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Differential effects of an anti-inflammatory diet on TNF-α and IL-6 methylation: systemic versus local inflammatory levels

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Veronika Victoria Samel

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AA…….Arachidonic acid

AGES……...Österreichische Agentur für Gesundheit und Ernährungssicherheit

ALA…….α-linolenic acid

BMI……...…Body mass index

DHA……..Docosahexaenoic acid

DNA…….Deoxyribonucleic acid

EPA……...Eicosapentaenoic acid

HRM……High-resolution melting analysis

IL-6……..Interleukin-6

IQR……..Interquartile range

LA……Linoleic acid

OLP……..Oral lichen planus

OSCC……...Oral squamous cell carcinoma

PUFA…….Polyunsaturated fatty acids

SPMs……..Specialized pro-resolution mediators

TNF-α……..Tumor necrosis factor-alpha

TLRs…….....Toll-like receptors

ω-3……...Omega-3-fatty-acids

ω-6……...Omega-6-fatty-acids

WHO……..World Health Organization

ABSTRACT

According to the World Health Organization, approximately 3.5 billion people were affected by oral diseases in 2023. This represents a growing public health concern, as oral diseases contribute to the development of secondary diseases and can impair general health in the long term. As a result, this incurs high costs, not only for the affected individuals but healthcare systems. Therefore, there is a growing interest in establishing biomarkers in periodontal medicine, as such markers could be useful for diagnosis, treatment evaluation and prognosis. Epigenetic therapeutic strategies are receiving increasing scientific attention, as oral diseases are predominantly driven by inflammatory processes that can be monitored via the cytokines TNF-α and IL-6, both of which are regulated by DNA methylation—a well-established epigenetic mechanism.

In the search for biomarkers, the present study investigated the influence of diet on oral health, given evidence suggesting that nutrition plays a significant role in epigenetic regulation. However, studies investigating the connection between DNA methylation and dietary quality remain scarce and often yield contradictory results. Accordingly, this study examined the association between TNF-α and IL-6 methylation and adherence to a Mediterranean diet and coffee intake. The aim was to determine whether these parameters could potentially serve as biomarkers in dentistry and the extent of diet's impact on TNF-α and IL-6 methylation levels. It was hypothesized that an anti-inflammatory diet would have a positive effect on local and systemic methylation patterns. To explore this, TNF-α and IL-6 methylation levels in capillary blood and buccal swabs were compared among 40 healthy participants. Additionally, the impact of fish, fruit, vegetables, and coffee consumption on TNF-α and IL-6 methylation was assessed. It was hypothesized that a diet rich in these components would positively influence methylation in the oral cavity.

The results showed that fish consumption affected TNF-α and IL-6 methylation levels in capillary blood, but not in the oral mucosa. No significant differences were found in TNF-α or IL-6 methylation between capillary blood and buccal swabs and increased fish intake was associated with higher IL-6 methylation and lower TNF-α methylation in blood. It should be considered that oral mucosa cells are short-lived, and long-term effects are likely more difficult to detect, which complicates the interpretation of the results. It should also be noted that this does not rule out a potential influence of fish consumption on local inflammation, as there is increasing evidence suggesting that the exposure time of foods also plays an important role in epigenetic regulation, which was not taken into account in this study.

Furthermore, the results regarding vegetables, fruit, and coffee intake were not statistically significant and were therefore not further analyzed.

In summary, it remains unclear to what extent an anti-inflammatory diet influences oral health and whether TNF-α and IL-6 methylation could serve as biomarkers in periodontal medicine. However, the observed effect of fish consumption on systemic inflammation does not rule out this possibility. Further studies with larger sample sizes and fewer limitations are needed to better assess the influence of a Mediterranean diet and coffee consumption on methylation and to clarify the potential of TNF-α and IL-6 methylation as biomarkers in the prevention of oral diseases and the reduction of existing inflammation in the oral cavity.

ZUSAMMENFASSUNG

Laut der WHO waren im Jahr 2023 rund 3,5 Milliarden Menschen von oralen Erkrankungen betroffen. Dieses Problem wurde mittlerweile auch vom öffentlichen Gesundheitswesen erkannt, da es die Grundlage für Sekundärerkrankungen bildet und in weiterer Folge zu einem verschlechterten Gesundheitszustand beiträgt. Dies führt in weiterer Folge zu hohen Kosten für das Gesundheitssystem wie auch für Betroffene selbst. Demnach wächst das Interesse an der Etablierung von Biomarkern in der parodontalen Medizin, die hilfreich bei Diagnosen, Therapieerfolgen und Prognosen sein könnten.

Oralen Erkrankungen liegen Entzündungen zugrunde, die anhand der Zytokine TNF-α und IL-6 gemessen werden können und deren Expression durch DNA-Methylierung beeinflusst wird, weshalb epigenetische Behandlungsansätze immer häufiger in den Fokus rücken.

Auf der Suche nach Biomarkern, analysierte die vorliegende Studie den Einfluss von Ernährung auf orale Gesundheit, da Studien darauf hinweisen, dass Ernährung einen großen Einfluss auf die epigenetische Regulation nimmt. Studien, die dem Zusammenhang zwischen DNA Methylierungen und Ernährungsqualität nachgingen, sind jedoch rar und widersprüchlich in ihren Ergebnissen. Aus diesem Grund hat die vorliegende Studie den Zusammenhang zwischen TNF-α- sowie IL-6-Methylierungen und mediterraner Ernährung sowie Kaffeekonsum untersucht. Ziel dieser Studie war es herauszufinden, ob diese Parameter in Zukunft als potenzielle Biomarker in der Zahnmedizin eingesetzt werden könnten und wie groß der Einfluss von Ernährung auf TNF-α- sowie IL-6-Methylierungen ist. Es wurde die Hypothese aufgestellt, dass eine antiinflammatorische Ernährung Einfluss auf lokale Entzündungen nimmt. Hierfür wurden TNF-α- und IL-6-Methylierungen zwischen dem Kapillarblut und der Mundschleimhaut bei 40 gesunden Proband:innen verglichen und der Einfluss von Fisch-, Obst-, Gemüse- sowie Kaffeekonsum auf TNF-α- und IL-6-Methylierungen gemessen. Die Hypothese lautete, dass sich ein Ernährungsstil, der sich durch hohen Fisch-, Obst-, Gemüse- sowie Kaffeekonsum auszeichnet, positiv auf Methylierungen im Mundbereich auswirkt.

Die Ergebnisse der Studien zeigen, dass der Konsum von Fisch TNF-α- und IL-6-Methylierungen im Kapillarblut beeinflusste, nicht jedoch jene in der Mundschleimhaut. Es konnte kein signifikanter Unterschied von TNF-α- und IL-6-Methylierungen zwischen dem Kapillarblut und der Mundschleimhaut nachgewiesen werden und ein erhöhter Fischkonsum wurde mit erhöhten IL-6- und niedrigeren TNF-α-Methylierungen im Blut assoziiert.

Hierbei sollte jedoch berücksichtig werden, dass Mundschleimhautzellen kurzlebig sind und langfristige Effekte schwer nachzuweisen sind, was die Interpretation der Ergebnisse erschwert. Aus den Ergebnissen kann auch nicht abgeleitet werden, dass lokale Entzündungen durch Fischkonsum unbeeinflusst bleiben, da es vermehrt Hinweise darauf gibt, dass die Einwirkzeit von Lebensmitteln ebenfalls eine wichtige Rolle in der epigenetischen Regulation spielt, die in dieser Studie nicht berücksichtigt wurde.

Weiters waren die Ergebnisse des Gemüse-, Obst- und Kaffeekonsums nicht signifikant und wurden aus diesem Grund nicht näher analysiert.

Zusammenfassend lässt sich sagen, dass es unklar bleibt, wie groß der Einfluss von antiinflammatorischer Ernährung auf die Mundgesundheit ist und ob TNF-α- and IL-6-Methylierungen zukünftig als Biomarker in der parodontalen Medizin eingesetzt werden könnten. Der aufgezeigte Effekt von Fischkonsum auf systemische Entzündungen lässt dies nicht ausschließen. Weitere Studien mit größeren Stichproben und weniger Limitierungen sind notwendig, um den Einfluss von mediterraner Ernährung und Kaffeekonsum auf DNA-Methylierungen besser abschätzen zu können und die Eignung der TNF-α- und IL-6-Methylierung als Biomarker sowohl zur Prävention oraler Erkrankungen als auch für die Reduktion bestehender Entzündungen im oralen Gewebe zu bewerten.

1. INTRODUCTION

1.1. ORAL HEALTH

The World Health Organization (WHO) defines oral health as a state of being free from "oral and facial pain, oral and throat cancer, oral infection, periodontal disease, tooth decay, tooth loss and other diseases and disorders that limit an individual's capacity in biting, chewing, smiling, speaking, and psychosocial well-being" (FDI World Dental Federation, 2015).

Around 3.5 billion people worldwide are estimated to be affected by oral health diseases (World Health Organization, 2023). Meanwhile, it has even been recognized as a public health problem. The crux however is, that it affects especially vulnerable groups with a low income (Alarcón-Sánchez et al., 2024).

Nutrition is one of many parameters that have a major influence on our health. The mouth is not only the first part of our body where the comminution process of food starts, but also the place where notable inflammation occurs. Therefore, a human's oral health condition tells a lot about his general health too. The oral cavity is a major gateway into the human body, which is why the saliva microbiota is crucial in maintaining not only the systemic, but also the oral health (Manzoor et al., 2021).

In recent days science pays greater emphasis on personalized nutrition and its influence on the oral microbiome (Gomez & Nelson, 2017). Periodontal diseases and dental caries are the most prevalent diseases among humans. Both are complex chronic diseases, that share common risk factors and can cause nutritional compromises. They are the primary cause of tooth loss (Chapple et al., 2017). Tooth loss has a strong influence on a human being's life as it leads to an unhealthy diet and malnutrition. Around 30% of people between the ages of 65 und 74 lose all their natural teeth worldwide (FDI World Dental Federation, 2015).

Many diseases of the oral cavity can undergo malignant transformation, e.g. oral squamous cell carcinoma (OSCC), which is one of the most frequent oral cancers (Rodríguez-Molinero et al., 2021). Generally, oral cancer is the 8th most common cancer worldwide (FDI World Dental Federation, 2015) and should therefore not be neglected.

The root of oral diseases is often related to malnutrition and other unhealthy behavioral factors. While it is commonly known that caries is a diet-related disease, this is not the case with diseases like gingivitis and periodontitis, which are influenced by diet too. Therefore, it can be clearly said that oral diseases are caused by malnutrition that is characterized by high-sugar consumption, pro-inflammatory fats and a lack of fiber and micronutrients.

This dietary composition is typically for the average Western-style diet (Woelber & Vach, 2023).

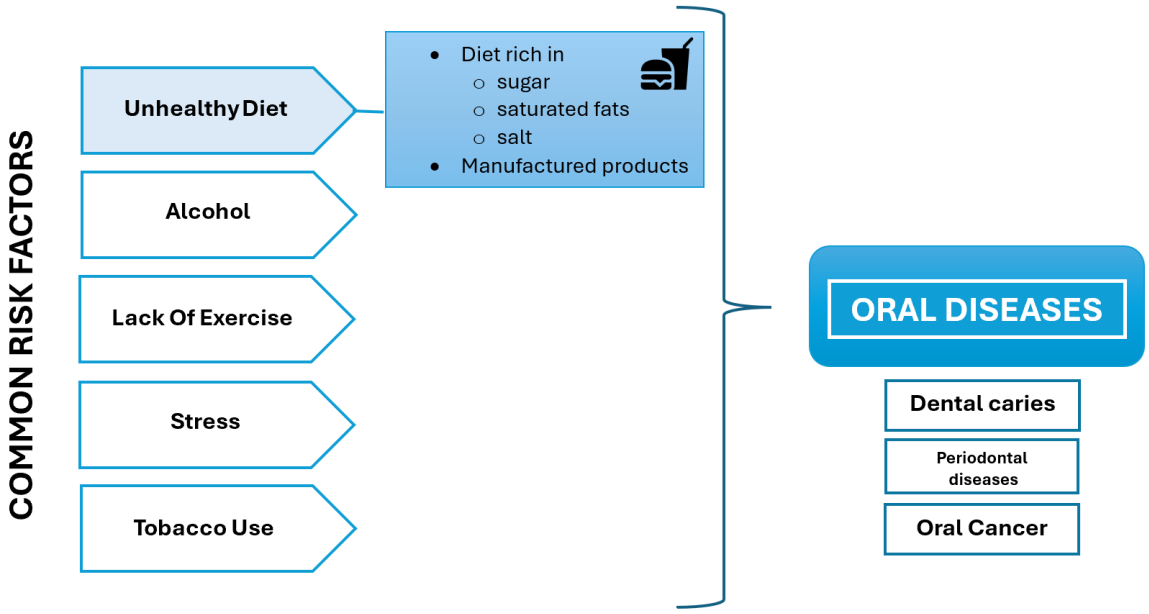

Figure 1: Common risk factors for oral diseases adapted after FDI World Dental Federation (2015)

It was shown that obesity, which is related to a diet high in carbohydrates, alters oral homeostasis, e.g. make changes to bacterial composition and pH, which, in further consequence, leads to a pro-inflammatory status and contributes to dental caries progression (Bizjak et al., 2022).

Tooth decay, the most common chronic childhood disease, is of dietary and bacterial origin, which occurs due to cariogenic diets, caused by the metabolism of specific bacteria on dietary sugars in sensitive hosts. The process of enamel demineralization appears to be facilitated by a shift in biofilm populations towards acid-producing and acid-tolerant cariogenic bacteria, likely triggered by low pH following sucrose fermentation (Gomez & Nelson, 2017). Based on over a century of research, there exists unambiguous evidence that dietary fermentable carbohydrates, such as sugars and starch, are a significant reason, however, they are not a sufficient cause for the initiation and progression of caries. Nutrition functions locally and systemically which is why micronutrients such as vitamin D, calcium, phosphates and vitamin K are of great importance for tooth mineralization. A lack of those has a negative impact on the quality of teeth (Chapple et al., 2017). A balanced salivary microbiota is therefore essential for oral health, whereas a dysbiosis can cause dental caries (Manzoor et al., 2021).

The prevalence of caries varies greatly between different parts of the world. A high prevalence is reported in many developing countries in Asia and Africa, as well as some Central and Eastern countries. The etiology of caries is intricate and multifactorial, encompassing lifestyle factors such as dietary habits, particularly frequent consumption of dietary sugars, oral hygiene, utilization of antibiotics, and other factors (Manzoor et al., 2021).

While dental caries is prevalent at all ages (Chapple et al., 2017), periodontitis is rather observed in people between the age of 55 and 59 (Wu et al., 2022). Nevertheless, younger individuals are not immune to periodontitis, as an increasing prevalence is also observed in this group (Wu et al., 2022). The risk of periodontitis increases due to poor nutrition, the quality of nutritional components as well as obesity, physical inactivity and tobacco smoking (Chapple et al., 2017). The stadium of periodontitis emerges if the case is given that gingivitis is not treated promptly, thus it has the potential to progress. The problem with periodontitis is that it can cause tooth loss and poor nutritional status among a lot of other things. This results in a reduced quality of life and can be an enormous economic burden for the healthcare system (Tonetti et al., 2017). In the field of periodontal health, a symbiosis exists between a health-associated biofilm and a proportionate host immune-inflammatory response. Periodontitis is caused by the emergence of dysbiosis in susceptible individuals, which is associated with dysregulation of the immune-inflammatory response. This leads to host-mediated connective tissue damage as well as alveolar bone loss (Chapple et al., 2017).

The impact of oral health on our general health can also be demonstrated by the great impact it has on our cognitive health. The link between cognitive health and oral health has been extensively investigated. Many studies have suggested that oral inflammation, contributes to cognitive health decline due to reduced sensory input related to the loss of masticatory contacts as well as tooth pain or tooth loss (Liang & Gomaa, 2023).

All that points out the importance of nutrition on oral health. Therefore, dentists and physicians should use nutritional dentistry to initiate healthier diets early on, before other secondary diseases manifest themselves (Woelber & Vach, 2023), which not only cause high costs for the health care system, but, above all, causes poor health in the individual. The best way forward would be the avoidance of oral diseases through prevention as the treatment of oral diseases remains unaffordable or inaccessible for large parts of our society, especially in low and middle-income countries (FDI World Dental Federation, 2015). Nutrition could therefore provide a remedy before oral diseases occur.

1.2. NUTRITION, INFLAMMATION AND EPIGENETICS

Several studies have demonstrated that the epigenome can be altered by exposure to a range of nutritional factors. The body of evidence that epigenetic is one of the mechanisms by which nutrients and bioactive compounds have an impact on metabolic traits are growing. Intricate interactions among food components, histone modifications, DNA methylation, non-coding RNA expression as well as chromatin remodeling factors result in a dynamic regulation of gene expression that governs the cellular phenotype (Milagro et al., 2013). Nutrition has a significant impact on the inflammatory response, as it regulates diverse genetic and epigenetic mechanisms involved in this process (Mecca et al., 2024). Generally, inflammation is defined as a biological response of the immune system that can be triggered by a variety of factors, including pathogens, damaged cells and toxic compounds (Chen et al., 2018).

When it comes to inflammatory response, there are especially two major classes of well-known polyunsaturated fatty acids (PUFAs), Omega-3 (ω-3) and omega-6 (ω-6) fatty acids. Eicosapentaenoic acid (EPA), docosapentaenoic acid (DHA) and arachidonic acid (AA) serve as substrates for the synthesis of lipid mediators, such as eicosanoids, which are involved in inflammatory processes. In these processes, immune mediators produced from DHA and EPA shift the immune balance towards resolution of inflammation (Tingö et al., 2022). Eicosanoids are mainly derived from AA, which are membrane phospholipids released upon the activation of phospholipase A2 by an inflammatory insult. The intensity of an inflammation response is proportional to the amount of AA presence in the membrane (Mecca et al., 2024). The production of AA depends on the activity and regulation of two enzymes: delta-6-desaturase and delta-5-desaturase. Both are key enzymes for ω-6 fatty acids and responsible for the conversion of linoleic acid (LA) into AA (Akash et al., 2018). Increasing ω-3 and decreasing ω-6 may serve as a mechanism to modulate the immune response towards the resolution of inflammation (Arnardottir et al., 2020).

The inflammatory response depends on modulatory genes, specialized pro-resolution mediators (SPMs) and the activity of eicosanoid hormones (Serhan, 2014). Inflammatory response aims to maintain homeostasis by identifying and eliminating the cause of imbalance. The type and degree of activated inflammatory response relies on the nature of the inflammatory trigger and its dimension, once identified, pathogens cause, inter alia, the production of inflammatory cytokines (Mohammed et al., 2022).

Furthermore, it has been discovered that increased concentrations of saturated fatty acids in human blood, especially those of palmitic acid, interact with the toll-like receptors TLR-2 and TLR-4. This results in an activation of NF-kB, which, in further consequence, also leads to inflammation (Hwang et al., 2016).

Inflammatory pathways have an impact on the pathogenesis of several chronic diseases and include inflammatory mediators and regulatory pathways. Inflammatory stimuli activate intracellular signaling pathways that consequential lead to an activation of the production of inflammatory mediators. Mainly inflammatory stimuli, including microbial products and cytokines, e.g. IL-6 and TNF-α, impart inflammation through interaction with TLRs (Chen et al., 2018). It has been revealed that IL-6 and TNF-α show diverse functions that may lead to tissue destruction, including chronic inflammation such as periodontitis (Ertugrul, 2017).

