adults), which could be a confounding factor.

Our results also revealed significant differences in the number of bacterial genera among the PKU, T1D, and control groups. In particular, two genera – *Alloprevotella* and *Leptotrichia* – were differentially abundant throughout all comparisons. The relative abundance of *Alloprevotella* was significantly higher in the control group compared to both the PKU and T1D groups, whereas *Leptotrichia* was significantly more abundant in the T1D group. Both genera have been associated with oral pathologies, including dental caries, periodontitis, and oral cancer (Rodrigues et al., 2021; Ulger Toprak et al., 2021). However, in our study, no association with oral health status was observed, which may be explained by the relatively young age of the patients.

The highest number of differentially abundant bacterial genera in the salivary microbiome (n = 17 bacterial species) was detected between the PKU and control groups. When comparing patients with PKU with different levels of dietary adherence, such genera as *Actinomyces*, *Capnocytophaga*, *Haemophilus*, and *Porphyromonas* were enriched in those who strictly followed the diet, compared to control individuals. Furthermore, an increased abundance of *Capnocytophaga* was observed in patients with PKU with partial adherence to the diet, while *Porphyromonas* was enriched in patients with both partial adherence and no dietary regimen. Among these genera, only *Haemophilus* has been described as a classical oral

microbiome exemplar, with an increased abundance previously being reported in healthy individuals (Caselli et al., 2020; Chattopadhyay et al., 2019), which contrasts with our findings. Moreover, recent studies have linked *Actinomyces* to the initiation of suppurative and granulomatous inflammatory lesions, while *Capnocytophaga* and *Porphyromonas* have been associated with periodontal disease (Idate et al., 2020; Thukral et al., 2017). A study by Bingöl and colleagues indicates reduced microbial diversity in the oral cavities of patients with PKU. His findings emphasise that both diet-induced dysbiosis and inflammation-driven alterations are critical variables influencing the unique oral microbiota in PKU (Bingol et al., 2024). A research study by Ostrowska and colleagues offers significant insight into the oral health of patients with PKU in relation to microbiome alterations. Increased abundance of *Streptococcus*, *Neisseria, Rothia*, and *Lactobacilli* are frequently linked to caries development in the presence of sugar-rich diets (Ostrowska et al., 2024).

A smaller number of differentially abundant bacterial genera in the salivary microbiome was observed between the T1D and control groups. Differential analysis identified six species with significantly different abundances between the two groups. *Leptotrichia* was enriched in T1D patients, whereas others, including *Alloprevotella* and *Fusobacterium*, were reduced. In addition to *Capnocytophaga*, the genera *Leptotrichia, Alloprevotella*, and *Fusobacterium* were identified as common constituents of the oral microbiome. Changes in the abundance of these taxa may be related to dietary restrictions, particularly reduced carbohydrate intake in T1D patients. Furthermore, many species showed significant positive correlations with patient-specific characteristics in the T1D group; for instance, nine species showed a positive correlation with the CPITN index. Previous studies have associated increased abundances of these bacteria with oral cancer and periodontitis (Li et al., 2014), highlighting the importance of continued monitoring. If future studies confirm our results, increased frequency of oral hygienist visits should be strongly recommended for patients with T1D.

Previous differential analyses have identified several bacterial genera, particularly *Porphyromonas* and *Fusobacterium*, that correlate with patient-specific characteristics in individuals without dietary restrictions (Ganesh et al., 2019; Keshary & Hagan, 2020). In our study, no significant correlations were found between bacterial genera and patient-specific characteristics in the control group, suggesting that this sample set served as an appropriate reference population.

We found that the presence of Porphyromonas was significantly higher in patients with PKU and T1D patients and was associated with several patient-specific parameters, including the number of extracted teeth and the Greene-Vermillion index. Cervino and colleagues previously reported that patients with both periodontitis and diabetes are prone to developing

renal, cardiovascular, and ocular diabetes complications (Cervino et al., 2019). From a clinical perspective, reducing the persistence of pathogenic bacteria such as *Porphyromonas* is therefore of considerable importance. Chlorhexidine, a commonly used antiseptic agent with broad-spectrum antibacterial activity, has been shown to reduce plaque accumulation, gingival inflammation, and bleeding (Varoni et al., 2012). However, due to adverse effects such as tooth discoloration, dysgeusia, and bacterial recolonisation, it is not recommended for long-term use (Butera et al., 2022). Based on this information, the use of herbal mouthwashes as a prophylactic measure for home care are strongly recommended (Haffajee et al., 2008). For instance, mouthwashes containing curcumin or peppermint have demonstrated antibacterial activity and inhibitory effects on bacterial growth when used to treat periodontal disease (Siddharth et al., 2020).