1.2.1. Anti-inflammatory Diet

When inflammation persists for a prolonged period, there is a possibility that it ends in chronic condition with further consequence of triggering a cascade of inflammatory events that can lead to permanent cellular harm and tissue injury as well as organ dysfunction (Ramos-Lopez et al., 2021).

The Mediterranean diet contains food groups and nutrients with desirable anti-inflammatory properties. It removes pro-inflammatory factors and enriches diet with whole grains, nuts, legumes, vegetables, fruits, skimmed dairy, eggs, fish and vegetable oils. These food groups are rich in antioxidants, flavonoids, fiber and folate (Polak-Szczybyło & Tabarkiewicz, 2024). Important dietary components of this diet are for example (Román et al., 2019):

- Long-chain ω-3 fatty acids, e.g. from fresh fish and almonds
- Polyphenols, including flavonoids, e.g. vegetables and fruits
- Bioactive compounds, e.g. fiber
- Antioxidants

Based on the Mediterranean diet with its anti-inflammatory effect, this master thesis will focus on fish, vegetables, fruits and coffee.

1.2.1.1. Fish

Essential fatty acids are not synthesized by mammals and therefore must be considered in diet. ALA is essential for the synthesis of longer ω-3 fatty acids including EPA and DHA. While humans can convert ALA into EPA, it only synthesis small amounts of

DHA. That is why dietary consumption of fish, seafood or fish oil is required to supply EPA and DHA (Román et al., 2019).

1.2.1.2. Fruits and Vegetables

It has been reported that plant-derived polyphenols have an influence on anti-inflammatory properties by interfering with immune cell regulation and synthesis of pro-inflammatory cytokine. These are associated with health benefits for different chronic diseases related to inflammation (Yahfoufi et al., 2018). Polyphenols naturally occur in fruits and vegetables (Román et al., 2019).

1.2.1.3. Coffee

Just like fruits and vegetables, coffee also contains polyphenols, which are known for having strong antioxidant activity (Román et al., 2019). Studies conducted in recent time suggest that coffee plays an important role in strengthening the immune system and protecting the body against different diseases e.g. type 2 diabetes or osteoporosis thanks to the compounds it contains such as caffeine and micronutrients (e.g. magnesium, potassium and niacin) (Açıkalın & Sanlier, 2021).

1.2.2. Proinflammatory Markers: Cytokines

Markers are used in clinical applications to show the comparison of normal to pathogenic biological processes. Furthermore, markers are also valuable tools for evaluating responses to treatment. Inflammatory markers have the potential to serve as a predictor of inflammatory diseases and correlate with the causes and consequences of various inflammatory diseases. Stimuli activate inflammatory cells and induce the production of inflammatory cytokines, such as IL-6 and TNF-α (Chen et al., 2018), which are produced locally in the inflamed tissues (Mohammed et al., 2022).

Cytokines are small proteins that mediate cell communication and regulate the processes of cell differentiation, migration, proliferation, and death (Florescu et al., 2023). They play a significant role in the initiation, regulation and prolongation of natural immune response (Ertugrul, 2017). These molecules can possibly be used as biomarkers for disease diagnosis, prognosis, and, in further consequence, for therapeutic decision making (Chen et al., 2018). The basic mediators of chronic inflammatory disease are IL-6 and TNF-α, which have the potential to destroy tissue and initiate bone loss. Both cytokines occur due to an immune-inflammatory response that develops in periodontal tissue which coincides with periodontal pathogen microorganisms. These proinflammatory mediators are hold responsible

predominantly for the destruction in periodontal disease (Ertugrul, 2017). Altered promoter methylation profiles of IL-6 and TNF have been observed in gingival tissue, peripheral blood or buccal mucosa from patients with periodontitis, correlating with changes in expression and disease severity (Jurdziński et al., 2020).

1.2.2.1. TNF-α

TNF-α is synthesized largely by macrophages and monocytes but also by neutrophils, fibroblasts as well as T and B lymphocytes. It plays a decisive role in mediating resistance against infections, stimulating innate and adaptative immunity in chronic inflammatory diseases, and plays a role in the pathogenesis of autoimmune disease (Florescu et al., 2023). TNF-α molecules induce the proliferation and differentiation of osteoclast pioneer cells, thereby stimulating bone resorption through indirect activation of matured osteoclasts. Furthermore, it induces IL-6 production, which in turn stimulates osteoclast formation, direct osteoclastic bone resorption, and T-cell differentiation (Ertugrul, 2017). The heightened expression of TNF-α in oral cavity fluids and tissues in periodontitis suggests their potential utilization as biomarkers for its occurrence and progression (Melguizo-Rodríguez et al., 2020).

1.2.2.2. IL-6

IL-6 is synthesized primarily by B and T lymphocytes, but also by macrophages and monocytes, with an important role in adaptive immunity and a verified role in chronic inflammation (Florescu et al., 2023). IL-6 is a cytokine that is frequently analyzed in oral cavity diseases, which is produced by numerous cells of the periodontium in response to TNF-α secretion. It is enormously important in the activity of immune cells, in osteoclasts and the inflammatory response to bacterial plaque formation (Melguizo-Rodríguez et al., 2020).

1.3. NON-INVASIVE METHOD: SALIVA

Scientific evidence has shown that levels of TNF-α and IL-6 change in the saliva of subjects with different oral pathologies such as dental caries or periodontitis (Rodríguez-Molinero et al., 2021). What makes saliva attractive as a potential biomarker, is that it is non-invasive and therefore an attractive alternative to blood when it comes to the diagnosis and prognosis of oral diseases (Rodríguez-Molinero et al., 2021). Further advantages as a clinical tool over the serum are the simplicity of collection, pain-freeness, storing and cost-effectiveness and real-time results, to mention just a few (Saxena et al., 2017) (Rodríguez-Molinero et al., 2021). Furthermore, buccal samples have indicated to be better surrogates than blood for epigenome-wide association studies (San-Cristobal et al., 2016). Nevertheless, it should also be mentioned that some biomarkers detected in saliva are not specific to particular diseases and therefore can be used for the diagnosis of various pathologies (Melguizo-Rodríguez et al., 2020). Therefore, the question is how well changes in the saliva reflect the course of a disease (Sikorska et al., 2018).

1.4. DNA METHYLATION AND ORAL HEALTH

The most studied epigenetic marker in human is DNA methylation (Carlberg & Molnár, 2023). It is the chemical addition of a methyl group to the cytosine residue, usually occurring at the site of cytosine–phosphate–guanosine (CpG). DNA methylation can also be observed at cytosines followed by a non-guanine base, such as adenine, cytosine, or thymine. This non-CpG methylation is a prevalent modification in neural tissues and exhibits an increase during development (Aristizabal et al., 2020). It has been proven that epigenetic modifications (such as DNA methylation in CpG islands) occur after environmental stimuli and play a fundamental role in inflammatory gene transcription (Bayarsaihan, 2011). DNA methylation can be modulated through diet and specific nutrients, deficiency or overnutrition and causes hypo- or hypermethylation, which can conduce the development of metabolic disorders (Frankhouser et al., 2022).

A comparison of TNF-α and IL-6 methylation levels in the gingival tissue between patients with chronic periodontitis and healthy control showed that methylation levels of one CpG island region in the IL-6 promoter was significantly lower in the gingival tissues of patients with chronical periodontitis, compared with the control group (Abasijiang et al., 2021). Decreased IL-6 promoter methylation (Ishida et al., 2012) have been observed in the peripheral blood from patients with periodontitis also in another study as well as increased

methylation levels of TNF promoter region (Kojima et al., 2016), though the latter observation has not been confirmed in an independent study (Kobayashi et al., 2016). Similarly, increased TNF promoter methylation (based on gingival biopsies) found in periodontitis patients, was not observed in experimental gingivitis (S. Zhang et al., 2013). Methylation patterns of the IL-6 promoter gene (DNA isolated from peripheral blood) was differentially hypomethylated in individuals with periodontitis and rheumatoid arthritis, which could indicate that the hypomethylated state of a single CpG in the IL-6 promoter region may promote higher serum levels of IL-6, supporting an important role for this cytokine in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and periodontitis (Ishida et al., 2012).

It is important to note the discrepancies between some studies. The differences in promoter methylation in IL-6 and TNF between patients with periodontitis and healthy individuals, which had been observed in some studies, have not been reproduced in independent analyses (Jurdziński et al., 2020). It should be kept in mind, that the application of techniques assessing only site-specific DNA methylation, such as bisulfite-conversion PCR methods, may partly explain divergent results regarding promoter methylation of the same genes analyzed in independent studies (Kurdyukov & Bullock, 2016).

Consequently, studies suggest that healthy control groups exhibit increased IL-6 methylation and decreased TNF-α methylation when compared to individuals with periodontitis.

2. OBJECTIVES

Oral health is affecting around 3.5 billion people worldwide (World Health Organization, 2023) and is recognized as a public health problem that affects especially vulnerable groups with a low income (Alarcón-Sánchez et al., 2024) concerning both younger and older individuals (Wu et al., 2022). A balanced salivary microbiota is essential for oral health that is partly influenced by nutrition which functions locally and systemically. This points out the importance of nutrition on oral health and why nutritional dentistry is important in the avoidance and manifestation of secondary diseases (Woelber & Vach, 2023). Some studies provide evidence that diet is important in epigenetic regulation, but studies linking DNA methylation and general diet quality are still scarce (Frankhouser et al., 2022). This study therefore aims to investigate to what extent nutrition affects TNF-α and IL-6 methylation in the oral cavity and to explore their potential as biomarkers.

Accordingly, the study pursues the following objectives:

The primary objective of this study is to analyse whether methylation levels of TNF-α and IL-6 are similarly reflected between capillary blood and oral mucosa tissues taking nutrition into account.

The secondary objective is to examine differences in methylation levels in relation to the consumption of fish, vegetables, and fruits, as they are part of the Mediterranean diet, as well as coffee intake, which is also known for its anti-inflammatory effects. Helping patients to reduce existing oral inflammation before undergoing treatment is a desirable goal in dentistry, which is why the impact of nutrition on oral inflammation shall be investigated.

It is hypothesized that a diet rich in those components has a positive influence on inflammatory methylation levels in the oral cavity. As oral diseases are recognized as a public health problem associated with high costs for the state as well as the individual, this study aims to provide insights into potential non-invasive markers for the prevention and treatment of oral diseases and the influence of nutritional components on inflammation in this regard.

3. MATERIALS AND METHODS

3.1. OVERVIEW

The following illustration gives an overview of the study process and shows the performed analyses.

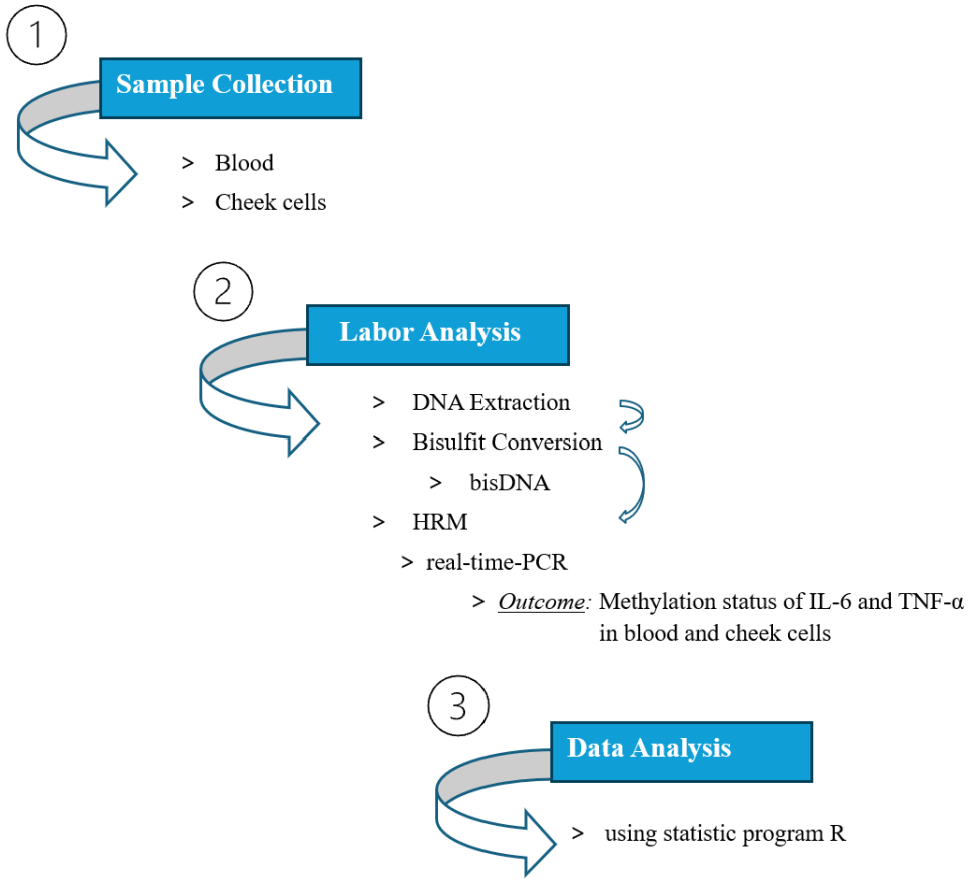

Figure 2: Overview of the analyses performed

3.2. COHORT RECRUITMENT AND DATA COLLECTION

The study was initially designed to include participants with impaired oral health. However, owing to an insufficient number of samples, only healthy individuals were ultimately recruited, which led to equal distribution across the groups.

At the beginning of the study a questionnaire was created which is based on a questionnaire that HealthBioCare uses for their analysis. The questionnaire that was used for this study contained, in addition to lifestyle and nutrition questions, also questions regarding oral health. The questionnaire is attached under 9.1 “Questionnaire”.

The aim of the study was to compare methylation levels between capillary blood and buccal swabs samples, taking diet and coffee consumption into account, for potential

markers. It was not easy to find subjects who wanted to participate in this study without any compensation and who agreed to provide capillary blood samples as personal DNA was collected. Furthermore, it was difficult for some participants to visit the lab besides their daily obligations. For this reason, the cohort recruitment was undertaken in the circle of acquaintances of the labor staff. The blood and buccal swab samples were taken from the probands in the laboratory of HealthBioCare. Single-serving sterile cervical brushes from "Teqler" were used to ensure that the buccal swabs samples become not contaminated and supply enough cells for the DNA extraction. Every subject had to collect their buccal swabs by using these brushes with circular movements. This procedure took 30 seconds and was repeated at each cheek to ensure that enough cells would be taken. After that, the brushes were closed anew in the protective cover and air-dried for 24 hours (see appendix under section 9.2). For the blood samples a Whatman® protein saver card was used to collect capillary blood with the aid of a safety lancet Extra 18G and previous disinfection of the fingertip.

3.3. LABORATORY ANALYSIS

3.3.1. DNA Extraction

DNA from capillary blood and buccal swabs were extracted to analyze the methylation levels of IL-6 and TNF-α. The extraction was performed following the instruction of the manufacturer's protocol (see appendix under section 9.3).

3.3.2. Methylation Analysis

A high-resolution melting analysis (HRM) is a sensitive and specific method for the detection of methylation (Wojdacz & Dobrovic, 2007). A HRM curve was conducted to analyze the methylation levels of the samples. For this purpose, the extracted DNA was used. Before that was possible, it had to be bisulfite converted to conduct further analyses.

3.3.2.1. Bisulfite Conversion

Bisulfite conversion is a chemical process used to distinguish between methylated and unmethylated DNA. Methylated DNA and unmethylated DNA acquire different sequences after bisulfite treatment resulting in PCR products with markedly different melting profiles (Wojdacz & Dobrovic, 2007). The bisulfite conversion was

performed following the instruction of the manufacturers protocol (see appendix under section 9.4).

3.3.2.2. *High-resolution Melting Analysis (HRM)*

Unmethylated cytosines (C) are converted into uracil (U), while methylated cytosines remain unchanged due to the methyl group protecting them. This conversion enables the distinction between methylated and unmethylated DNA. During PCR, methylated Cs remain Cs, which is not the case for originally unmethylated Cs that occur as Ts during PCR (Patterson et al., 2011) and are converted into U. For the analysis of the methylation levels a high-resolution melting curve was performed with a Rotor Gene Q (Qiagen, Germany) (see appendix under section 9.5).

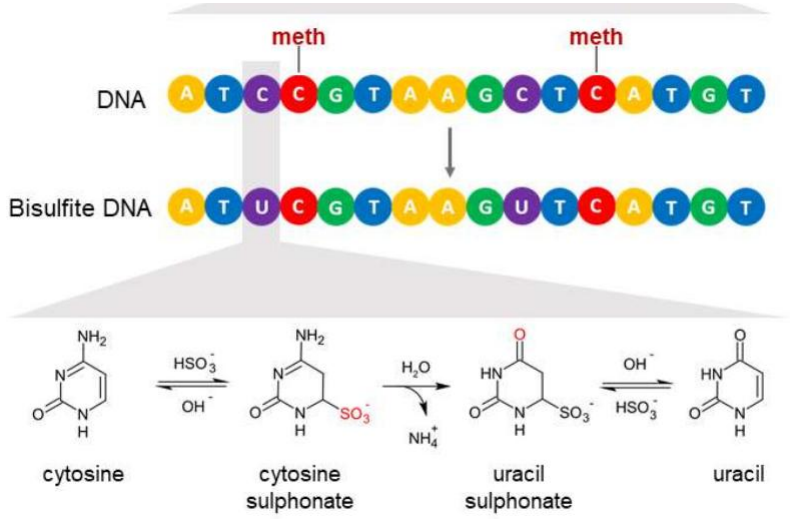

Figure 3: Methylated and unmethylated DNA (Ochoa et al., 2022)

3.4. STATISTICS

Data from laboratory was evaluated using the statistical program R. The Shapiro–Wilk test showed that TNF-α results in blood as well as in the buccal swabs were not normally distributed. Since the data was not normally distributed, a Mann–Whitney *U* test was used to test whether there was a significant difference or not. A Student's *t*-test could not be carried out for IL-6 as IL-6 results were only distributed normally in blood, but not in buccal swabs.

Furthermore, the Kruskal–Wallis test, a one-factor ANOVA, was used for testing the effects of fish consumption on the inflammation markers, as TNF-α is a metric variable and was not normally distributed, whereas fish is a categorical variable with more than two

categories. The Kruskal–Wallis test is an extent of the Mann–Whitney *U* test. To find out which groups differ from each other, Dunn's test was used.

4. RESULTS

4.1. CHARACTERISTICS OF THE STUDY POPULATION

A total of 40 adults were included in the study, half of whom were women. The participants were asked about their gender in an open question format. Only the answers “man” and “woman” had been written down, which is why no other gender identity is mentioned in the results. Exclusion criteria were chronic diseases and medication as well as an age under 18 and over 65. There was no person who was excluded due to not meeting the inclusion criteria. The average age and BMI of participants were respectively 26.27 (± 4.25) years and 22.48 (± 2.66) kg/m2.

	Study Population
Total [n]	40
Gender ♀	20
Age [in years]	26.27 ± 4.25
BMI [kg/m²]	22.48 ± 2.66
TNF-α (capillary blood)	29.38 ± 5.23
TNF-α (buccal swabs)	19.64 ± 10.03
IL-6 (capillary blood)	67.94 ± 23.92
IL-6 (buccal swabs)	86.18 ± 27.22
Coffee drinker [%]	72.5
Regular fish consume [%]	22.5
Data are presented in n (%), mean ± SD	

Table 1: Descriptive characteristics of the 40 study participants

4.2. OUTCOME

4.2.1. TNF-α and IL-6 in Blood and Buccal Swabs

Figure 4 shows that the median of TNF-α in blood is higher than that of TNF-α in the buccal swabs. Furthermore, two outliers for TNF-α in blood and one outlier for TNF-α in the buccal swabs are visible. The Interquartile Range (IQR) is significantly narrower for TNF-α in blood which shows less dispersion. The Mann–Whitney *U* test showed that there is significant difference (p=2.34e-10) between TNF-α methylations levels in blood and those in buccal swabs.

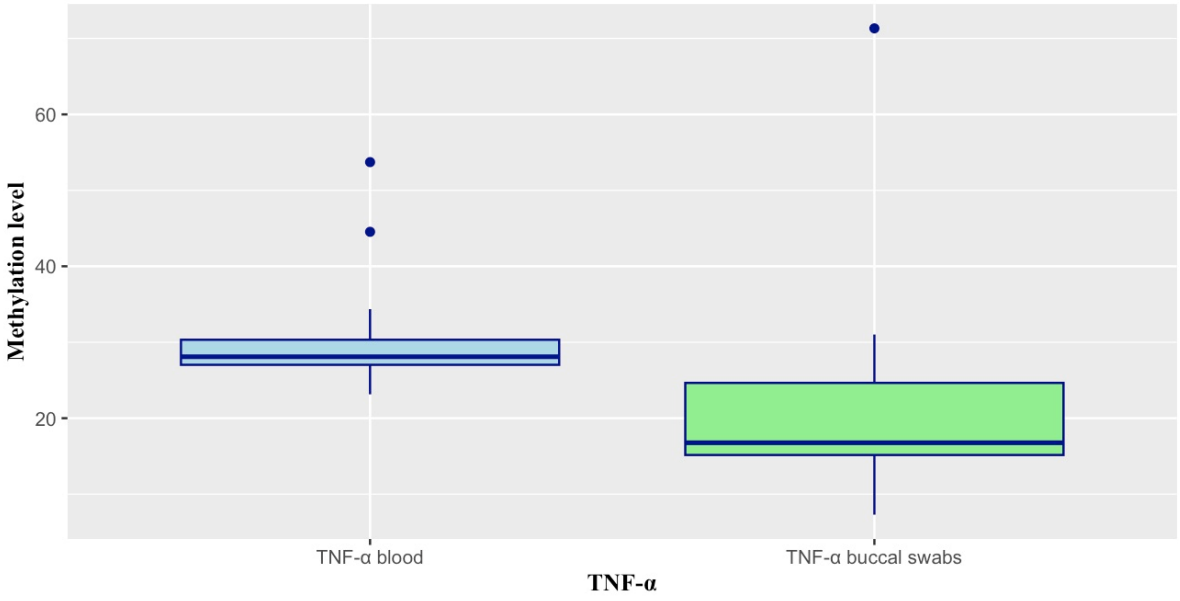

Figure 4: Comparison of methylation levels between TNF-α blood and TNF-α buccal swabs

Figure 5 shows a relatively high standard deviation. There is a significant (p=0.0175) positive correlation between TNF-α in blood and TNF-α in buccal swabs (ρ=0.4002) as the yellow line shows. The blue dots represent each participant in the study. The methylation level of TNF-α in blood does increase as well as the methylation level of TNF-α in the buccal swabs does, but not to the same extent. Figure 5 shows clearly that the increase in both media does not occur at a ratio of 1:1.

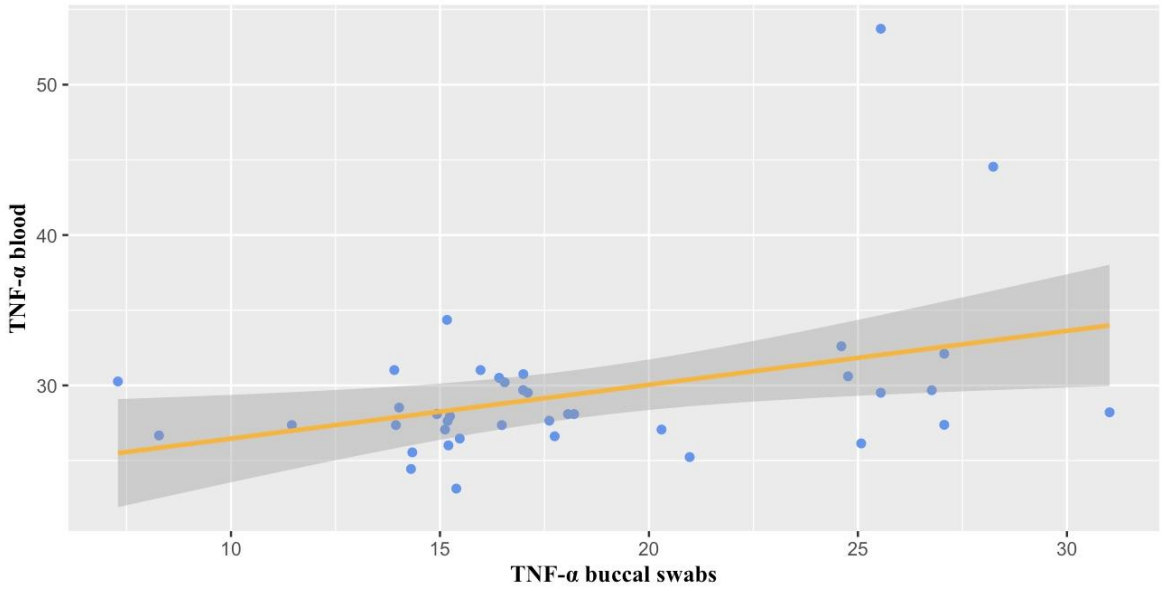

Figure 5: Scatterplot between TNF-α blood and TNF-α buccal swabs. The yellow trend line highlights the type of relationship, whereas the gray zone indicates the standard deviation

Figure 6 shows that the median in oral mucosa of IL-6 methylation is higher than IL-6 methylation in blood. The median of IL-6 methylation in blood is 67.62, while that of oral mucosa is 94.72. The variance is quite similar. The IL-6 methylation results in blood are normally distributed, while those in oral mucosa are not. The Mann–Whitney *U* test showed that there is a significant difference between both (p=0.0004).

Nevertheless, there is no positive correlation (ρ= -0.0356) between the IL-6 results in blood and those in buccal swabs. The results are not significant (p=0.848), which is why they will not be explained in more detail.

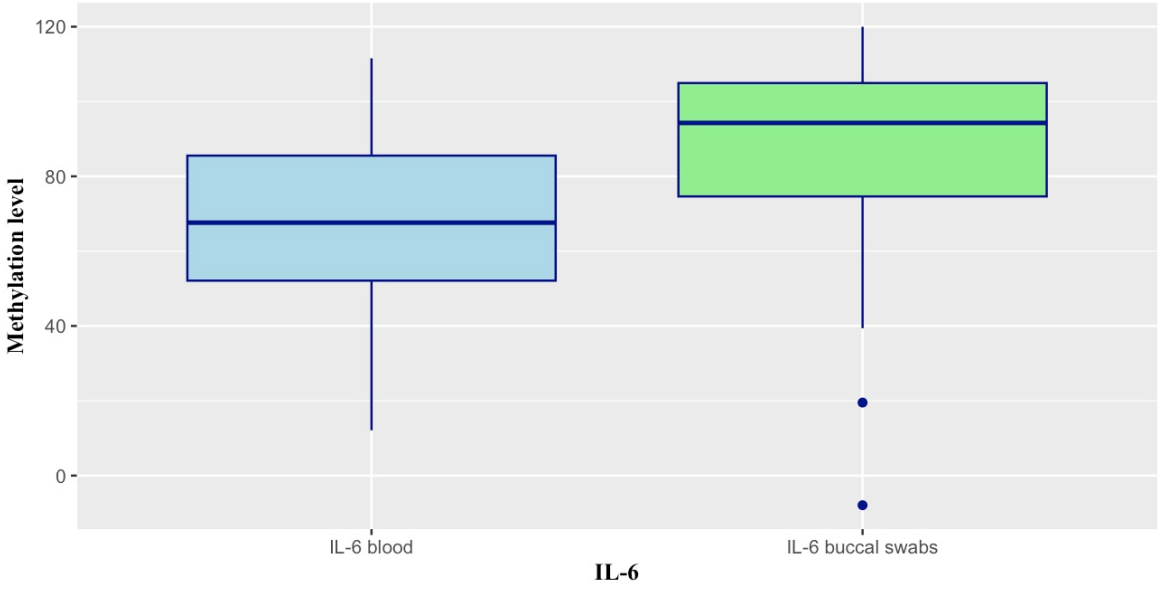

Figure 6: Comparison of methylation levels between IL-6 blood and IL-6 buccal swabs

4.2.2. Fish Consumption

Participants were asked how often they consume fish per week. Group 1 shows those who never or rarely eat fish. Group 2 consumes fish once a week, whereas group 3 eats fish 2-3 times per week. Group 4 consumes fish at least 4 times per week. Figure 4 shows that there was no one in group 3 and only one single person in group 4.

There is a significant difference (p=0.0212) in TNF-α methylation between all groups. The median is significantly higher in group 1 in comparison to the other two groups. Dunn's test showed that group 1 differs significantly (p= 0.0093) from group 2. No significant difference could be demonstrated between group 1 and group 4 (p= 0.7314) or group 2 and group 4 (p=1.0).

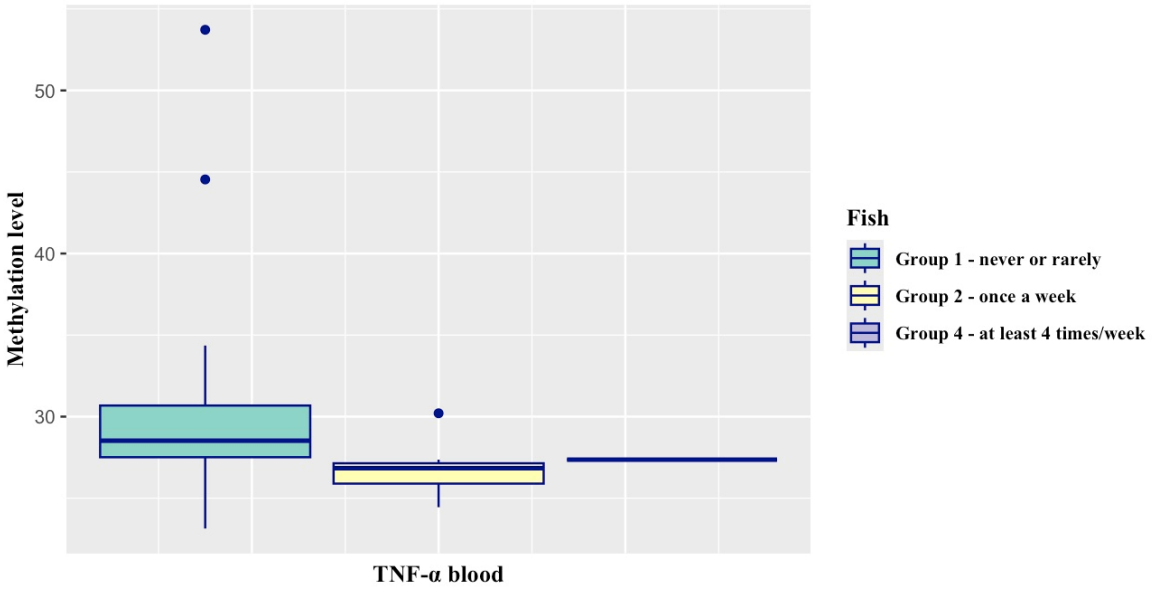

Figure 7: Methylation level of TNF-α in blood according to fish consumption

Figure 8 shows methylation levels of TNF-α in buccal swabs. No significant difference (p=0.0685) could be demonstrated.

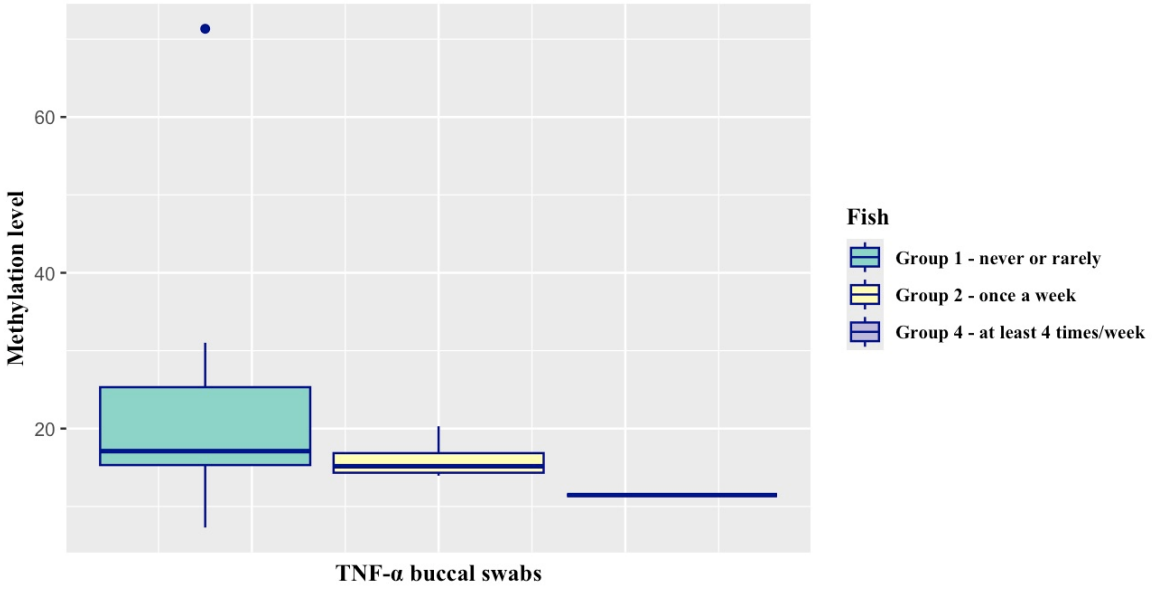

Figure 8: Methylation level of TNF-α in buccal swabs according to fish consumption

Figure 9 shows methylation levels of IL-6 in blood according to fish consumption. The Kruskal Wallis test has shown a significant result (p=0.0026). Group 2 had the highest methylation levels of IL-6 in blood. There was a significant difference between group 1 and group 2 (p=0.0016), but no significant difference between group 1 and group 4 (p=0.6205) as well as no significant difference between group 2 and group 4 (p=0.0673).

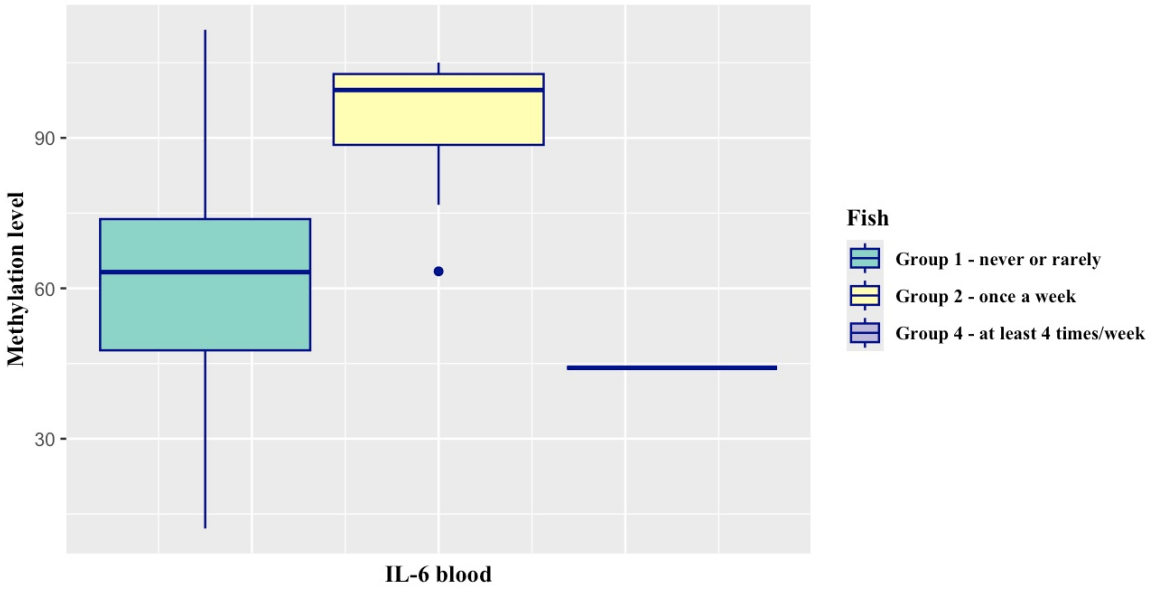

Figure 9: Methylation level of IL-6 in blood according to fish consumption

Figure 10 shows methylation levels of IL-6 in buccal swabs and no significant result (p=0.3511).

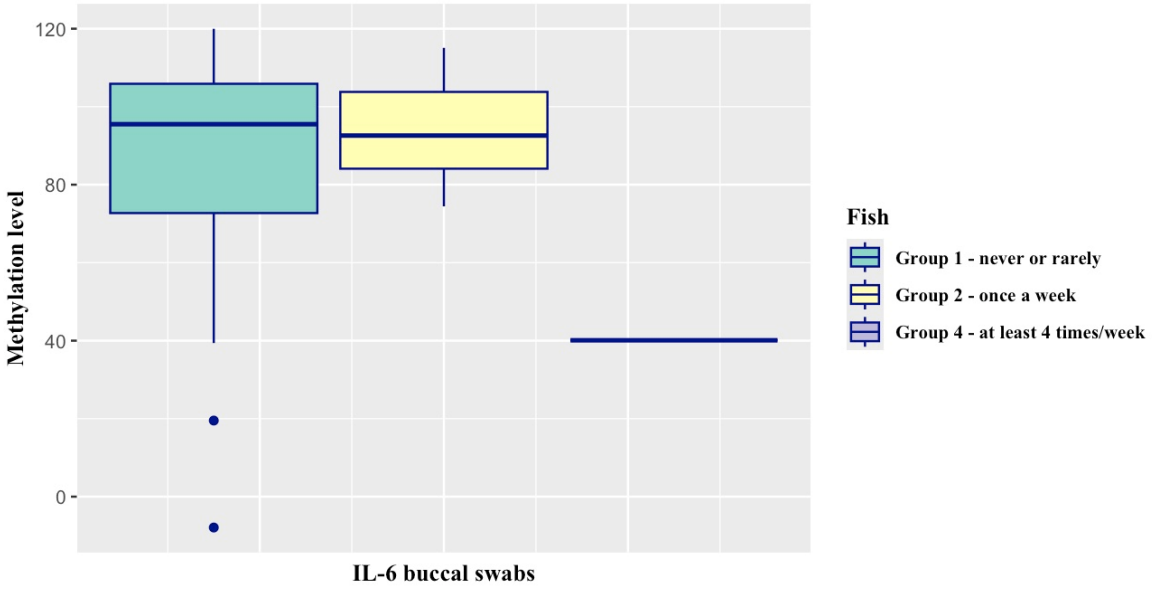

Figure 10: Methylation level of IL-6 in buccal swabs according to fish consumption

4.2.3. Vegetable Consumption

Participants were asked about the frequency of their vegetable consumption. No one was assigned to group 1 (seldom or never) or group 2 (once per week). Group 3 consumes vegetables 2-3 times a week, whereas group 4 eats vegetables 4-6 times per week. Group 5 consumes vegetables daily. Group 6 consumes vegetables several times a day.