4.5 Genetic predisposition on oral health parameters in phenylketonuria, type 1 diabetes, and control group

The genetic analysis performed in the current study aimed to explore associations between specific allelic variants and oral hygiene indices across the study groups. For the *DEFB1* rs11362 variant, a statistically significant association was found between the minor allele (A) and a higher Silness-Löe index score. This suggests that individuals with this genetic variant may be more predisposed to gingival inflammation and plaque accumulation. Similarly, the Greene-Vermillion index was associated with the minor allele (A) in a dominant manner. These findings imply that this genetic variant may affect gingival health and plaque control. The *IL1B* rs1143634 variant was proven significant with the T1D group, particularly when the minor allele (T) was inherited recessively, implying that T1D patients with the *IL1B* rs1143634 variant required calculus removal less frequently. This suggests that individuals with T1D and this genetic variant may have a decreased risk of calculus formation and, therefore, a decreased periodontal disease risk.

Current research results on the associations of genetic variants with periodontal disease risk severity are highly variable. Although we did not find any prior studies that looked at periodontal disease risk in conjunction with oral hygiene indices and PKU/T1D patients specifically, the role of genetic variants is a widely studied subject in a variety of populations and study designs. Regarding *DEFB1* rs11362 and periodontal disease, the current research shows variable results. While some authors have been able to prove a role of *DEFB1* rs11362 in increased periodontal disease severity (Ikuta et al., 2015; Zhong et al., 2019; Zupin et al., 2017), other studies did not find any such association (Shao et al., 2019), and some authors argue that *DEFB1* rs11362 is linked to an increased caries development risk, rather than periodontal disease (Navarra et al., 2016; Ozturk et al., 2010). The inconsistency in these results

could be due to the multifactorial nature of periodontal disease, as it is very difficult to account for all aetiological factors that could be influencing the disease severity in each studied population. In our study, we included individuals with well-controlled T1D. There are several examples in the existing research where the *IL1B* rs1143634 variant was not proven to increase periodontal disease risk (Isaza-Guzmán et al., 2016; Mesa et al., 2017). However, some authors have found a statistically significant association between *IL1B* rs1143634 and periodontal disease severity (Brodzikowska et al., 2019; Yin et al., 2016).

The *CD36* gene encodes a multifunctional receptor involved in fatty acid transport and taste perception, particularly of dietary fats. A significant association was observed between the rs1761667 variant and caries risk in the T1D group under a recessive model ($p = 0.033$). Minor allele homozygotes were more prevalent in the high caries risk group (60 %) compared to medium (13 %) and low (16 %) risk groups. While this association is intriguing, its interpretation is not straightforward. The *CD36* variant may influence dietary preferences or lipid sensing; however, sugar intake rather than fat consumption is the primary dietary risk factor for caries (Chapple et al., 2017). Moreover, this variant has not previously been associated with oral health parameters. Given that the association was observed only in the T1D group, it may also reflect sample size effects or specific metabolic interactions within this population. Further research is needed to clarify the nature and robustness of this association.

The *TAS2R38* gene is known for encoding a bitter taste receptor that detects bacterial byproducts and influences oral innate immunity. Although rs1726866 did not reach statistical significance across groups, a marginal association was noted with the CPITN index in controls when the major allele was dominant. In the PKU group, the rs713598 variant of *TAS2R38* was associated with the Green-Vermillion Index, a measure of oral hygiene. Major allele homozygotes were disproportionately represented in individuals with higher index scores, implying poorer hygiene status. Additionally, rs713598 showed a significant relationship with basal saliva viscosity; individuals carrying at least one minor allele had more watery saliva. As mentioned in the literature review section dietary patterns that are affected by genetic variants may also influence oral microbial composition, increasing the risk for dental caries (de Jesus et al., 2022). Although contradictory results show that probably diet itself has a stronger impact than genetic variants in the taste receptors themselves (Sandell & Collado, 2018). The results about association with hygiene indices and saliva viscosity is not possible to compare as we did not identify any published study.

The absence of association in our study may reflect population-specific dietary habits, environmental influences, or the limited sample size. These findings underscore the complex

relationship between genetics, taste function, and oral health, particularly in populations with metabolic disorders.

4.6 Targeted recommendation effects on daily oral hygiene habits in phenylketonuria patients

Our study focused especially on patients with PKU because there are not many useful guidelines for their regular dental care. We conducted a second survey of patients with PKU and offered specific suggestions based on the findings relating to dental health and daily routines among those over 12 years old.

There are various reports suggesting that targeted education is leading to improvement on oral health because of improved oral hygiene habits, but those are mainly targeted to school age children without focus on PKU (Stein et al., 2018).

Regarding adults with PKU, it has been proposed that the increased risk of oral health problems may be due to social burdens (Bingol et al., 2023; Cazzorla et al., 2018). It is important to mention that when interviewing patients with PKU, the level of education was determined only for adult patients, the results showed that 20 % of adult patients with PKU have a higher education and 23 % have special education, but the education level for parents of patients with PKU was not determined. Patients with higher education are more likely to practice oral hygiene, brushing their teeth more often, and having regular dental check-ups (Minervini et al., 2023). This study showed that in the group ≥ 18-year, there were no significant improvements in daily oral care habits even after receiving recommendations. The families of children with PKU face several social issues throughout their childhood, as well as difficulties obtaining the recommended amino acid mixture and providing proper nutrition.