Figure 11 shows methylation levels of TNF-α in blood according to vegetable consumption. The results are not significant (p= 0.5996). The median of group 3, group 4 und group 5 show a quite similar median. Group 6, the group with the highest vegetable consumption, has the highest median of the methylation levels of TNF-α.

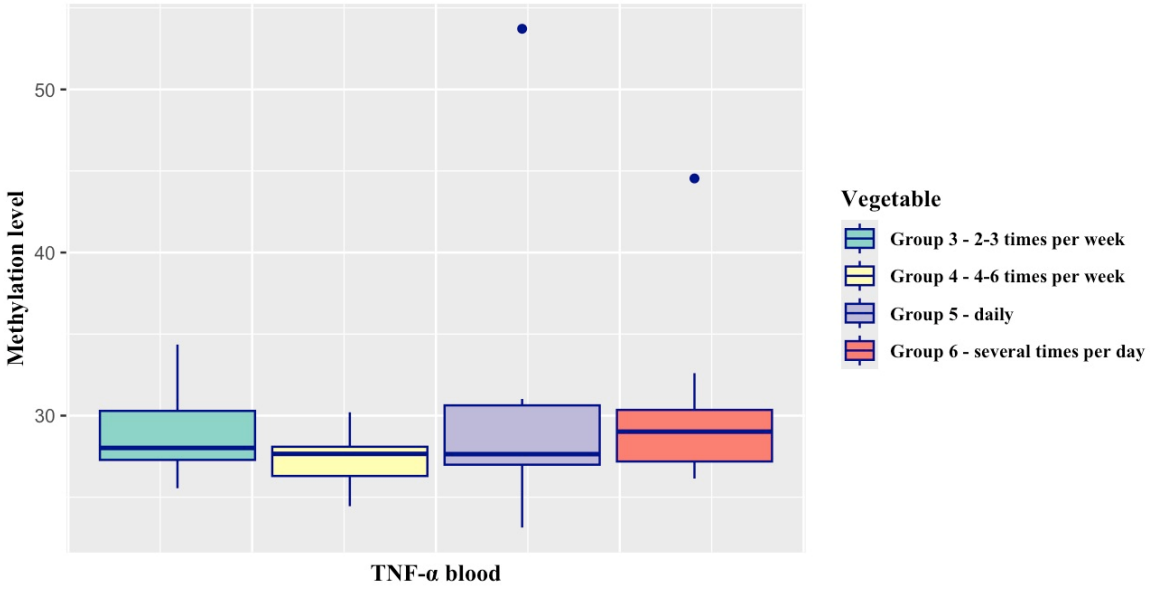

Figure 11: Methylation level of TNF-α in blood according to vegetable consumption

Figure 12 shows the methylation level of TNF-α in buccal swabs according to the vegetable consumption. Methylation levels are not significant (p= 0.6335). The median is noticeably higher in group 6 compared to the other groups. The median of group 3, group 4 and group 5 is similar.

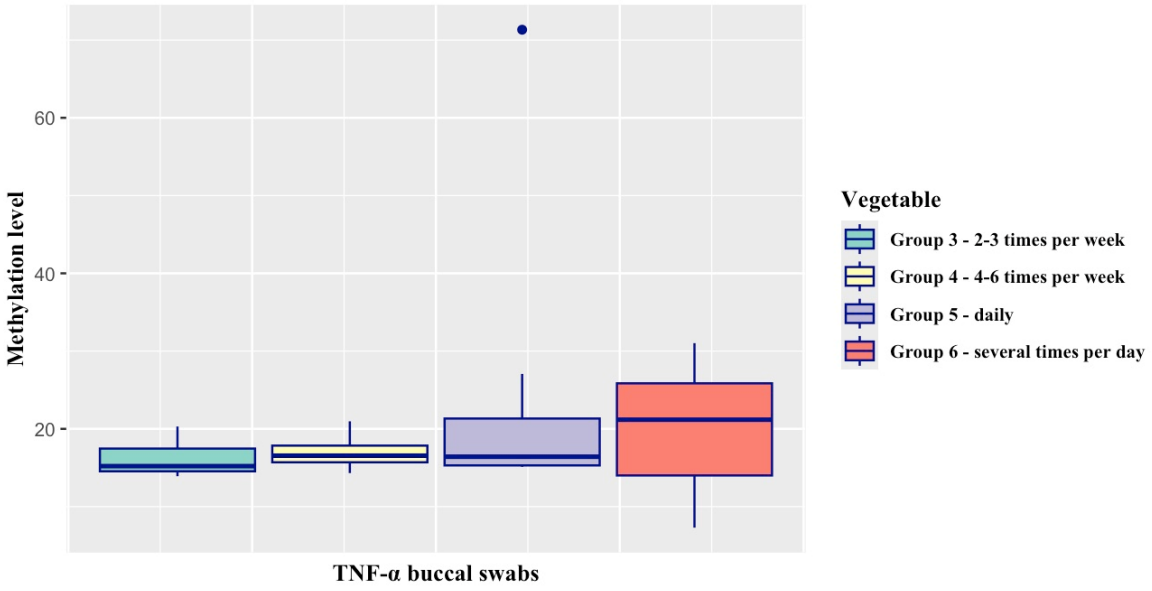

Figure 12: Methylation level of TNF-α in buccal swabs according to vegetable consumption

Figure 13 shows the methylation level of IL-6 in blood according to vegetable consumption. The results are not significant (p= 0.3136). In comparison to TNF-α methylation levels in figure 12, figure 13 shows that IL-6 methylation levels vary widely within the different groups, especially in group 3.

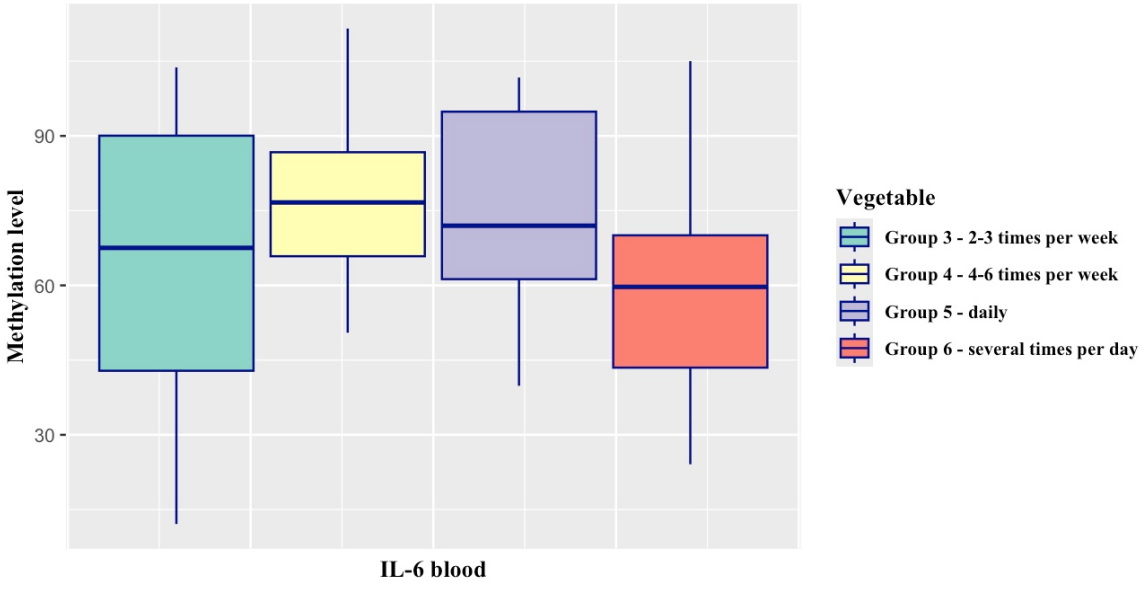

Figure 13: Methylation level of IL-6 in blood according to vegetable consumption

Figure 14 shows the methylation level of IL-6 in buccal swabs according to vegetable consumption. Also, those boxplots show that methylation levels vary widely within the different groups, however the variation is not as strong as in blood. These results are not significant (p=0.5125). The median does not show a trend, or another pattern.

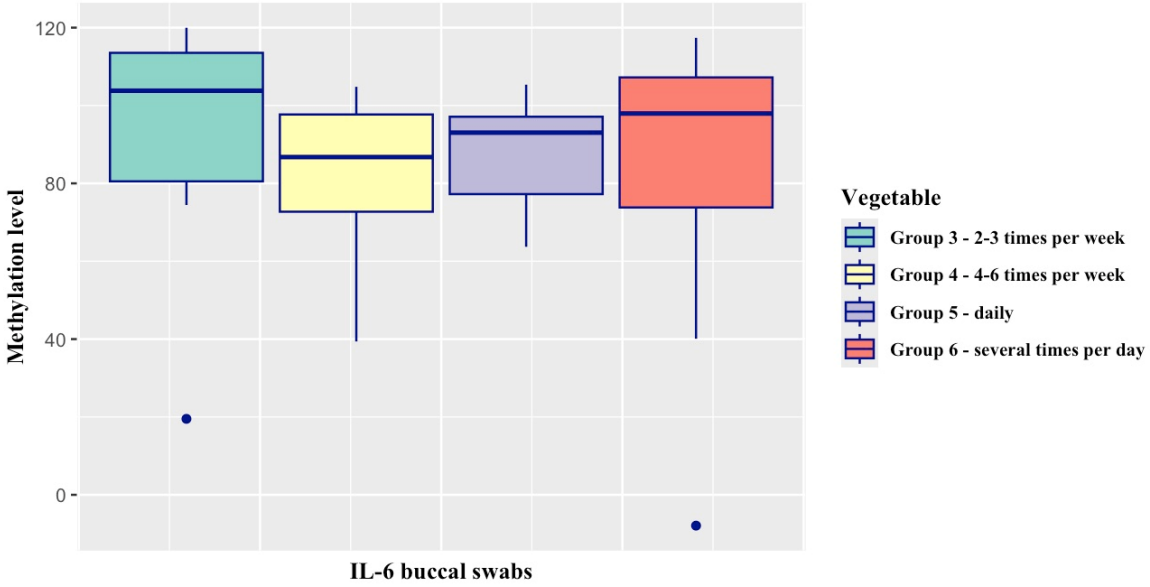

Figure 14: Methylation level of IL-6 in buccal swabs according to vegetable consumption

4.2.4. Fruit Consumption

Participants were asked about the frequency of their fruit consumption. No one was assigned to group 1 (seldom or never). Group 2 consumes fruits once per week. Group 3 consumes fruits 2-3 times a week, whereas group 4 eats fruits 4-6 times per week. Group 5 consumes fruits daily. Participants in Group 6 consume fruit several times daily. Figure 15 shows methylation levels of TNF-α in blood according to fruit consumption. There is no recognizable trend, and the results are not significant (p=0.6143).

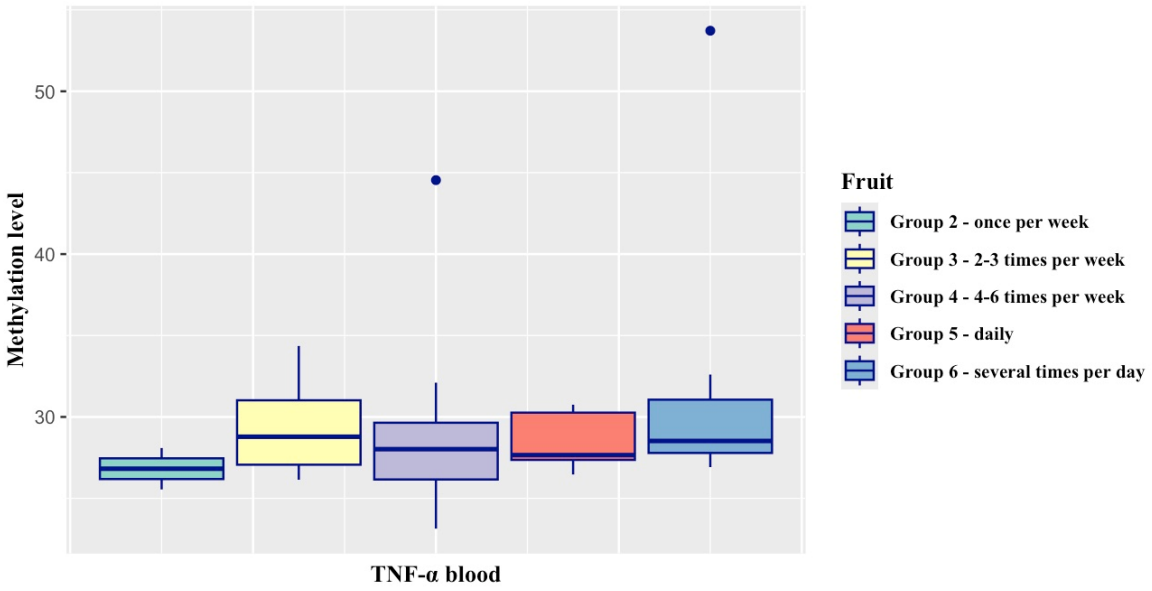

Figure 15: Methylation level of TNF-α in blood according to fruit consumption

Figure 16 shows the methylation level of TNF-α in buccal swabs according to fruit consumption. The results are not significant (p= 0.6386)

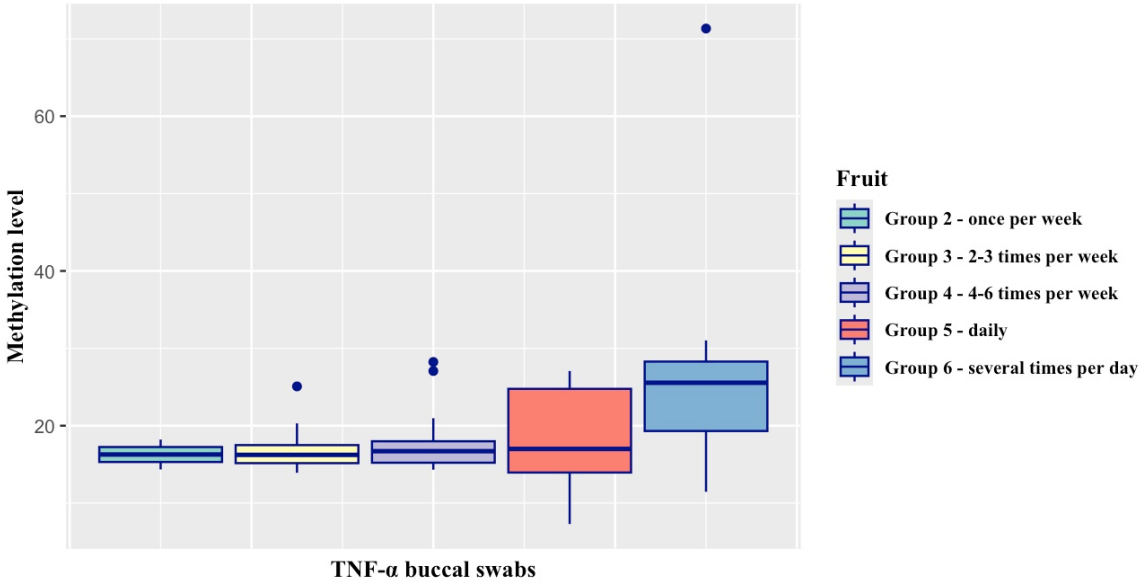

Figure 16: Methylation level of TNF-α in buccal swabs according to fruit consumption

Figure 17 shows the methylation level of IL-6 in the blood according to fruit consumption. The results are not significant (p=0.1275). A downward trend is recognizable. Higher fruit consumption goes along with lower IL-6 methylation levels.

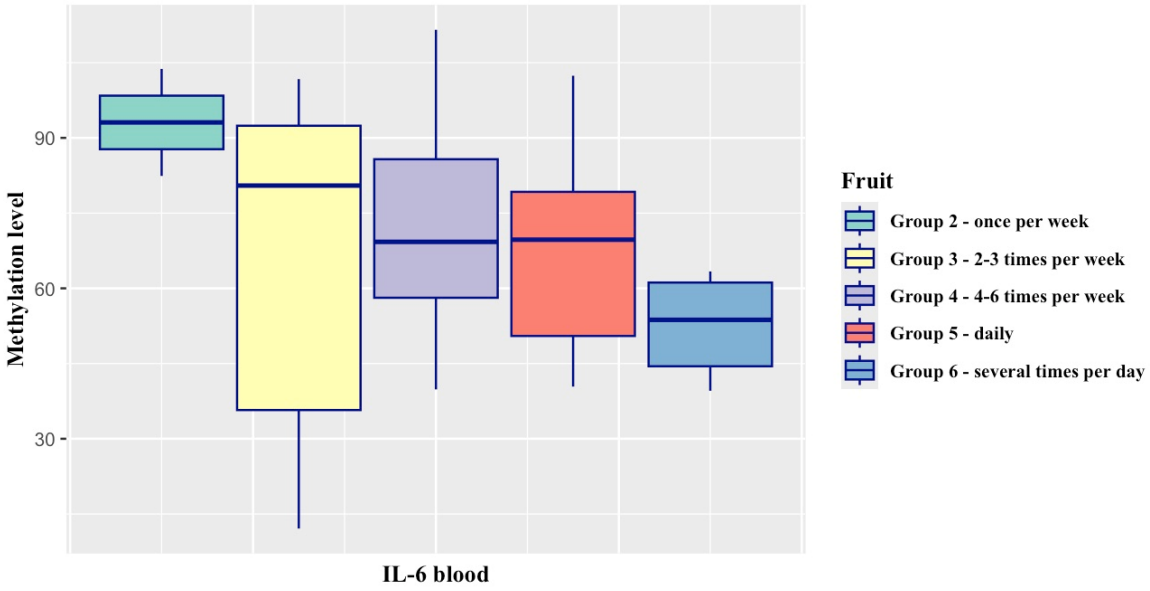

Figure 17: Methylation level of IL-6 in blood according to fruit consumption

Figure 18 shows the methylation level of IL-6 in buccal swabs according to fruit consumption. The results are not significant (p=0.6169).

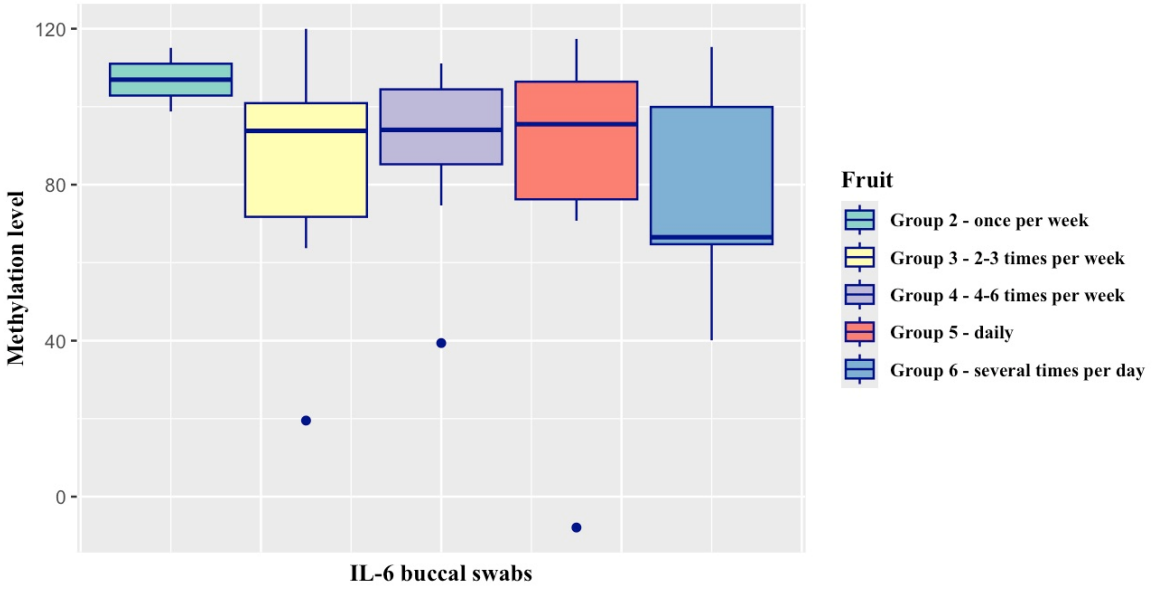

Figure 18: Methylation level of IL-6 in buccal swabs according to fruit consumption

4.2.5. Coffee Intake

Participants were asked if they consume coffee. Group 1 consumes coffee, whereas group 2 does not. Figure 19 shows methylation levels of TNF-α in blood according to coffee consumption. The median of group 1 is slightly higher than in group 2. The results are not significant (p=0.2215).

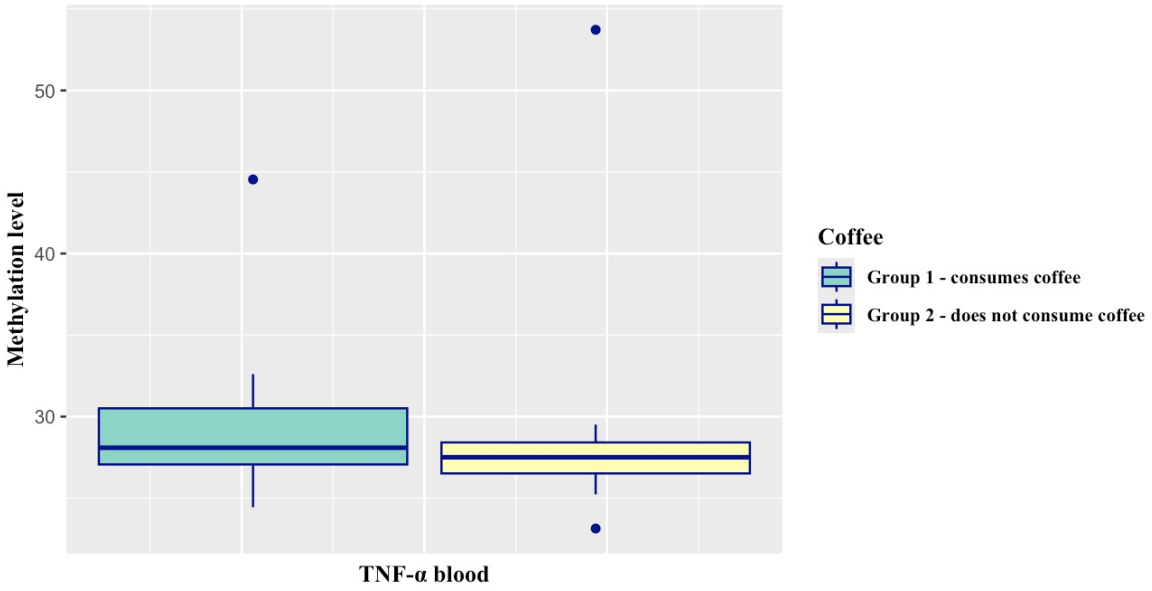

Figure 19: Methylation level of TNF-α in blood according to coffee consumption

Figure 20 shows methylation levels of TNF-α in buccal swabs according to coffee consumption. The median of both groups is almost identical. The results are not significant (p=0.5956).

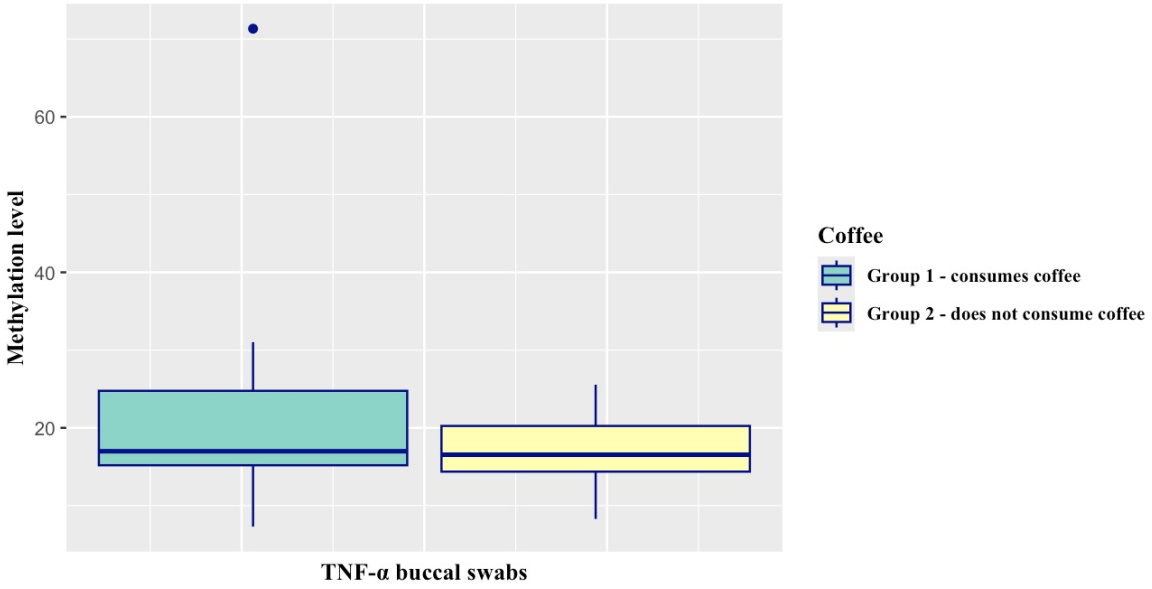

Figure 20: Methylation level of TNF-α in buccal swabs according to coffee consumption

Figure 21 shows methylation levels of IL-6 in blood according to coffee consumption. This graphic shows again that the range of IL-6 is large within the groups. The results are not significant (p=0.5305).

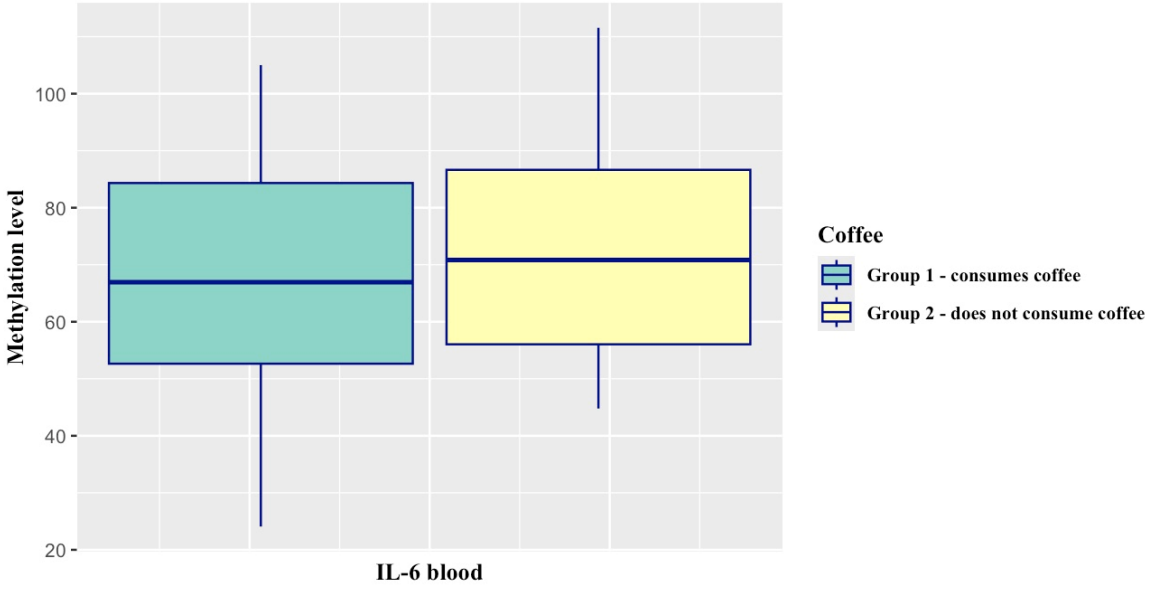

Figure 21: Methylation level of IL-6 in blood according to coffee consumption

Figure 22 shows methylation levels of IL-6 in buccal swabs according to coffee consumption. The median of group 1 is slightly higher than in group 2. The results are not significant (p=0.5735).

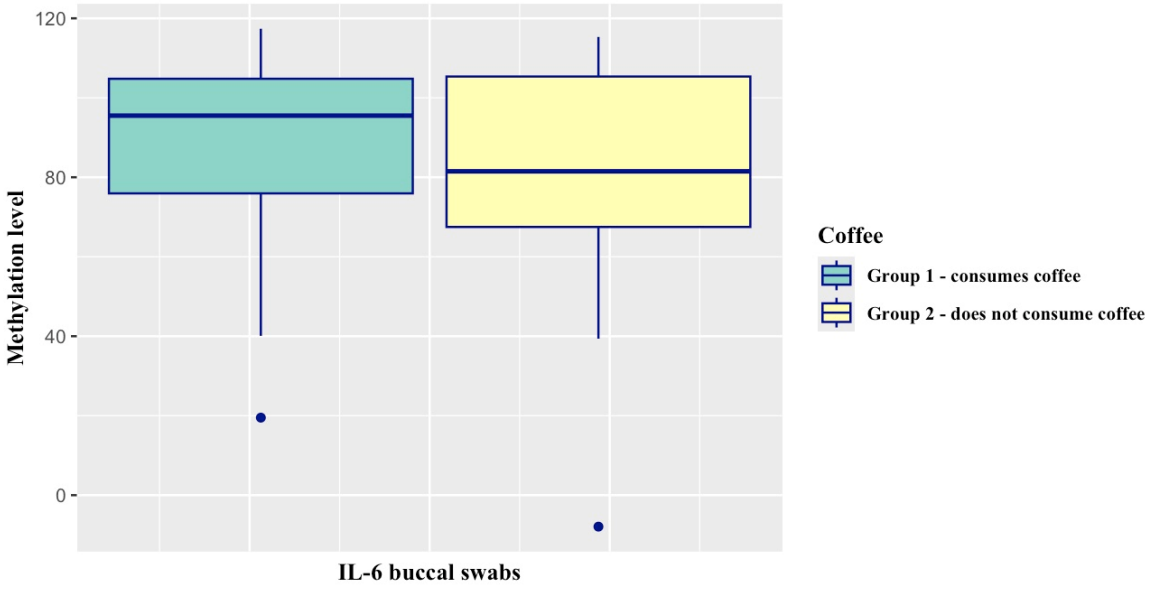

Figure 22: Methylation level of IL-6 in buccal swabs according to coffee consumption

5. DISCUSSION

After adjusting for dietary habits, only fish consumption was associated with a statistically significant outcome, whereas the remaining results did not reach statistical significance. For this reason, the discussion will primarily focus on the influence of fish consumption on TNF-α and IL-6 methylation levels.

Despite that fact, the discussion will, although the results are not statistically significant, discuss some noticeable trends of vegetable consumption and TNF-α methylation levels as other studies show significant correlations between the two. No trend was observed between IL-6 methylation levels and vegetable consumption. The same applies to IL-6 as well TNF-α methylation levels and fruit consumption. Finally, the results of coffee intake and methylation levels were also not significant, and methylation levels were similar between coffee drinkers and non-drinkers, thus no trend was noticeable here either.

5.1. TNF-α METHYLATION

The results show that the methylation levels of TNF-α are significantly higher in capillary blood than those in buccal swabs. Nevertheless, TNF-α methylation levels in buccal swabs did not increase to the same extent as TNF-α methylations did in capillary blood. Furthermore, the results in blood and buccal swabs correlated, but did not increase to the same extent. This indicates that TNF-α was more strongly suppressed in blood than locally in the oral cavity, since higher TNF-α methylation levels generally lead to reduced TNF-α expression.

Furthermore, it should be considered that the tissues differ between each other, which can lead to different results, although measuring the same methylations. Buccal swabs are locally exposed to the oral microbiome and are directly influenced by various factors such as smoking or diet, especially as food is chewed and broken down directly in the oral cavity.

In addition, TNF-α could also be more active in the oral cavity which could indicate a local inflammatory process and suggests that epigenetic regulation of TNF-α is tissue-specific. It should be considered that the cohort of this study consisted entirely of healthy young participants. Therefore, significantly lower TNF-α methylation levels in the oral region could point out increased propensity for an inflammatory response and show possible active inflammation. However, whether local inflammatory activity was indeed present or not cannot be determined, as no dental examination (e.g. for periodontitis or other oral diseases) was conducted in this study, nor TNF-α was measured to confirm this.

Data regarding high and low TNF-α methylation levels between study groups and control groups also proved to be contradictory in the conducted literature review. Various studies have reported conflicting results regarding TNF-α methylation in patients with periodontitis. In some studies, participants with periodontitis showed lower TNF-α methylation levels compared to the control group (Abasijiang et al., 2021) or demonstrated that participants with apical periodontitis showed also lower TNF-α methylation levels, compared to healthy individuals (Fernández et al., 2025). Nevertheless, there are also studies that found the opposite, reporting higher TNF-α methylation levels in the study group (Kobayashi et al., 2016).

Nonetheless, only healthy participants and no control group was included in the present study, whose results show lower TNF-α methylation levels in the oral cavity compared to capillary blood. Comparisons with other studies as well as the interpretation of the results are difficult, as data on this topic is highly variable and inconsistent, as mentioned before.

5.2. IL-6 METHYLATION

IL-6 shows the contrary of TNF-α results: the methylation levels were significantly higher in the oral mucosa compared to those in capillary blood, but there is no correlation between IL-6 methylation levels in these tissues. This indicates that an increase in one of the two values does not necessarily correspond to an increase in the other, suggesting that IL-6 methylation levels in blood are not correlated with those in oral mucosa.

This is quite interesting as another study showed different results, where IL-6 promoter methylation levels in blood were significantly higher in comparison to gingival tissue (Kobayashi et al., 2016). Similarly, other studies have found that participants with oral diseases—particularly periodontitis—exhibit lower IL-6 methylation levels compared to the control group: Abasijiang et al. (2021) compared IL-6 methylation levels between patients with chronic periodontitis and control group in peripheral blood, Kobayashi et al. (2016) did so as well and also Ishida et al. (2012) compared the IL-6 methylations between a study group with chronic periodontitis and the control group.

As methylation levels of IL-6 were higher in oral mucosa, it can be suggested that participants, all of whom were healthy, may not have had any acute inflammation in the oral cavity. It could be assumed that higher IL-6 methylation represents a normal regulation of IL-6 in the mouth to limit inflammation, as the oral cavity is constantly exposed to factors such as oral bacteria and other environmental influences. Nevertheless, it remains uncertain

whether all study participants were orally healthy, as no dental examination was conducted in the study.

It should also be considered that, compared to TNF-α methylation levels, IL-6 methylation levels showed considerable variability. A high variability between individual subjects regarding IL-6 methylation was also observed in other studies (F. F. Zhang et al., 2012) and depends on various lifestyle factors (Mao et al., 2017).

5.3. ↑ IL-6 AND ↓ TNF-α METHYLATIONS LEVELS

Unfortunately, this study did not include a separate control group, and the participants did not undergo a dental oral examination. Therefore, it remains unclear what the actual oral health status of the participants was, and it cannot be said with certainty that study participants indeed were orally healthy. It is possible that some had early-stage periodontitis or other oral diseases that had not been manifested clinically but may have already influenced methylation patterns in the oral mucosa. In this study participants showed lower TNF-α, but higher IL-6 methylation levels in buccal swabs compared to capillary blood. For example, low TNF-α methylation in the oral cavity could indicate an initial inflammatory process that had not yet produced noticeable symptoms and therefore remained undetected by the participants. Additionally, even minor microtraumas in the oral area could have been present, potentially leading to decreased TNF-α methylation levels as TNF-α is a cytokine known for its rapid response to inflammation. At the same time, higher IL-6 methylation levels suggest that any potential inflammation in the oral region had not persisted for long and that the gene may have been methylated more strongly to prevent the development of chronic inflammation. However, all of this remains unclear, as an accurate interpretation of the methylation results is difficult without a control group or prior dental examination.

5.4. TNF-α AND IL-6 METHYLATION LEVELS: FISH CONSUMPTION

The consumption of fish had a significant effect on TNF-α as well as on IL-6 blood methylation levels, but not on the methylation levels in buccal swabs. On first impression, TNF-α and IL-6 methylation status in the oral mucosa seems not to be influenced by fish consumption in this study, but it should be taken into account that oral mucosa cells are short-lived, and long-term effects are likely more difficult to detect, which complicates the interpretation of the results. Furthermore, it cannot be concluded from this that fish consumption does not influence local inflammation, as there is increasing evidence

suggesting that the exposure time of foods also plays an important role in epigenetic regulation, which was not considered in this study.

Study participants who rarely or never consumed fish had significantly higher TNF-α methylation levels in blood than people who consumed fish more frequently. Group 1, which did not consume fish, demonstrated the highest TNF-α methylation levels. Accordingly, no effect was observed with the consumption of fish more than once per week. These findings contrast with those of other studies as findings related to TNF-α methylation levels and PUFA demonstrate that the consumption of the ω-3 PUFA DHA, which is known to be anti-inflammatory and primarily found in fatty marine fish, results in higher methylation of TNF (Hussey et al., 2021), whereas higher ω-6 PUFA intake, known as precursors of series 2 prostaglandins and therefore proinflammatory, was associated with lower TNF promoter methylation levels (Hermsdorff et al., 2013).

Also, AGES recommends consuming fish 1–2 times per week as it is rich in ω−3 fatty acids, proteins, iodine, and vitamin D. Fish consumption is characterized especially by the omega-3 fatty acids DHA and EPA, making it an essential part of the diet. The results of these studies show that consuming fish more than once per week did not influence TNF-α methylation. However, these findings oppose previous studies and contradict current recommendations, since the highest TNF-α methylation levels was observed in participants who did not consume fish. Additionally, methylation levels declined with higher fish consumption.