Through consultations and interviews patients with PKU reported limited awareness among dentists and dental hygienists of the specific challenges associated with PKU and its impact on oral health and hygiene.

The observed PKU group made few visits to dentists and dental hygienists for caries prevention and gingival health. Specifically, 40 % of the younger group and 39 % of the older PKU group visit the dentist rarely, while 48 % and 55 % visit the hygienist, respectively. Six months is the most widely advised revisiting interval for dental treatment in the literature (Fee et al., 2020). Dental professionals are convinced that frequent examinations allow disease to be detected and treated in time and preventive interventions to be delivered. A clinical guideline recommends that the longest period between examinations for both children and adults should be 12 months. For adults who maintain good oral health and appropriate home care habits, this can be extended to 24 months (Pitts et al., 2004).

It is important that the metabolic team, dentists, general practitioners, and psychologists encourage, motivate, and support patients with PKU to follow the recommendations of oral hygiene procedures, as 90 % of patients with PKU < and ≥ 18 years of age believe that this will improve their oral health and sequentially overall quality of life. To increase the awareness among healthcare professionals about oral hygiene habits in patients with PKU, oral care recommendations for patients with PKU were published in a local journal "Doctor of Latvia" (in Latvian "Latvijas Ārsts"). Patients who visit the dentist regularly are more willing to maintain good care of their oral health, which is why it is very important to motivate patients with PKU to visit their dentist and dental hygienist every 3 to 6 months. Periodontal disease should be diagnosed and treated as early as possible with professional oral hygiene procedures and periodontal treatment (Bendoraitiene et al., 2017).

Patients with PKU must carefully select their toothpaste considering its formulation. Toothpaste must not contain aspartame, a sweetener (Cury & Tenuta, 2014; Hu et al., 2021). The answers of the questionnaire show that 87 % of patients with PKU < 18 years of age and 42 % ≥ 18 years of age claim that the toothpaste they use does not contain aspartame. Unfortunately, this leads to the conclusion that patients with PKU and their parents do not pay attention to such important details and recommendations about proper daily oral care, using toothpastes without aspartame. Admittedly extra aspartame usage through toothpaste is not reported among accidental consumption of aspartame in patients with PKU, but using aspartame containing chewing gum is (Newbould et al., 2021). Chewing gum stimulates salivation. Saliva influences oral health both through its nonspecific physiochemical properties and through more specific effects (Dodds et al., 2005). An investigation of the oral health of patients with PKU in Latvia revealed that 60 % of patients with PKU had a lower basal saliva secretion rate compared with healthy counterparts and T1D patients. Salivation can be induced by gustatory or masticatory stimulation, such as using chewing gum (Olsson et al., 1991). Other desirable properties of chewing gums are adequate buffer capacity and the use of non-cariogenic sweeteners such as xylitol, sorbitol, and mannitol. These sweeteners will not result in an increase in the incidence of caries due to fermentation by oral microorganisms but will instead decrease the caries activity (J & Bamashmous, 2022; Kandelman & Gagnon, 1987; Kashket et al., 1989; Liang et al., 2024; Luo et al., 2024). Patients with PKU and their parents should know that chewing gum must not contain aspartame (Wu et al., 2022).

According to the questionnaire, only 39 % of patients with PKU ≥ 18 years of age and 17 % of patients with PKU < 18 years of age use dental floss daily. It makes one wonder about the knowledge, role, and modelling of daily hygiene procedures by parents, as well as the constant reminders by the dentist of proper daily oral health care. A daily mouthwash is

recommended for patients with PKU to reduce dental plaque. The results concluded that still many patients can be introduced to natural mouthwashes, as more than 80 % of patients with PKU have not heard about them or did not include them in their daily hygiene procedures. It is not possible to compare data from other populations with patients with PKU, as they have published information about the frequency of brushing teeth, but missing information on mouth rinsing after use of amino acid formula and flossing (Ballikaya et al., 2020; Bingol et al., 2023; Ghasemi et al., 2023).

Neves and colleagues in their study demonstrated the crucial role of the family in the oral health of their children (Neves et al., 2020). Parents of children with PKU prioritise maintaining optimal general health and daily functioning rather than focusing on prevention of oral disease. Children with chronic medical conditions and their families face many pressures and are often delayed in seeking dental care as it is simply not a priority (Moursi et al., 2010). By motivating parents and regularly reminding them of the oral care of children, this study confirmed that parents play an important role in maintaining their children's oral health, as significant improvement in oral hygiene after receiving recommendations was observed in patients under 18 years of age, suggesting the influence of parents on the oral care of their children.

Gingivitis is the only stage of periodontal disease that is reversible, as long as the patient implements and maintains an impeccable oral hygiene routine (Wolf et al., 2011). To avert severe periodontal disease development, it is crucial to implement preventive measures, such as patient information and motivation on how and why to improve their oral hygiene routine (Forbes-Haley et al., 2016; Petersen & Ogawa, 2012).

4.7 Limitations of the study

The study's limitation is the small number of nonrandomised patients. However, it is important to acknowledge that the total number of patients is 79, which is 78 % of all patients with PKU aged 12 years and older in Latvia who routinely conduct counter examinations.