In contrast, the situation is different for IL-6, where fish consumption (once a week) compared to no fish consumption is associated with higher IL-6 methylation levels, which in turn indicates a lower level of inflammation. Group 2, which consumed fish once per week, demonstrated the highest methylation levels. No increase was observed in group 4 where increased fish consumption does not seem to be associated with either beneficial or adverse effects, probably due to the fact this group included only one participant.

Consequently, it remains unclear whether a higher frequency of fish consumption per week would have led to increased IL-6 methylation levels or not. A larger sample size in group 4 would have been required to clarify this. Other studies, however, showed that higher ω-3 PUFA was associated with lower IL-6 methylation (Ma et al., 2016), which could not be observed in the present study.

Therefore, the expected outcome that fish consumption results in higher TNF-α methylation levels could not be proven in this study. However, this does not apply to the results for IL-6

methylation levels. Deviating results could be related to the large number of limitations, which are explained in more detail in section 6. It should also be mentioned that the distribution of the test subjects within the fish consumption groups varied. For example, there was not a single test subject in group 3 (fish consumption 2-3 times per week), whereas group 1 included 31 study participants and group 2 only 8 participants (these are also those two groups that differed significantly). Unfortunately, the uneven distribution of participants across the groups likely had an impact on the results, as this makes it difficult to compare the groups reliably. Although group 1 and group 2 differed significantly, they should be viewed with caution in terms of their significance, as it must also be borne in mind that the type of fish was not surveyed, which is also discussed in more detail in the limitations. As Jurdziński et al. (2020) pointed out, it is important to note the discrepancies between some studies, which examined methylation levels. Various studies have already been cited in this work to illustrate the variability in results regarding methylation analyses. Also, Hermsdorff et. al (2013) notes that the modulation of specific nuclear factors still underlies unclear mechanisms and further studies are necessary to investigate the precise relationship between dietary fat and DNA methylation. Unfortunately, this aspect limits the interpretability of the results, but nevertheless, provide insight into epigenetic regulation and nutrition.

5.5. TNF-α Methylation Levels: Vegetable Consumption

All groups consumed vegetables regularly and frequently. There was no group that consumed little or no vegetables. Although the results for vegetable consumption were not significant, the highest median was observed in group 6 (including 16 participants), which was the group with the highest vegetable intake (multiple times daily). A positive effect on TNF-α was only seen with multiple daily servings of vegetables, whereas the methylation results in both blood and buccal swabs showed that there was no difference between consuming vegetables 2–3 times per week and eating them daily. Only multiple daily servings resulted in a noticeably higher median. The findings did not reach significance but nevertheless showed a trend.

Another study also showed positive association between TNF-α methylation levels and vegetable consumption (Boonrong et al., 2024). Furthermore, an increased vegetable consumption was also associated with a significant decrease in TNF-α in a study from 2023, where participants who consumed more vegetables showed lower TNF-α compared to those with a low consumption (Gariballa et al., 2023). Both studies also assessed vegetable

consumption using a food frequency questionnaire.

Thus, a high vegetable intake appears to have a beneficial effect on TNF-α. Although this association could not be confirmed with statistical significance in the present study, a trend in that direction was still observable. However, since the number of studies investigating vegetable consumption and TNF-α methylation, or methylation in general, is limited, further research is needed to clearly determine the extent of the impact of vegetable intake on TNF-α methylation.

6. Limitations and Future Research

As already pointed out in the results, this study had some limitations, which are explained in more detail below.

6.1. Questionnaire

First, the questionnaire assessed the frequency of fish, vegetable, and fruit consumption, but not the exact quantity of these foods. If someone reported consuming vegetables several times a day, it remains unclear how much is actually consumed. Perception is a very individual thing and there are several things that have an influence on the perception of portion size. These include, among other things, the presentation of the food. For example, consumers perceive portions of food as smaller when they are presented vertically (Szocs & Lefebvre, 2017). It is known that small portion sizes tend to be overestimated whereas large ones are rather underestimated (Szenczi-Cseh et al., 2017).

AGES recommends 5 portions of fruit and vegetables per day, whereby a portion is defined as follows: cooked vegetables (200 - 300 g), raw vegetables (100-200 g), salad (75-100 g), pulses (raw approx. 70-100 g, cooked approx. 150-200 g), fruit (125-150 g), vegetables (125-150 g), vegetable or fruit juice (200 ml). The questionnaire defines a vegetable and fruit portion as “handful”, which is at least an approximate indication, but is not clearly defined and may differ in the perception from person to person. Some may consider a handful to be an overflowing hand, while for others a few berries could already be considered as a handful. Similarly, the exact amount of fish consumed was not assessed. The Austrian dietary guidelines by AGES recommend one portion of fish per week, with an optional second portion. While the questionnaire records how often fish is consumed, it does not provide information on the quantity. Normally, a portion of fish corresponds to a finger-thick, palm-sized piece (AGES, 2024), which was unfortunately not asked for in the

questionnaire. Therefore, it is possible that some participants reported daily fish consumption, but one individual might eat an entire trout each day, while another might consume only a couple of spoonfuls of tuna in a salad. Unfortunately, this information was not included in the questionnaire but would be important for the outcome. An exact definition of a portion should be given in the beginning of the questionnaire to make sure that a comparison between the quantity of consumption among study participants is conclusive. In this study it is unclear what a test person understands by a portion exactly, which is why it is difficult to compare the results.

Moreover, not only does the amount of food consumed appear to play a role in epigenetic regulation, but also the exposure time to food, which was not considered in this study.

Furthermore, the types of fish, vegetables, and fruits consumed were not recorded. This information would be particularly relevant for fish, as ω−3 fatty acid content varies significantly between different species. Oily fish, such as sea fish, mackerel, salmon, tuna, herring or local cold-water fish such as char are a good source of ω−3 fatty acids, which are essential in diet.

Also, the term “seldom” was not explained in detail, and it is unclear where the exact difference lies between “seldom” and “seldom or never”. This could have been perceived differently by the respondents, which is why it is not possible to compare the answers reliably here either.

In general, it can be said that the reliance on self-reported lifestyle data introduces notable biases and inaccuracies that do not point out the usual eating habits as over- and underreporting is unavoidable at questionnaires without additional measurements. The evaluation of nutritional status and caloric intake is complicated by the need for comprehensive dietary data, underscoring the need for more accurate and impartial data collection techniques. This might potentially produce false negative results that may have an impact on the outcome.

6.2. PARTICIPANTS

First and foremost, it should be noted that the study was originally planned to also include participants who were not orally healthy, which unfortunately was ultimately not the case.

As this was a pilot study, 40 healthy participants were included. Furthermore, there were also major differences within the individual groups (e.g. regarding the fish consumers, there were 31 people in group 1 but only 8 people in group 2). If a representative group had been

included, the results might have differed.

It should also be mentioned that the participants were quite young, on average 27 years old, which also does not speak for a representative group. In addition, all the study participants came from close environment of the study organizers. All of them were Austrians or Germans, mostly athletic with a healthy lifestyle, primarily students and, as already mentioned, young. So, the study's dataset predominantly features adult German and Austrian participants, limiting its generalizability across different demographics. To enhance the informative value, future research must employ more extensive, more diverse datasets that include a variety of populations, ethnicities and genders.

Aside from that, there was no separate (disease) control group included, making it more difficult to interpret methylation levels of TNF-α and IL-6 in the oral cavity and the effects of nutrition and coffee intake on these parameters. A comparison with a control group would have provided more reliable results regarding the influence of diet and coffee on both methylations in oral cavity, as other studies that have been identified in the literature review reported opposing results in TNF-α and IL-6 methylation levels.

Furthermore, the lack of dental examination in this study complicates the interpretation of the results. Although all study participants were young and reported to be healthy, it was not previously verified by a dentist whether they were orally healthy and e.g were not affected by diseases such as caries or periodontitis. For interpreting the varying results in methylation patterns, such an examination would have been of high value.

6.3. SAMPLING

There is a possibility of application errors during sampling. While the blood sample was always taken by trained laboratory staff, the study participants had to collect the oral mucosa cells themselves using a brush under the supervision of the laboratory staff. It is possible that the collection of oral mucosal cells worked better for some study participants than for others. After sample collection, the amount of DNA collected from each subject was not tested individually. This did only take place in pretests to ensure that buccal swabs generally contain enough DNA to be able to carry out the subsequent tests. As a result, there is a possibility that sampling errors occurred and the number of cells varied greatly, which would also have an influence on methylation levels. Although care was taken to ensure that enough cells were taken, due to using the brush with circular movements, it was not possible to determine whether the participants pressed the brush well enough and thus removed an

adequate number of cells. The cell counts were not normalized, although it is possible that some study participants collected more cells than others. Despite everything, undetected things like this can always occur in the lab.

6.4. Evaluation

No standardized method was used for the analysis of the results, which may be one reason for the high variability observed in this and other studies as well as for the different outcomes.

6.5. Enhancing Research Quality in Future Studies

Due to the numerous limitations in this study, which were mentioned above, it cannot be ruled out that diet has a positive effect on TNF-α and IL-6 methylation in the oral cavity. For future studies, the inclusion of a control group, a prior dental examination, a better distribution of participants across groups and clearly defined quantity specifications in the questionnaire as well as exposure time of dietary components for better comparison are necessary to achieve more conclusive results.

7. Conclusion

This study aimed to examine whether the consumption of fish, vegetables, fruit, and coffee influences TNF-α and IL-6 methylation in buccal swabs, and to evaluate the potential of these epigenetic markers as biomarkers in dentistry for assessing the inflammatory status in the oral cavity and whether inflammation can be positively modulated by anti-inflammatory dietary factors in the mouth.

This study demonstrated that fish consumption, as part of the Mediterranean diet, had an influence on the methylation levels of IL-6 and TNF-α in capillary blood, but not in buccal swabs. No statistically significant differences in TNF-α and IL-6 methylation levels were observed between blood and buccal swabs in this context. According to the results, it remains unclear to what extent an anti-inflammatory diet influences oral health and whether TNF-α and IL-6 methylation levels could serve as biomarkers in periodontal medicine. It should be taken into account that oral mucosa cells are short-lived, and long-term effects are likely more difficult to detect. However, the observed effect of fish consumption on systemic inflammation does not rule out this possibility, especially when considering that there is increasing evidence that the length of exposure to food-derived bioactive compounds may also influence epigenetic regulation.

However, TNF-α and IL-6 methylation in capillary blood were significantly affected by fish consumption, suggesting that systemic inflammation is influenced by the consumption of fish. While higher fish consumption was associated with increased IL-6 methylation in blood, this was not the case for TNF-α methylations in blood, where lower methylation levels were observed. Due to the lack of dental examination and an uneven distribution of participants across study groups, the interpretability of the findings is limited.

As mentioned in the introduction, research on the relationship between fatty acids and DNA methylation is still limited and requires further investigation. The literature review showed that different studies have reported varying results regarding hypo- or hypermethylation, highlighting the need for further studies to enhance the validity of the findings.

Furthermore, the results for vegetables, fruit, and coffee consumption were all not statistically significant and were therefore not considered further in the study.

To sum up, no clear conclusions can currently be drawn regarding the influence of an anti-inflammatory diet und coffee intake on TNF-α and IL-6 methylation levels and their

potential for biomarkers in dentistry. However, certain limitations of this study may have influenced the results, which is why further research is needed.

8. References

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9. APPENDIX

9.1. QUESTIONNAIRE

Fragebogen: Mundgesundheit / Questionnaire: Oral health

Allgemeines / General information

Datum / Date

Vorname / First Name

Nachname / Last Name

Alter / Age

Geschlecht / Gender

Gewicht (kg) / Weight (kg)

Größe (cm) / Height (cm)

1. Wo sind Sie aufgewachsen? / Where did you grow up?
   - ☐ Stadt / City
   - ☐ Land / Country
2. Wo liegt Ihr aktueller Wohnort? / Where is your current place of residence?
   - ☐ Stadt / City
   - ☐ Land / Country
3. Pendeln Sie **regelmäßig**? / Do you commute **regularly**?
   - ☐ Nein / No
   - ☐ Ja, vom Land in die Stadt / Yes, from the countryside to the city
   - ☐ Ja, von der Stadt aufs Land / Yes, from the city to the countryside

Gesundheit / Health

1. Leiden Sie **häufiger als zwei Mal** im Jahr unter folgenden Beschwerden? / Do you suffer from the following complaints **more than twice** a year?
   - ☐ Keine / None
   - ☐ Zahnfleischbluten/Zahnfleischentzündung / Bleeding gums/gum inflammation
   - ☐ Sodbrennen / Heartburn
   - ☐ Reflux / Reflux
   - ☐ Grippale Infekte / Flu-like infections
   - ☐ Entzündungen der Haut und/oder Schleimhaut / Inflammation of the skin and/or mucous membranes
   - ☐ Muskel- oder Gelenkerkrankungen / Muscle or joint diseases
   - ☐ Blasenentzündung / Cystitis
   - ☐ Prostatabeschwerden / Prostate problems
   - ☐ Pilzinfektionen im Genitalbereich / Fungal infections in the genital area
   - ☐ Pilzinfektionen von Haut oder Füssen / Fungal infections of the skin or feet

1

☐ Sonstiges: / Other: _____

Wenn ja, nehmen Sie Medikamente zur Behandlung (bitte nennen)? / If yes, do you take medication for treatment (please specify)? _____

2. Wurde eine der folgenden Stoffwechselerkrankungen bei Ihnen diagnostiziert? / Have you been diagnosed with any of the following metabolic diseases?
   - ☐ Keine / None
   - ☐ Diabetes mellitus Typ 1 / Type 1 diabetes mellitus
   - ☐ Diabetes mellitus Typ 2 / Type 2 diabetes mellitus
   - ☐ Schilddrüsendysfunktion / Thyroid dysfunction
   - ☐ Pankreasleiden / Pancreatic disorders
   - ☐ Gicht / Gout
   - ☐ Fettstoffwechselstörung / Lipid metabolism disorder
   - ☐ Gestörte Glukosetoleranz / Impaired glucose tolerance

   Wenn ja, nehmen Sie Medikamente zur Behandlung (bitte nennen)? / If yes, do you take medication for treatment (please specify)? _____

3. Leiden Sie unter einer der folgenden Erkrankungen oder Beschwerden? / Do you suffer from any of the following illnesses or complaints?
   - ☐ Keine / None
   - ☐ Bluthochdruck / High blood pressure
   - ☐ Entzündliche Erkrankungen im Magen-/Darmbereich / Inflammatory diseases in the gastrointestinal tract
   - ☐ Depressionen / Depression
   - ☐ Parodontose / Periodontal disease
   - ☐ Osteoporose / Osteoporosis
   - ☐ Chronische Rhinitis / Chronic rhinitis
   - ☐ Rheuma / Rheumatism
   - ☐ Hormonelle Störungen / Hormonal disorders
   - ☐ Sonstige Erkrankungen: / Other diseases: _____

   Wenn ja, nehmen Sie Medikamente zur Behandlung (bitte nennen)? / If yes, do you take medication for treatment (please specify)? _____

4. Haben Sie Allergien oder Unverträglichkeiten? / Do you have any allergies or intolerances?
   - ☐ Nein / No
   - ☐ Ja / Yes

   Wenn ja, welche? / If yes, which ones? _____

5. Bei Lebensmittelallergien bzw. Unverträglichkeiten: Nehmen Sie diätetische Lebensmittel zu sich, um betreffende Lebensmittel trotzdem essen zu können (z.B. Lactase, Daosin,...) ? / In the case of food allergies or intolerances: Do you consume dietary supplements so that you can still eat the food in question (e.g. Lactase, Daosin, ..)?
   - ☐ Nein, ich vermeide diese Lebensmittel / No, I avoid these foods
   - ☐ Nein, aber ich esse die unverträglichen Lebensmittel nur in geringen Mengen / No, but I consume the foods in question only in small quantities
   - ☐ Ja / Yes

     Wenn ja, welche/s? / If yes, which one(s)? _____

     Wenn ja, wie oft?/ If yes, how often?
     - ☐ Seltener als 1x pro Woche / Less than 1x per week
     - ☐ 1x pro Woche / 1x per week
     - ☐ 2-3x pro Woche / 2-3x per week
     - ☐ Täglich / Daily

6. Wie oft haben Sie **im letzten Monat** Schmerzmittel/entzündungshemmende Medikamente (Kopfschmerzen, Gliederschmerzen, Verkühlung etc.) eingenommen? / How often have you taken painkillers/anti-inflammatory medication (headaches, aching limbs, colds, etc.) in **the last month**?
   - ☐ Nie / Never
   - ☐ 2x pro Monat / 2x per month
   - ☐ 1x pro Woche / 1x per week
   - ☐ Öfter als 1x pro Woche / More than once a week

     Wenn ja, welche/s (wenn möglich genaue Produktbezeichnung)? / If yes, which one(s) (if possible, exact product name)? _____

7. Haben Sie in den **letzten drei Monaten** ein Antibiotikum eingenommen? / Have you taken an antibiotic in **the last three months**?
   - ☐ Nein / No
   - ☐ Ja / Yes

     Wenn ja, welches (wenn möglich genaue Produktbezeichnung)? / If yes, which one (if possible, exact product name)? _____

Mundgesundheit / Oral Health

1. Wie oft putzen Sie Ihre Zähne am Tag? / How often do you brush your teeth per day?

   … Mal / Times

2. Welche Zahnbürste verwenden Sie? / Which toothbrush do you use?
   - ☐ Manuelle / Manual
   - ☐ Elektrische / Electrical
   - ☐ Ultraschall / Ultrasound

2.1. Zahnbürstenstärke / Bristle thickness

   - ☐ Weich / Soft
   - ☐ Mittel / Medium
   - ☐ Hart / Hard

3. Welche Zahnpasta verwenden Sie momentan? / Which toothpaste do you currently use?
   - ☐ Universal-Zahnpasta / Universal toothpaste
   - ☐ Sensitiv-Zahnpasta / Toothpaste for sensitive teeth
   - ☐ Weißmacher-Zahnpasta / Whitening toothpaste
   - ☐ Fluorid-Zahnpasta / Fluoride toothpaste
   - ☐ Zahnpasta ohne Fluorid / Toothpaste without fluoride
   - ☐ Ölzahncreme / Oil toothpaste

4. Verwenden Sie Produkte mit hohem Fluorid-Gehalt (z.B. Elmex Zahngel, Zymafluor etc.)? / Do you use products with a high fluoride content (e.g. Elmex tooth gel, Zymafluor etc.)?
   - ☐ Nein / No
   - ☐ Ja / Yes

   Wenn ja, wie oft? / If yes, how often?
   - ☐ Seltener als 1x pro Woche / Less than 1x per week
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ Täglich / Daily

5. Verwenden Sie momentan eine Mundspülung? / Are you currently using a mouthwash?
   - ☐ Nein / No
   - ☐ Ja / Yes

   Wenn ja, welche Art von Mundspülung? / If yes, what type of mouthwash?
   - ☐ Antibakteriell / Antibacterial
   - ☐ Für weiße Zähne / Whitening
   - ☐ Für sensitive Zähne / For sensitive teeth
   - ☐ Gegen Zahnstein / Anti-tartar
   - ☐ Gegen Mundgeruch / Against bad breath
   - ☐ Zur Entgiftung / Detoxifying

Wenn ja, wie oft? / If yes, how often?

- ☐ Seltener als 1x pro Woche / Less than 1x per week
- ☐ 1x pro Woche / 1x per week
- ☐ 2-3x pro Woche / 2-3x per week
- ☐ Täglich / Daily

6. Verwenden Sie Zahnseide/Interdentalbürsten? / Do you use dental floss/interdental brushes?
   - ☐ Nein / No
   - ☐ Ja / Yes

   Wenn ja, wie oft? / If yes, how often?
   - ☐ Seltener als 1x pro Woche / Less than 1x per week
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ Täglich / Daily

7. Verwenden Sie Zahnöl? / Do you use dental oil?
   - ☐ Nein / No
   - ☐ Ja / Yes

8. Kauen Sie Kaugummi? / Do you chew gum?
   - ☐ Nein / No
   - ☐ Ja / Yes

   Wenn ja, welche/n ? / If yes, what kind?
   - ☐ Mit Zucker / With sugar
   - ☐ Zuckerfrei / Sugar-free
   - ☐ Probiotisch / Probiotic
   - ☐ Mit Xylitol / With Xylitol
   - ☐ Antiviral / Antiviral

   Wenn ja, wie oft? / If yes, how often?
   - ☐ Seltener als 1x pro Woche / Less than 1x per week
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ Täglich / Daily

9. Wie oft gehen Sie zum Zahnarzt? / How often do you visit a dentist?
   - ☐ Nie / Never
   - ☐ 1x pro Jahr / 1x per year
   - ☐ 2x pro Jahr/ 2x per year
   - ☐ Mehr als 2x pro Jahr / More than 2x per year

10. Lassen Sie **regelmäßig** eine Mundhygiene beim Zahnarzt durchführen? / Do you have professional dental cleaning performed by a dentist **regularly**?
   - ☐ Nein / No
   - ☐ Ja / Yes

     Wenn ja, wie oft? / If yes, how often?
     - ☐ 1x pro Jahr / 1x per year
     - ☐ 2x pro Jahr / 2x per year
     - ☐ Mehr als 2x pro Jahr / More than 2x per year

     Wann war Ihre letzte Mundhygiene? (Monat & Jahr) / When was your last professional dental cleaning? _____

11. Sind Ihre Zähne gebleicht? / Are your teeth bleached?
   - ☐ Nein / No
   - ☐ Ja / Yes

     Wenn ja, wie oft wurden sie gebleicht? / If yes, how often were they bleached? _____

     Wenn ja, wann wurden sie zum letzten Mal gebleicht? / If yes, when was the last time they were bleached? _____

12. Trifft Folgendes auf Sie zu: / Does the following apply to you:
   - ☐ Nein / No
   - ☐ Zahnspange / Braces

     Wenn ja, welche Art von Zahnspange? / If yes, what type of braces?
     - ☐ Festsitzend/fix / Fixed braces
     - ☐ Herausnehmbar/Nacht-Spange / Removable/night braces
     - ☐ Aligner / Aligners
   - ☐ Anti Knirsch-Schiene / Anti-grinding splint
   - ☐ Funktionsschiene / Functional splint
   - ☐ Schnarchschiene / Anti-snoring splint
   - ☐ Prothese / Dental prosthesis
   - ☐ Kronen / Crowns

     Aus welchem Material ist/sind die Krone/n? / What material(s) is/are the crown(s) made of? _____
   - ☐ Implantate / Implants

     Aus welchem Material ist/sind die Implantate? / What material(s) is/are the implant(s) made of? _____

- ☐ Zahnlücke/n / Tooth gap/s
- ☐ Wurzelgefüllte Zähne / Root-filled teeth

13. Hat Folgendes in der Vergangenheit auf Sie zugetroffen? / Has the following applied to you in the past?
    - ☐ Nein / No
    - ☐ Zahnspange / Braces
      Wenn ja, welche Art von Zahnspange? / If yes, what type of braces?
        - ☐ Festsitzend/fix / Fixed braces
        - ☐ Herausnehmbar/Nacht-Spange / Removable/night braces
        - ☐ Aligner / Aligners
    - ☐ Anti Knirsch-Schiene / Anti-grinding splint
    - ☐ Funktionsschiene / Functional splint
    - ☐ Schnarchschiene / Anti-snoring splint
    - ☐ Prothese / Dental prosthesis
    - ☐ Kronen / Crowns
      Aus welchem Material ist/sind die Krone/n? / What material(s) is/are the crown(s) made of? _____
    - ☐ Implantate / Implants
      Aus welchem Material ist/sind die Implantate? / What material(s) is/are the implant(s) made of? _____
    - ☐ Zahnlücke/n / Tooth gap/s
    - ☐ Wurzelgefüllte Zähne / Root-filled teeth

14. Haben Sie derzeit Zahnschmerzen/Zahnprobleme? / Do you currently have toothache/tooth problems?
    - ☐ Nein / No
    - ☐ Ja / Yes
      Wenn ja, wie viele Zähne sind davon betroffen? / If yes, how many teeth are affected? _____

15. Haben Sie Zahnfleischbluten? / Do you have bleeding gums?
    - ☐ Nein / No
    - ☐ Ja / Yes
      Wenn ja, sind Sie wegen Zahnfleischbluten in Behandlung? / If yes, are you being treated for bleeding gums?
        - ☐ Nein / No
        - ☐ Ja / Yes
          Wenn ja, wie/womit werden Sie behandelt? / If yes, how are you being treated? _____

16. Bildet sich über Nacht Zahnbelag auf Ihren Zähnen? / Does plaque build up on your teeth overnight?
    - ☐ Nein / No
    - ☐ Ja, aber sehr wenig / Yes, but very little
    - ☐ Ja, aber mäßig / Yes, but moderately
    - ☐ Ja, viel / Yes, a lot
    - ☐ Weiß ich nicht / I don't know

17. Sind Sie von Zähneknirschen betroffen? / Are you affected by teeth grinding?
    - ☐ Nein / No
    - ☐ Ja / Yes

18. Haben Sie Probleme mit Zahnsteinbildung? / Do you suffer from tartar build-up?
    - ☐ Nein / No
    - ☐ Ja / Yes

19. Wurden Sie in den **letzten sechs Monaten** wegen Karies behandelt (Bohren, Inlay, Füllung etc.)? / Have you been treated for caries in the **last six months** (drilling, inlay, filling, ect.)?
    - ☐ Nein / No
    - ☐ Ja / Yes

20. Leiden Sie unter Mundgeruch? / Do you suffer from bad breath?
    - ☐ Nein / No
    - ☐ Ja / Yes
    - ☐ Weiß ich nicht / I don't know

21. Haben Sie starken Speichelfluss? / Do you have a high saliva production?
    - ☐ Nein / No
    - ☐ Ja / Yes

22. Fühlen sich Ihre Zähne locker an? / Do your teeth feel loose?
    - ☐ Nein / No
    - ☐ Ja / Yes

Ernährung / Nutrition

1. Wie oft essen Sie Gemüse (eine Portion = ca. eine Handvoll)? / How often do you eat vegetables (one portion = approx. one handful)?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ 4-6x pro Woche / 4-6x per week
   - ☐ Täglich / Daily
   - ☐ Mehrmals täglich / Several times per day

2. Welche Gemüsesorten essen Sie bevorzugt? / Which vegetables do you prefer?
   - ☐ Grünes Blattgemüse (Salat, Spinat, Mangold, Pak Choi, ...) / Green leafy vegetables (lettuce, spinach, chard, pak choi, …)
   - ☐ Kohlgemüse (Brokkoli, Kohl, Kohlsprossen, …) / Cruciferous vegetables (broccoli, cabbage, sprouts, …)
   - ☐ Hülsenfrüchte (Erbsen, Linsen, …) / Legumes (peas, lentils, …)
   - ☐ Stärkehaltiges Gemüse (Kartoffel, Süßkartoffel, …) / Starchy vegetables (potato, sweet potato, …)
   - ☐ Sonstiges Gemüse (Tomate, Paprika, Sellerie, Melanzani, Zucchini, …) / Other vegetables (tomatoes, bell pepper, eggplant, zucchini, …)

3. Wie oft essen Sie Obst (eine Portion = ca. eine Handvoll)? / How often do you eat fruit (one portion = approx. one handful)?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ 4-6x pro Woche / 4-6x per week
   - ☐ Täglich / Daily
   - ☐ Mehrmals täglich / Several times per day

4. Welche Obstsorten essen Sie bevorzugt? / Which type of fruit do you prefer?
   - ☐ Beerenobst (Erdbeere, Brombeere, Himbeere, ...) / Berries (Strawberry, blackberry, raspberry, …)
   - ☐ Steinobst (Kirsche, Pflaumen, Nektarine, Pfirsich, ...) / Stone fruit (cherry, plum, nectarine, peach, …)
   - ☐ Kernobst (Apfel, Birne, Quitte, …) / Pome fruit (apple, pear, quince, …)

5. Wie oft in der Woche konsumieren Sie Vollkornprodukte und Samen (=Ballaststoffe)? / How many times per week do you consume whole grain products and seeds (=fiber)?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1-3x pro Woche / 1-3x per week
   - ☐ 4-6x pro Woche / 4-6x per week
   - ☐ Täglich/mehrmals täglich / Daily/several times per day

6. An wie vielen Tagen in der Woche konsumieren Sie Milchprodukte? / How many times per week do you consume dairy products?

   tierische (inkl. Schaf- und Ziegenmilchprodukte) / **animal products** (incl. sheep and goat milk products)
   - ☐ Nie / Never
   - ☐ Gelegentlich bis jeden zweiten Tag / Occasionally to every other day
   - ☐ Jeden Tag / Every day
   - ☐ Mehrmals am Tag / Several times per day

   pflanzliche / plant-based
   - ☐ Nie / Never
   - ☐ Gelegentlich bis jeden zweiten Tag / Occasionally to every other day
   - ☐ Jeden Tag / Every day
   - ☐ Mehrmals am Tag / Several times per day

7. Wie oft in der Woche konsumieren Sie fermentierte Produkte (z.B. Sauerkraut, Tempeh, Miso, (Soja-)Joghurt, …)? / How many times per week do you consume fermented products (e.g. sauerkraut, tempeh, miso, (soy-)yogurt, …)?
   - ☐ Nie / Never
   - ☐ Gelegentlich bis jeden zweiten Tag / Occasionally to every other day
   - ☐ Jeden Tag / Every day
   - ☐ Mehrmals am Tag / Several times per day

8. Wie oft in der Woche essen Sie Fisch? / How many times per week do you eat fish?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1x pro Woche / 1x per week
   - ☐ 2-3x pro Woche / 2-3x per week
   - ☐ 4x und mehr pro Woche / 4x and more per week

9. Wie oft in der Woche essen Sie Fleisch? / How many times per week do you eat meat?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1-3x pro Woche / 1-3x per week
   - ☐ 4-6x pro Woche / 4-6x per week
   - ☐ Täglich/mehrmals täglich / Daily/several times per day

10. An wie vielen Tagen essen Sie verarbeitete Lebensmittel (Wurstwaren, Fast Food, Fertiggerichte, Fruchtjoghurt, abgepacktes Brot, …)? / How many times per week do you eat processed food (sausages, fast food, fruit yogurt, packaged bread, …)?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ Mehrmals in der Woche / Several times per week
   - ☐ Jeden Tag in der Woche / Every day of the week

11. Welche der angegebenen Süßigkeiten/Snacks konsumieren Sie öfter als **3 Mal** in der Woche? / Which of the listed sweets/snacks do you consume more than **3 times** per week?
   - ☐ Bonbons/Zuckerl / Sweets
   - ☐ Dunkle Schokolade / Dark chocolate
   - ☐ Milchschokolade/Pralinen / Milk chocolate
   - ☐ Kuchen/Gebäck/Kekse / Cakes/pastries/biscuits
   - ☐ Eis/Pudding/Cremes / Ice cream/puddings/creams
   - ☐ Gummibären/Gummisachen / Gummy bears
   - ☐ Chips/Popcorn/Gesalzene Nüsse / Crisps/popcorn/salted nuts
   - ☐ Müsli Riegel/Proteinriegel / Muesli bar/protein bar

12. Verwenden Sie täglich eines der folgenden Süßungsmittel (z.B. Honig im Tee, Zucker im Kaffee, …)? / Do you use any of the following sweeteners every day (e.g. honey in tea, sugar in coffee, …)?
   - ☐ Nein / No
   - ☐ Haushaltszucker / Regular sugar
   - ☐ Honig / Honey
   - ☐ Agaven Dicksaft / Agave syrup
   - ☐ Ahornsirup / Maple syrup
   - ☐ Kokosblütensirup/-zucker / Coconut syrup/sugar
   - ☐ Reissirup / Rice syrup
   - ☐ Stevia / Stevia
   - ☐ Birkenzucker (Xylit) / Birch sugar (xylitol)
   - ☐ Erythrit / Erythritol
   - ☐ Aspartam / Aspartame
   - ☐ Sonstige: / Other: _____

13. Nehmen Sie **regelmäßig** Nahrungsergänzungsmittel und/oder Präparate zu sich? / Do you take dietary supplements **regularly**?

Vitamine / Vitamins

- ☐ Multivitamin / Multivitamin
- ☐ Biotin / Biotin
- ☐ Folsäure / Folic acid
- ☐ Vitamin B12 / Vitamin B12
- ☐ Vitamin B3 / Vitamin B3
- ☐ Vitamin C / Vitamin C
- ☐ Vitamin D / Vitamin D
- ☐ Vitamin E / Vitamin E
- ☐ Sonstige: / Other: _____

Mineralstoffe / Minerals

- ☐ Eisen / Iron
- ☐ Kalzium / Calcium
- ☐ Magnesium / Magnesium
- ☐ Selen / Selenium
- ☐ Zink / Zinc
- ☐ Sonstige: / Other: _____

Lifestyle / Lifestyle

- ☐ Aminosäuren/Protein / Amino acids/proteins
- ☐ Ballaststoffe / Dietary fiber
- ☐ Omega-3 / Omega-3
- ☐ Grüntee Kapseln / Green tea capsules
- ☐ Knoblauch Präparate / Garlic powder
- ☐ L-Carnitin / L-carnitine
- ☐ Präbiotische Präparate / Prebiotics
- ☐ Probiotische Präparate / Probiotics
- ☐ Timeblock / Timeblock
- ☐ Sonstige: / Other: _____

Pflanzliches / Plant supplements

- ☐ Kurkuma / Turmeric
- ☐ Ingwer / Ginger
- ☐ Thymian / Thyme
- ☐ Salbei / Sage
- ☐ Erden/Algen / Soils/algae
- ☐ Zeolith / Zeolite

☐ Bentonit / Bentonite
☐ Chlorella / Chlorella
☐ Spirulina / Spirulina
☐ Sonstige: / Other: _____

14. Wie viel Flüssigkeit nehmen Sie pro Tag zu sich (alle Getränke)? / How much liquid do you drink per day (all drinks)?
    - ☐ Weniger als 1 Liter / Less than 1 liter
    - ☐ 1-2 Liter / 1-2 liters
    - ☐ 2-3 Liter / 2-3 liters
    - ☐ Mehr als 3 Liter / More than 3 liters

15. Welche Art(en) von Getränken nehmen Sie durchschnittlich pro Tag zu sich? / What type(s) of drinks do you consume on average per day?

	Nein / No	Ja / Yes	
Wasser / Water	☐	☐	ca. / approx. … L
Gezuckerte Getränke (Limonade, Saft, Sirup, …) / Sweetened drinks (lemonade, juice, syrup, …)	☐	☐	ca. / approx. … L
Light Getränke (Limonade Light, Sirup light, …) / Light Drinks (Light lemonade, syrup light, …)	☐	☐	ca. / approx. … L
Tee / Tea Wenn ja, welcher (Früchte, Grün/Schwarztee, Kräuter …)? / If yes, which one (fruit, green/black, herbal, …) _____	☐	☐	ca. / approx. … L
Kaffee / Coffee	☐	☐	ca. / approx. … L
Andere / Other Wenn ja, welche? / If yes, which one? _____	☐	☐	ca. / approx. … L

Sport & Stress / Sport & stress

4. Wie oft in der Woche sind Sie körperlich aktiv (Einkäufe zu Fuß, Spazieren, Gartenarbeiten etc.)? / How many times per week are you physically active (walks, gardening, shopping, …)?

- ☐ Selten bis Nie / Rarely to never
- ☐ 1x pro Woche / 1x per week
- ☐ 2-5x pro Woche / 2-5x per week
- ☐ 6-7x pro Woche / 6-7x per week

5. Wie oft in der Woche betreiben Sie Sport? / How many times per week do you exercise?
   - ☐ Selten bis Nie / Rarely to never
   - ☐ 1-2x pro Woche / 1-2x per week
   - ☐ mindestens 2x pro Woche / At least 2x per week

6. Wie viele Stunden in der Woche betreiben Sie: *(bitte runden Sie auf)* / How many hours per week do you engage in: *(please round up)*

   Ausdauersport / Endurance training … Std. pro Woche / Hours per week
   Kraftsport / Strength training … Std. pro Woche / Hours per week

7. Wie hoch schätzen Sie Ihren negativen Stress in Arbeit, Freizeit oder Familie rückblickend auf den letzten Monat ein? / Looking back over the last month, how much negative stress do you think you have experienced at work, in your free time or with your family?
   - ☐ Gering / Low
   - ☐ Mäßig / Moderate
   - ☐ Hoch / High
   - ☐ Sehr hoch / Very high

8. Wie oft trinken Sie Alkohol? / How often do you drink alcohol?

	Bier / Beer	Wein / Wine	Hochprozentiges / Liquors
Nie / Never	☐	☐	☐
Nur zu Anlässen / only on occasions	☐	☐	☐
1-3x pro Monat / 1-3x per month	☐	☐	☐
1-3x pro Woche / 1-3x per week	☐	☐	☐
4-6x pro Woche / 4-6x per week	☐	☐	☐
Täglich / Daily	☐	☐	☐

9. Rauchen Sie oder haben Sie früher Zigaretten geraucht? / Do you smoke or have you smoked cigarettes in the past?
   - ☐ Nein, ich rauche nicht und habe nie geraucht / No, I do not smoke and have never smoked
   - ☐ Früher (vor mehr als 10 Jahren) / In the past (more than 10 years ago)
   - ☐ Früher (vor weniger als 10 Jahren) / In the past (less than 10 years ago)
   - ☐ Ja, zurzeit weniger als eine Schachtel Zigaretten am Tag / Yes, currently less than one pack of cigarettes per day
   - ☐ Ja, zurzeit mehr als eine Schachtel Zigaretten am Tag / Yes, currently more than one pack of cigarettes per day

10. Wenn Sie keine Zigaretten rauchen/geraucht haben, verwenden Sie oder haben Sie früher eine andere Art von Tabak- oder Nikotinprodukten verwendet? / If you do not smoke/have not smoked cigarettes, do you use or have you previously used any other type of tobacco or nicotine product?

    - ☐ Nein / No
    - ☐ Ja, früher (vor mehr als 10 Jahren) / Yes, in the past (more than 10 years ago)
    - ☐ Ja, früher (vor weniger als 10 Jahren) / Yes, in the past (less than 10 years ago)
    - ☐ Ja, gelegentlich / Yes, occasionally
    - ☐ Ja, regelmäßig / Yes, regularly

    Wenn ja, welche? / If yes, which ones?