It should be noted that there is low self-motivation, weak response of patients with PKU, reluctance to go to dental examinations and answer questionnaires. Patients with PKU should be addressed at all times and motivated to take care of their health.

The limitation of this study was the low number of included T1D patients, as the aim of the study was to include individual T1D patients with good glycaemic control. The low number of patients could explain, the striking difference in the number of genera significantly associated with the patient-specific characteristics between the PKU and T1D groups which could at least partly be attributed to the uneven number of subjects in each study group, thus affecting

the statistical power. The results of this study illustrate the need for further studies with larger sample sizes to understand the full picture of how PKU dietary restrictions and treatments affect patients over time.

The limitation of this study is the potential unreliability of PKU patients' self-reported adherence to the strict diet. Patients with PKU provide blood tests once a month that can't reveal the true situation. It is difficult to make valuable conclusions from the small patient count; we hope that our study will encourage other colleagues to perform similar studies to help provide the best possible care for these patients.

Comparison of clinical parameters between groups, including those with T1D, may have been affected by significant differences in age. However, as the PKU group ranged from 12 to 52 years, it was not possible to enrol well controlled T1D individuals of comparable age.

In analysing genetic predisposition, we selected only a limited number of genetic variants for genotyping. This approach did not allow for the calculation of haplotype associations or polygenic risk scores, which would have been feasible with the use of next-generation sequencing.

4.8 Strengths of the study

Rare conditions are not typical areas of focus, especially in terms of prevalence. Ordinarily the dental profession focuses on the actionable steps that a medical specialist can take within their practice or solely discusses oral health manifestations for patients with PKU (Ballikaya et al., 2020). A large proportion of all patients with PKU in Latvia were included in the study, accounting for 78 %, and this makes the study unique.

There is a deficiency in information and publications on oral health in PKU. Numerous publications exist about PKU, however, those addressing oral health are few.

Additionally, future research is suggested to place greater emphasis on preventive measures customised to the oral health needs of patients with PKU with the aim of reducing the occurrence and severity of oral health complications. In this study, the chosen data collection method involved a survey before and after implementing preventive measures. The proposed method for future studies is to conduct research that allows long-term patient monitoring and increased in-person visits throughout the study period. Any survey conducted is susceptible to the tendency of the patient to ignore complete candour in their responses. This concern can be effectively mitigated by incorporating personal consultations following the survey, thus reducing the likelihood of patient bias, or additionally free toothpastes, dental floss, and mouthwashes could be distributed.

This study highlighted the attitude of patients with PKU and the lack of knowledge of oral hygiene, especially in the group containing patients 18 years of age and older. This problem was previously underestimated. Future activities should be carried out, as recommendations alone are insufficient to achieve the goal.

This study provides an important overview of the clinical situation of PKU and T1D patients, especially since there are very few sources in the literature discussing the oral health of patients with PKU and there are no specific recommendations for their oral hygiene.

To our knowledge, studies investigating the association between genetic predisposition and the oral microbiome in relation to oral health have not incorporated such detailed oral hygiene parameters as assessed in this study. Our approach uniquely combines a comprehensive oral health evaluation with multiple laboratory methodologies – including genotyping of selected genetic variants and next-generation sequencing of the oral microbiome – to explore potential associations with greater resolution.

Conclusions

1 Patients with PKU and T1D have a higher prevalence of carious teeth and increased risk of periodontal disease, higher Silness-Löe plaque index, CPITN index and Greene-Vermillion index compared to a control group. The PKU group exhibited the worst oral health outcomes among all three study groups.

2 Survey results indicate that knowledge of oral care and hygiene is insufficient both among adult patients and caregivers of paediatric patients. The specifically developed recommendations led to significant improvements only in the group of patients under 18 years of age.

3 No major or systemic differences in overall microbiome composition were found between the groups; however, within the context of these specific diets, certain individual genera showed statistically significant differences that were associated with oral health characteristics.

4 Genetic associations between *DEFB1* rs11362, *IL1B* rs1143634, *CD36* rs1761667, and *TAS2R38* rs713598 variants and oral health characteristics were observed in the PKU and T1D groups, suggesting that genetic factors may contribute to oral health differences in these population groups.

Proposals

This study has the potential to serve as a reference for future research aimed at developing recommendations for the prevention of periodontal disease and the reduction of caries risk in patients with PKU and T1D. We have identified the importance of regularly reminding patients to maintain good oral health. One effective strategy could be the annual administration of a questionnaire that not only monitors oral hygiene behaviours but also reinforces daily oral care recommendations. These reminders could be seamlessly incorporated into the routine annual visits patients with PKU have with their dietitians.

An essential step forward would be the formal inclusion of a dentist in the multidisciplinary care team – specifically one with expertise in PKU – to ensure comprehensive oral health management. Regular encouragement from healthcare professionals, emphasising the importance of dental check-ups, can significantly enhance patient awareness and adherence to good oral hygiene practices.

To evaluate the long-term impact of these recommendations, it would be valuable to repeat the survey after three years.

Publications and reports on topics of the Thesis

Publications:

1. Abola, I., Emulina, D. E., Skadins, I., Brinkmane, A., Gailite, L., and Auzenbaha, M. 2022. "Dental Status and Periodontal Health of Patients with Phenylketonuria in Latvia," *Acta Stomatol. Croat.*, , doi: 10.15644/asc56/2/2.
2. Abola, I., Gudra, D., Ustinova, M., Fridmanis, D., Emulina, D. E., Skadins, I., Brinkmane, A., Lauga-Tunina, U., Gailite, L., Auzenbaha, M. 2023. "Oral Microbiome Traits of Type 1 Diabetes and Phenylketonuria Patients in Latvia," *Microorganisms*, , doi: 10.3390/microorganisms11061471.
3. Emulina, D. E., Abola, I., Brinkmane, A., Isakovs, A., Skadins, I., Moisejevs, G., Gailite, L., Auzenbaha, M. 2024. "The Impact of *IL1B* rs1143634 and *DEFB1* rs11362 Variants on Periodontitis Risk in Phenylketonuria and Type 1 Diabetes Mellitus Patients in a Latvian Population," *Diagnostics*, , doi: 10.3390/diagnostics14020192.
4. Abola, I., Intlere, N. A., Brinkmane, A., Laktina, S., Zarina, A., Vasilevska, L., Skadins, I., Moisejevs, G., Gailite, L., Auzenbaha, M. 2024. "Oral health care knowledge among Phenylketonuria patients in the Latvian population," *Molecular Genetics and Metabolism Reports, 2024,* doi: 10.1016/j.ymgmr.2024.101167

Reports and theses at international congresses and conferences:

1. 35th ESPKU Virtual Conference, 15–17 October 2021, "Oral health in Latvian patients with Phenylketonuria".
2. Theses for the 9th Congress of Latvian Doctors, "Orālā mikrobioma raksturojums fenilketonūrijas un 1. tipa cukura diabēta pacientiem Latvijas populācijā".

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Annexes

Approval of Central Medical Ethics Committee (No 1/19-03-26)

Centrālā medicīnas ētikas komiteja

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Rīgā

26.03.2019. Nr.1/19-03-26

Rīgas Stradiņa universitātes
Molekulārās Ģenētikas Zinātniskajai laboratorijai

Atzinums par pētījumu
"Mutes dobuma veselības izvērtēšana
pacientiem ar fenilketonūriju un
1.tipa cukura diabētu"

Centrālā medicīnas ētikas komiteja 2018.gada 6.decembrī ir izskatījusi Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas iesniegto pētījumu *"Mutes dobuma veselības izvērtēšana pacientiem ar fenilketonūriju un 1.tipa cukura diabētu"*.

Pamatojoties uz Centrālās medicīnas ētikas komitejas 2018.gada 6.decembra sēdes protokola Nr.2018-5 punktu Nr.3 un iesniegtajiem labojumiem, tiek izsniegts atzinums, ka Rīgas Stradiņa Universitātes Molekulārās Ģenētikas Zinātniskās laboratorijas pētījums *"Mutes dobuma veselības izvērtēšana pacientiem ar fenilketonūriju un 1.tipa cukura diabētu"* nav pretrunā ar bioētikas normām.

Centrālās medicīnas ētikas
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Genoma izpētes padome

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20.05.2019. Nr.A-10/19-05-20

Rīgas Stradiņa Universitātes,
Molekulārās Ģenētikas Zinātniskajai Laboratorijai,
Dr. Ivetai Ābolai,

Atzinums par pētījumu: *"Mutesdobuma veselības izvērtēšana pacientiem ar fenilketonūriju un 1. tipa cukura diabētu"*

Genoma izpētes padome izskatīja iesniegumu par Rīgas Stradiņa Universitātes, Molekulārās Ģenētikas Zinātniskajā Laboratorijā plānoto Dr. Ivetas Ābolas pētījumu *"Mutesdobuma veselības izvērtēšana pacientiem ar fenilketonūriju un 1. tipa cukura diabētu"*.

Pamatojoties uz Genoma izpētes padomes locekļu balsojumu, tiek izsniegts atzinums, ka Genoma izpētes padome atbalsta Dr. Ivetas Ābolas Rīgas Stradiņa Universitātes, Molekulārās Ģenētikas Zinātniskajā Laboratorijā plānotā pētījuma *"Mutesdobuma veselības izvērtēšana pacientiem ar fenilketonūriju un 1. tipa cukura diabētu"* īstenošanu.

Genoma izpētes padomes
Priekšsēdētājs J.Kloviņš

Informed consent for the study Participant

Information for study participation

Dear Sir/Madam!
We invite you to participate in the study "Evaluation of oral health in patients with phenylketonuria and type 1 diabetes mellitus", which will be carried out in collaboration with the Molecular Genetics Scientific Laboratory of Riga Stradiņš University and the Institute of Dentistry of Riga Stradiņš University.
Please read the information about the study before signing the consent form.

Aim of the study
The aim of the study is to assess whether patients with phenylketonuria and type 1 diabetes have a higher risk of more common oral diseases such as caries and periodontal disease. The hypoThesis is that selective diet and eating habits, which are also influenced by taste, are the cause of changes in dental and overall oral health. The study will include an analysis of medical history, such as tooth brushing. The bacteria present in your oral cavity will be analysed to assess the relationship with your diet and eating habits. Genetic variants in taste genes will be analysed to assess whether possible food preferences and between-meal snacking affect oral health. Finally, the risks associated with caries and periodontal disease will be analysed in order to draw conclusions on influencing factors and to provide recommendations for optimal oral care.

Project description
During the visit you will be asked questions about your eating habits, oral care habits and other oral health related questions. You will be examined with a mirror, probe and periodontal probe. During the examination, the dentist will mark healthy, damaged and missing teeth on the patient's chart. The dentist will assess oral hygiene, mucosal and tongue health. During the examination, you will be asked to spit into two barrels. One of the tubes will be used to analyse the bacteria living in your oral cavity. The amount of bacteria will be assessed, as well as their impact on your oral health. The saliva from the other barrel will be used to extract genetic information (DNA) and further analyse genes that affect taste, such as sweet and bitter taste sensations. As well as to analyse genes that put people at higher risk of developing tooth decay and periodontal disease. The saliva material will be analysed and tested at the Molecular Genetics Research Laboratory of Riga Stradiņš University.

Duration of the project
The project is planned for 5 years. Your consent document is kept for 75 years. With your consent, the material collected may be used in other projects at your discretion. If you change your mind about taking part in the project, it is possible to stop taking part in the project by contacting the research coordinator, in which case the material collected will be destroyed. Withdrawal from the project will not affect the course of treatment.

Potential risks
There are no risks to your health associated with participation in the study. You will have a saliva sample taken, which cannot affect your health or well-being.

Confidentiality and participant rights
The "Law on Human Genome Research" adopted by the Parliament of the Republic of Latvia, as well as the "Law on the Protection of Personal Data" guarantee the confidentiality of your personal data, health, heredity and the information obtained. The information will be stored in a restricted location and will not be shared with your family members, insurance companies or employer. The data and saliva samples collected will not be used for commercial purposes or illegally passed on to uninvolved third parties. Data stored about you in the Molecular Genetics Research Laboratory of Riga Stradiņš University will be released only in the following cases: to you personally, upon your written request, or upon discovery of clinically significant variations if you have consented to receive information about them. Information about your health status, the results of the studies (but not your personal data!) will also be made available to the researchers taking part in the study. By signing the consent document, you also have the right to prohibit the addition, updating or verification of your condition descriptions within the project. You have the right to withdraw your consent to participate in this project at any

time, in which case your tissue samples, health and heredity records and any personally identifiable information will be destroyed.

Voluntary participation
Your participation in the project is voluntary. There will be no adverse consequences for you if you refuse to participate.
Potential benefits
By participating in the project you will receive an oral health assessment. The study will assess the risk factors that contribute to poor oral health and will result in recommendations for oral care.
Contact
If you have any questions about this study, please contact
Iveta Abola - abola.iveta@gmail.com

Patient consent to participate in the study

1. I have received and read the written information on the study "Oral Health Assessment in Patients with Phenylketonuria and Type 1 Diabetes Mellitus". I have received answers to all my questions. I have had sufficient time to reflect on my decision to allow biological material for the study.
2. I agree to provide a sample of the biological material - free of charge - for genetic studies. I understand that the transfer of the samples does not pose a risk to my/my child's health, which has been explained to me.
3. I understand that any information identifying me/my child will be kept confidential and that all samples will be coded. I understand that I may stop participation in the study at any time without explanation, knowing that this will not affect my/my child's future treatment. I am aware that in this case the biological material and any identifying information will be destroyed.
4. Health Assessment:
   - o I authorise the completion, updating or review of the medical record
   - o I prohibit the completion, updating or verification of the medical record.
   - o I Authorise the use of images, photographs of the medical records for scientific purposes, subject to the provisions of Regulation (EU) 2016/679 of the European Parliament and of the Council on the protection of natural persons with regard to the processing of personal data and on the free movement of such data.
   - o I prohibit the use of images, photographs of medical records for scientific purposes.
5. In case the study of biological material reveals information about a risk to my/my child's health that I have not known before (tick as appropriate):
   - o I consent to being informed of this information
   - o I agree to be informed only if the health risk is avoidable
   - o I do not wish to receive any additional information.
6. Storage of biological material:
   - o I agree that the biological material will be stored and used in other studies at the discretion of the investigators without restriction
   - o I agree that the biological material will be stored
   - o I agree that the biological material is used only for this study and must be destroyed afterwards.

Name, surname:
Personal code/ person identifier:
Address: _____
Date:
Signature:

Researcher (Authorised Person)
Name, Surname: _
Personal code/ persona identifier _____
Adress:
Date:
Signature:

Registration no:

Consent of parents/legal guardians of minor children to participate in the study

1. I have received and read the written information about the study "Oral Health Assessment in Patients with Phenylketonuria and Type 1 Diabetes Mellitus." I have received answers to all my questions. I have had sufficient time to reflect on my decision to allow biological material for the study.
2. I agree to provide a sample of the biological material - free of charge - for genetic studies. I understand that the transfer of the samples does not pose a risk to the health of my child, which has been explained to me.
3. I understand that any information identifying my child will be kept confidential and that all samples will be coded. I understand that I may stop participation in the study at any time without explanation, knowing that this will not affect my child's future treatment. I understand that in this case, the biological material and any identifying information will be destroyed.
4. Health Assessment:
   - o I authorise the completion, updating or review of the medical record
   - o I prohibit the completion, updating or verification of the medical record.
   - o I Authorise the use of images, photographs of the medical records for scientific purposes, subject to the provisions of Regulation (EU) 2016/679 of the European Parliament and of the Council on the protection of natural persons with regard to the processing of personal data and on the free movement of such data.
   - o I prohibit the use of images, photographs of medical records for scientific purposes.
5. In case the study of biological material reveals information about a risk to my child's health that I have not known before (tick the box):
   - o I consent to being informed of this information
   - o I agree to be informed only if the health risk is avoidable
   - o I do not wish to receive any additional information
6. Storage of biological material:
   - o I agree that the biological material will be stored and used in other studies at the discretion of the investigators without restriction
   - o I agree that the biological material will be stored
   - o I agree that the biological material is used only for this study and must be destroyed afterwards.

Name, surname:
Personal code/ person identifier:
Adress: _____
Date:
Signature:

Researcher (Authorised Person)
Name, Surname: _
Personal code/ person identifier _____
Adress:
Date:
Signature:

Registration no:

Patient clinical card

Patient name and surname

Date

dd.mm.yyyy

Personal code

Phone no

(in case of minor giving mother, father or other legal guardian phone no)

Name, surname and contacts of family doctor_____
(specialist contacts, in case of specific disease)

Anamnesis

Disease	Yes	No	Description
Epilepsy			
Bleeding disorder			
Cardiovascular diseases (including high blood pressure)			
Endocrine systems (diabetes, etc.)			
Diseases of the gastrointestinal tract			
Allergies			
Congenital diseases			
Infectious diseases (hepatitis, HIV, TB)			
Stress (significant changes in life, e.g. starting school, new job, higher responsibility)			
Salivary gland diseases			
Oncological diseases			
Other illnesses			
For children (problems during pregnancy, birth injuries, illnesses in infancy)			
Use of medication (specify which ones)			
Pregnancy, lactation			

Oral health anamnesis

	Description
Fluoride toothpaste (concentration)	
Frequency of brushing teeth (times per day, per week when brushing)	
Use of other preventive measures (dental floss, interdental brushes, tongue cleaner, etc.)	
Frequency, regularity of visits to the dentist	
Frequency, regularity of dental hygienist visits	
Smoking	
For children up to 6 years of age - family caries experience (mother, father, guardian, brothers, sisters)	

Patient's complaints, wishes

Patient signature_____

Additional investigations

Nutritional analysis (fill in the table or mark which of the three situations corresponds)

	Frequency	Description
Meals		
Snack		
together		☐ Non-cariogenic ☐ Partly cariogenic ☐ Cariogenic nutrition
Drinking water per day (quantity and assessment)		

Assess which of the following best fits the patient's eating habits?

☐ I eat no more than 4 times a day, I don't eat sweets or I eat them no more than once a week. I usually drink water, unsweetened tea or coffee, but sweet drinks no more than once a week.

☐ I eat up to 4 times a day, and I tend to snack on sweet or salty snacks 1 or 2 times between meals. I drink tea, coffee with sugar, cocoa, chocolate drink, flavored milk (e.g. Rasens), lemonade and juice both from a package and freshly squeezed, but it is included in the mentioned meals and snacks

☐ It's hard to count how many times a day I eat something. I tend to snack several times between meals. Between meals, I often drink tea, coffee with sugar, cocoa, chocolate drinks, flavored milk (e.g. Rasens), lemonades and juices (both from a package and freshly squeezed).

Saliva investigations

Investigation	Result	Evaluation
Basal saliva secretion (determined by drying the mucous membrane of the lower lip and waiting for a drop of saliva to appear)	☐ < 60 sec ☐ > 60 sec	
Viscosity of basal saliva	☐ Waterly ☐ Foamy ☐ Sticky, stringy	
Basal saliva pH		
Speed of basal saliva secretion (determined by collecting saliva from the patient for 15 minutes at rest)	(ml / min)	☐ Norm ☐ Reduced secretion ☐ Xerostomia
Speed of stimulated saliva secretion (determined by patient collecting saliva for 5 minutes, with chewing stimulation)	(ml / min)	☐ Norm ☐ Reduced secretion ☐ Xerostomia
Saliva bufercapacity	☐ High ☐ Medium ☐ Low	
S. mutans amount in saliva		
Lactobacillus spp. amount in salive		

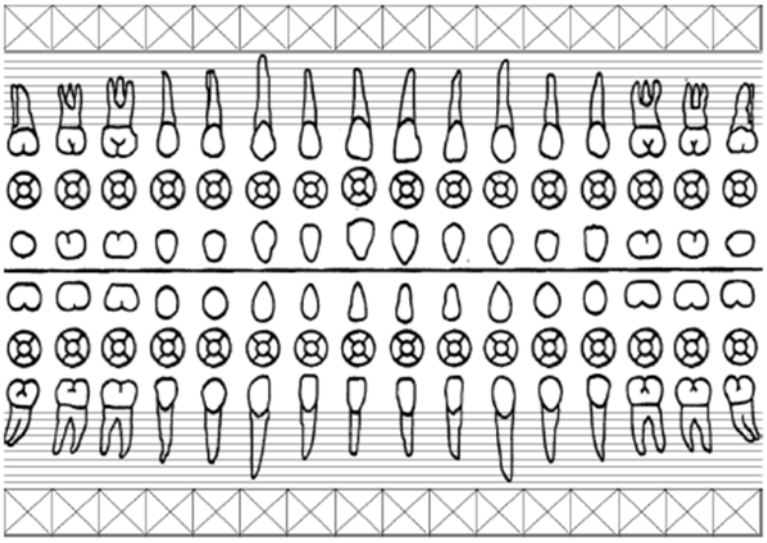
Other examinations

Extraoral examination	
Lymph nodes	
TML	
Bite anomalies (Angle classification, others)	
Pathologies of the mucous membrane	
History of existing orthodontic appliances	
Existing prostheses	

Clinical card

Name Surname

Date of examination



Teeth	Caries	Other pathology of hard tissue	CPI				Teeth	Caries	Other pathology of hard tissue	CPI			
			Bleeding	Tartar	Gum pocket 4-5 mm	Gum pocket > 6 mm	Bleeding			Bleeding	Tartar	Gum pocket 4-5 mm	Gum pocket > 6 mm
18							38						
17							37						
16							36						
15							35						
14							34						
13							33						
12							32						
11							31						
21							41						
22							42						
23							43						
24							44						
25							45						
26							46						
27							47						
28							48						

CPITN	Hygiene instruction	Tartar removal	Complex treatment	Plaque indekss	Silness-Löe indekss	Simple Green-Vermillion indekss
	Yes=1 No=0			%	(0-3)	AI= CI= MHI= (0-6)

$K_sPEz=$	$K_sPEz=$
$K_sPEv=$	$K_sPEv=$

The survey included the following sections and questions:

- demographics section (age, gender);
- how frequently you/ your child is tooth-brushing (responses: twice per day, once per day, less than once);
- do you or does your child use a toothpaste without aspartame (responses: yes, no);
- do you or does your child use a mouth rinse (responses: yes, no);
- do you or does your child do interdental cleaning (e.g. flossing)? (Responses: yes, no);
- how often do you/ does your child visit a dentist (responses: twice a year, once a year, less than once a year);
- how often do you/ does your child visit a dental hygienist (responses: twice a year, once a year, less than once a year);
- do you/ does your child rinse mouth every time after consuming PKU formula (responses: yes, no, including other, e.g. drinking water);
- additional question was asked for patients with PKU older than 18 years of age: do you clean a tongue? (responses: yes, no);

Oral health recommendations for patients with PKU

Daily regimen:	1) Brush teeth twice daily: once after breakfast and once before bedtime. 2) Use fluoride toothpaste (1000-1450ppm) that does not contain aspartame: Ages 0-2: Rice-grain-sized amount (1000 ppm) Ages 2-6: Pea-sized amount (1000 ppm) Ages 6+: Half-length of toothbrush bristle part (1450 ppm). 3. Use a toothbrush with soft bristles (labeled “soft” or “ultra-soft”).
Tooth brushing tips	Avoid rinsing after brushing; spit out excess toothpaste. Children under 10 need parental assistance; children aged 10+ can brush independently.
Interdental Cleaning	Use dental floss or an irrigator daily to clean between teeth. Suitable for both baby (deciduous) and adult (permanent) teeth.
Tongue cleaning	For adults (18+), clean the tongue each morning after brushing. Use a toothbrush, tongue scraper, or spoon, moving from back to front of the tongue.
Saliva stimulation	Use aspartame-free chewing gum to stimulate saliva, which helps to remove dental plaque.
After aminoacid mixture consumption care	After consuming amino-acid mixtures, rinse the mouth with plain water.
Mouth wash	If recommended, use chlorhexidine mouthwash (0.05 %-0.09 %) twice daily, 30 minutes after brushing. Use 10 mL of liquid and rinse for 30 seconds, avoiding rinsing with water afterward. Alternatives: Rinse with saltwater, coconut oil, or plant-based mouthwashes (Aloe Vera, Echinacea, Chamomile, Grapefruit seed oil) 2-3 times per day. Use for one month, then take a 3-month break. Avoid rinsing with water after use.
Dentist and oral hygienist visits	Schedule dental check-ups every six months as part of preventive care. Visit a dental hygienist every 3-6 months for professional cleaning as part of proactive oral care.