    - ☐ Zigarren / Cigars
    - ☐ E-Zigaretten / E-cigarettes
    - ☐ Vapes / Vapes
    - ☐ Shisha / Shisha
    - ☐ Snus / Snus
    - ☐ Kautabak / Chewing tobacco
    - ☐ Sonstige: / Other: _____

9.2. PROTOCOL: SAMPLE COLLECTION

*Anleitung: Probenentnahme Mundschleimhautzellen

1) Blood Card

- Die Proband:innen sollen ihr Blut auf der „**Five Spot Blood Card**" abgeben, da dieses für die Aging-Auswertung benötigt wird. Hier soll bitte darauf geachtet werden, dass **mindestens 4,** aber am besten alle der 5 Kreise mit dem Blut vollständig gefüllt sind
- Anschließend das Etikett (z.B. 001-BS) auf der Blood Card aufkleben

2) Vorgehensweise – Entnahme der Mundschleimhautzellen

1) Bei der Entnahme sollen Handschuhe getragen werden
2) Vor der Entnahme sollen die Proband:innen ihren Mund 30 Sekunden lang gründlich mit Wasser ausspülen
3) Stäbchen vorsichtig aus der Schutzhülle entnehmen.
   Die Schutzhülle aufbewahren.
4) Das Stäbchen gründlich mit Kreisbewegungen entlang der Wangeninnenseite 30 Sekunden lang reiben, um genug Mundschleimhautzellen zu gewinnen
   Diesen Vorgang pro Stäbchen (also insgesamt 3x) wiederholen:

   1. Stäbchen: Linke Wangeninnenseite

   2. Stäbchen: Rechte Wangeninnenseite

   3. Stäbchen: Beide Wangeninnenseiten (hier 15 Sekunden lang entlang der rechten Wangeninnenseite reiben, anschließend 15 Sekunden lang entlang der linken Wangeninnenseite)

   Die passenden Etiketten auf den Stäbchen anbringen *(z.B. 1. Stäbchen: 001-L; 2. Stäbchen: 001-R; 3. Stäbchen: 001-B).*
5) Stäbchen aufrecht in das Styropor stecken, sodass sie sich gegenseitig nicht berühren und für ca. **24h** gründlich an der Luft trocknen lassen.
6) Anschließend das Stäbchen zurück in die Schutzhülle stecken und mit den passenden Etiketten zukleben *(z.B. 001-L; 001-R; 001-B).*

Achtung: Bitte darauf achten, dass die Stäbchen vollkommen trocken sind, bevor sie in die Schutzhülle gesteckt werden!

7) Folgendes bitte postalisch zusenden:
   - ✓ Ausgefüllte Fragebogen
   - ✓ Unterzeichnete Einverständniserklärung
   - ✓ Stäbchen (jeweils 3 pro Person)
   - ✓ Blood Card

Adresse:
HealthBioCare GmbH
z.H. Hahnekamp/Samel
Nussdorferstraße 67
1090 Wien

**angelehnt an: Applied biosystems - Best Practices for Collection of Buccal Swabs for Genotyping Experiments*

9.3. PROTOCOL: DNA EXTRACTION

DNA/RNA Extraction 04.06.2024
Buccal Swabs

Instruments

- ThermoMixer
- Centrifuge
- Vortexer
- KingFisher DUO
- Pipettes
- Scissors
- Tweezers
- Bunsenbrenner/Kerze

Consumables

- 96 deep-well plates
- 12-tip comb
- 5ml Tubes
- 15ml Falcon Tubes
- 1.5 ml tubes
- pipette tips
- 5ml Stepper Syringes
- 10ml Stepper Syringes
- 96% ethanol
- 80% ethanol
- Isopropanol

Kits

- MagMAX FFPE DNA/RNA Ultra Kit

Before first use of the kit prepare Wash Solutions from the concentrates
• Add 46 mL of isopropanol to RNA Wash Solution Buffer Concentrate, mix, and store at room temperature.
• Add 168 mL of ethanol to Wash Solution 2 Concentrate, mix, and store at room temperature.

Day 1: Blood spots punching/stamping and incubation

- Prepare Protease Solution

Component	Volume per well	12 Rxns[1]
Protease	10 µl	130 µl
Protease Digestion Buffer	225 µl	2925 µl
Total Protease Solution	235 µl	3055 µl

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- Excise **4 x 4 mm Ø punches** from each patient dried blood spots, place them in a labeled 1.5 ml tube with the help of a metal rod and tweezers.
- Immerse the **puncher, metal rod and tweezers in ethanol** and hold it over the flame for 2 seconds between each sample.
- Add **235 µl protease Buffer** to each tube. Be sure that the liquid is covering the sample.
- Incubate the tubes on a ThermoMixer at 55°C over-night at 850 rpm.

Buccal Swabs incubation

- Prepare Protease Solution (make sure to include some overage)

Component	**Volume per well**	**12 Rxns** + 1 Reserve
Protease	20 µl	260 µl
Protease Digestion Buffer	450 µl	5850 µl
Total Protease Solution	470 µl	6110 µl

- Cut the head of the swab brushes from each patient and place them in a labeled 1.5 ml tube
- Immerse the **scissors and tweezers (if needed) in ethanol** and hold it over the flame for 2 seconds between each sample.
- Add **470 µl protease Buffer** to each tube. Be sure that the liquid is covering the sample.
- Incubate the tubes on a ThermoMixer at 55°C over-night at 850 rpm.

Day 2: Purification of nucleic acids

- Prepare the following solutions for the number of samples you have.

Storage of components	
- 20°C	Proteinase, DNase, DNase Buffer
4°C	Nucleic Acid Binding Beads
RT	All other components
Buffer 12 Rxns[1]	[1] Volumes include 10% overage

- Prepare **DNA Binding Buffer**

Component	**Volume per well**	**12 Rxns[1]**
Binding Solution	250 µl	3250 µl
Binding Beads	20 µl	260 µl
Total DNA Binding Buffer	270 µl	3510 µl

- Prepare **DNAse Solution**

Component	**Volume per well**	**12 Rxns[1]**
DNase	20 µl	260 µl
DNase Buffer	10 µl	130 µl
Nuclease-free Water	70 µl	910 µl
Total DNase Solution	100 µl	1300 µl

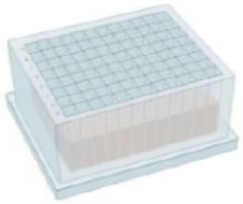
- Prepare **RNA Binding Buffer**

Component	Volume per well	12 Rxns[1]
Binding Solution	150 µl	1950 µl
Isopropanol	500 µl	6500 µl
Total RNA Binding Buffer	650 µl	8450 µl

- Prepare **RNA Rebinding Buffer**

Component	Volume per well	12 Rxns[1]
Binding Solution	200 µl	2600 µl
Isopropanol	250 µl	3250 µl
Total RNA Rebinding Buffer	450 µl	5850 µl

- Set up the processing plate **Label both plates with either "DNA" or "RNA"** on the side wall.
- Fill them according to the following sceme and mostly use the stepper using the appropriate syringes.



DNA plate setup

Row ID	Plate row	Reagent	Vol per well
Sample [1]	A	DNA Binding Buffer	270 µl
DNA Wash Buffer 1	B	DNA Wash Buffer	400 µl
DNA Wash Buffer 2	C	DNA Wash Buffer	400 µl
Wash Solution 2 - 1	D	Wash Solution 2	500 µl
Wash Solution 2 - 2	E	Wash Solution 2	500 µl
Tip Comb	F	Place a KingFisher™ Duo 12-Tip Comb	
Empty	G	Empty	
Elution	H	Elution Solution	50 µl

RNA plate setup

Row ID	Plate row	Reagent	Vol per well
DNase [1]	A	DNase Solution	100 µl
RNA Wash Buffer 1	B	RNA Wash Buffer	500 µl
RNA Wash Buffer 2	C	RNA Wash Buffer	500 µl
Wash Solution 2 – 1	D	Wash Solution 2	1000 µl
Wash Solution 2 – 2	E	Wash Solution 2	1000 µl
Empty	F	Empty	
Empty	G	Empty	
Elution	H	Elution Solution	50 µl

Do not use the 1000µl Pipette for this Step

[1] The instrument prompts the user to add 450 µL of RNA Rebinding Buffer to the DNase Row after the DNase treatment step.

- Ensure that the instrument is set up for processing with the deep-well magnetic head and select the "**DBS RNA and DNA extraction**" program on the instrument.
- Change magnetic head and holder if necessary
- When the **DNA and RNA plates** are completely prepared, centrifuge and squeeze out the punched paper with a 1000 µl pipette tip and add up to **200 µl** of sample to each well in Row A of the DNA plate.
- Do not forget the **KingFisher™ Duo 12-Tip Comb**
- Start the run and load the prepared processing plates when prompted by the instrument.
- When prompted by the instrument (approximately 15-20 minutes after initial start):
  Remove the **DNA plate** from the instrument.
  - Add 20 µl of **Nucleic Acid Binding Beads** to each sample well in **Row A**
  - Add 650 µl of **RNA Binding Buffer** to each sample in **Row A**
  - Load the DNA plate back to the instrument, then press **Start**
- When prompted by the instrument (approximately 50–55 minutes after initial start):
  Remove the **RNA plate** from the instrument.
  - Add 450 µl of **RNA Rebinding Buffer** to each sample in **Row A** (vortex !)
  - Load the RNA plate back to the instrument, then press **Start**
- Prepare Autoclaved 1,5ml Tubes for the DNA as well as the RNA samples. Label them with sample Nr, date and DNA/RNA
- Add 100µl of Nuclease free water to the prepared DNA tubes.
- At the end of the run, remove the two plates from the instrument and transfer the **eluted DNA (Row H of the DNA plate**) and the eluted **RNA (Row H of RNA plate)** to RNase free tubes. The purified DNA and RNA is ready for immediate use.
- Store at –20°C for long-term storage.
- Discard the liquid from the DNA and RNA plates as well as the deep well plates.

9.4. PROTOCOL: BISULFITE CONVERSION

SOP-LAB-002-01 28.03.22
Bisulfite conversion

Instruments

- SimpliAmp Endpoint PCR
- Centrifuge
- Vortexer
- Pipettes
- Thermomixer

Consumables

- 1.5 ml tubes
- 0.2 ml pcr 8 well tube strips and caps
- pipette tips
- 96% ethanol
- nfw

Kits

- EpiTect Bisulfit Kit Qiagen (ID: 59104)

Before start

- Which samples need a HRM analysis? (check Probenliste: Aging, Metabo, Kombi, Prevention, Intervention...)
- **Label the 8 well tube strips**: note on the cap the beginning and the end of the strip and write in the lab book the order in which samples are pipetted in the strip.
- Before starting dissolve the necessary **Bisulfite Mixes** with 800µl **NFW** each. To remove all precipitation the solution can be heated to 56°C and vortexed intensively. Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions (incl. surplus). If converting fewer than 8 DNA samples or there are leftovers, dissolved Bisulfite Mix can be stored at –30°C to –15°C for up to 4 weeks without any loss of performance.
- Before first use add **30 ml ethanol** (96–100%) to **Buffer BW** and store at room temperature (15–25°C). Invert the bottle several times before starting the procedure.
- Before first use add **27 ml ethanol** (96–100%) to **Buffer BD** and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the EpiTect spin column.

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Procedure

Day 1: Bisulfite conversion

1. Thaw DNA to be used in the bisulfite reactions.

2. Prepare the bisulfite reactions in 200 µl PCR 8-tubes-strips according to table. Add each component in the order listed. Note:

3. Mix each reaction by pipetting up and down.

Component	Volume per reaction
gDNA	20 µl
Bisulfite Mix (dissolved)	85 µl
DNA Protect Buffer	35 µl
Total volume	140 µl

Tip: Mix Bisulfite Mix and DNA Protect Buffer for all reactions in a separate tube beforehand and pipette this into the 8-tubes-strips. Then pipette the samples onto it and mix with the pipette.

4. Perform the **bisulfite DNA conversion** using a thermal cycler. Program the thermal cycler according to Table 3. The complete cycle should take approximately 5 h.

5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. (Note the balance - if only one 8-tubes-strip is used, use a 2nd empty strip to balance.) Start the thermal cycling incubation.

Table 3. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite†	20°C

† Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Day 2: Cleanup of bisulfite converted DNA

- Before first use add **310 µl RNase-free water to the lyophilized carrier RNA** (310 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing. Store at –30°C to –15°C until use. Aliquots can be stored for up to 1 year.
- Add **dissolved carrier RNA to Buffer BL**. Calculate the volume of Buffer BL and dissolved carrier RNA required for the number of samples to be processed. If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.

Table 1. Carrier RNA and Buffer BL volumes

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution†	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

* The volumes given contain a 10% surplus for pipetting inaccuracies.
† Resulting in a final concentration of 10 µg/ml carrier RNA in Buffer BL.

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to **clean labeled 1.5 ml** microcentrifuge tubes. Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. Add **560 µl freshly prepared Buffer BL** containing 10 µg/ml carrier RNA to each sample. Mix the solutions by vortexing and then centrifuge briefly.

8. Place the necessary number of spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding **labeled spin column**.

9. **Centrifuge** the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

10. Add **500 µl Buffer BW** to each spin column, and **centrifuge** at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

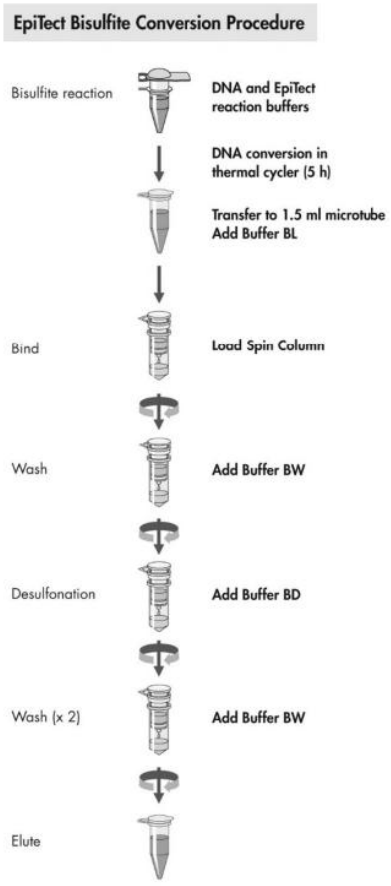
11. **Add 500 µl Buffer BD (fridge)** to each spin column and **incubate for 15 min** at room temperature (15–25°C). If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

IMPORTANT: The bottle containing Buffer BD should be **closed** immediately after use to avoid acidification from carbon dioxide in the air. Note: It is important to **close the lids** of the spin columns before incubation.

12. **Centrifuge** the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

13. **Add 500 µl Buffer BW** to each spin column and **centrifuge** at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

14. Repeat step 13 once.



15. Place the spin columns into new **2 ml collection** tubes and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

16. Recommended: Place the spin columns with open lids into **clean labeled 1.5 ml microcentrifuge** tubes and incubate the spin columns for **5 min at 56°C in a heating block**. This step enables evaporation of any remaining liquid.

17. Dispense **20 µl Buffer EB** onto the center of each membrane. Elute the purified DNA by **centrifugation** for 1 min at approximately 15,000 x g (12,000 rpm).

Note: If the purified DNA is to be stored for up to 24 h, we recommend storage at 2–8°C. For storage longer than 24 h, we recommend storage at –30°C to –15°C.

9.5. PROTOCOL: HRM

SOP-LAB-005-01 28.03.22
HRM Analysis

Instruments

- Rotor-Gene Q qPCR
- centrifuge
- vortexer
- pipettes
- PCR tubes loading rack

Consumables

- 4 well pcr strips + caps
- 1.5 ml tubes
- pipette tips

Reagents

- HRM methylation standards 0% and 100% stocks: different mixtures for each gene
- MasterMixes: different for each gene
- forward and reverse primers for each gene (Stock 100pmol/µl)
- bisulfite converted DNA (samples)
- bisulfite converted UM DNA (plate-to-plate control)
- nfw

Before start

- **Which samples** need which HRM analyses? (check Probenliste: Aging, Metabo, Kombi, Prevention, Intervention, Bluezones...)
- Turn on **laptop and the Rotor-Gene** instrument
- Prepare **pipetting layout** and note where each sample is to be pipette

1	S0	9	S100	17	Nr2	25		33		41		49		57		65	
2	S0	10	S100	18	Nr2	26		34		42		50		58		66	
3	S25	11	UM	19	Nr3	27		35		43		51		59		67	
4	S25	12	UM	20	Nr3	28		36		44		52		60		68	
5	S50	13	NFW	21	Nr4	29		37		45		53		61		69	
6	S50	14	NFW	22	Nr4	30		38		46		54		62		70	
7	S75	15	Nr1	23	Nr5	31		39		47		55		63		71	
8	S75	16	Nr1	24	Nr5	32		40		48		56		64		72	

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➢ Prepare **Methylation standards** from 0% and 100% stocks with final concentration 10ng/µl
Example: 1µl 0% (10ng/ µl) + 1µl 100% (10ng/ µl) = 2µl 50% (10ng/ µl)

Gene	MasterMix	Methylation Standards	Primer sequences
TNFα	(BIOZYM – white cap) HRM Mix (BIORAD as soon as Biozym is used up)	0, 7.5, 15, 25, 50 %	fw: 5'-ttt tgg aaa gga tat tat gag tat tga-3' rev: 5'-cta aaa ccc taa aac ccc cct at-3'
IL-6	(BIORAD – yellow cap) Precision Melt Supermix for HRM Analysis	0, 25, 50, 75, 100 %	fw: 5'-tta tgt agg aaa gag aat ttg gtt tag-3' rev: 5'-aaa aaa taa aat cat cca ttc ttc ac-3'

- Standards, and samples are analyzed in **duplicates** plate-to-plate control, no template control in one copy
- Prepare sufficient amount of **Master Mix** according to the table in 1.5 ml tubes

1 Rxn µl	TNFα	IL-6	
MM	5	5	
fw primer	0.1	0.1	← 1:10 dilution
rev primer	0.1	0.1	← dilution + pre-mix
Nfw	3.8	3.8	
Total volume	9 µl	9µl	

- Prepare enough 4 well pcr strips and caps and insert them into the **cooling block**
- Pipette **9 µl of the prepared Master Mix** to the well. Use reverse pipetting and the same pipette tip
- **Add 1 µl** of each standard, plate-to-plate control, no template control, and samples according to pipetting layout in duplicates. Use new pipette tip each time
- Put the **caps** on the strips
- Open the Rotor-Gene instrument, take the **metal ring out** and place the tubes on the rotor disc.
  Note the **numbering** on the disc and place the tubes according to pipetting layout.
  Important: **Fill the rotor disc completely** with tubes, leave no empty positions!
- Put the **metal ring back on**. Close the instrument
- Start program and **design run** (Cycling conditions...)

Gene	TNF/IL-6	
precycling	10min 95°C	
cycling	15sec 95°C	x 45 cycles
	60sec 60°C	
	-	
Hold 1	1min 95°C	
Hold 2	1min 45°C	
HRM	65°C-80°C with 2sec and 0,1°C per step, 90sec wait pre first step, gain optimization at 70	

- Or use the run profile in the quick start tab
- Select the 72-well rotor and confirm that the locking ring is attached then go to the next page
- Start the run and save it on the desktop in the appropriate folder with the date and the sample numbers. Then you can label the standards, plate-to-plate control, the ntc, and the samples in the program.
- After the run has finished – save it, copy it on a **USB drive** and upload it to the **server**: