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Effects of nutrition and lifestyle on miRNA markers in the aspect of oral health

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1 List of Abbreviations

BMI ..... *Body mass index*
cDNA ..... *Complementary DNA*
CVD ..... *Cardiovascular disease*
DNA ..... *Deoxyribonucleic acid*
GCF ..... *Gingival crevicular fluid*
GERD ..... *Gastroesophageal reflux disease*
hDPCs ..... *Human dental pulp cells*
IL ..... *Interleukin*
IRAK1 ..... *Interleukin-1 receptor-associated kinase 1*
LPS ..... *Lipopolysaccharides*
MAPK ..... *Mitogen-activated protein kinase*
miRNA ..... *MicroRNA*
mRNA ..... *Messenger ribonucleic acid*
NAFLD ..... *Non-alcoholic fatty liver disease*
NCD ..... *Noncommunicable diseases*
NFW ..... *Nuclease-free water*
NF-κB ..... *Nuclear factor kappa B*
omni ..... Omnivore
OPMD ..... Oculopharyngeal muscular dystrophy
OSCC ..... *Oral squamous cell carcinoma*
P. gingivalis ..... *Porphyromonas gingivalis*
PER1 ..... *Period circadian protein homolog 1*
pre-miRNAs ..... *Precursor microRNA*
pri-miRNA ..... *Primary microRNA*
PTEN ..... *Phosphatase and tensin homolog*
qPCR ..... *Real-time polymerase chain reaction*
RNA ..... *Ribonucleic acid*
RT-PCR ..... *Reverse transcription polymerase chain reaction*
TNF ..... *Tumor necrosis factor*
TRAF ..... *Tumor necrosis factor receptor*
UTR ..... *Untranslated regions*
veg ..... Vegetarian

2 Abstract

Nutrition and lifestyle are well-established factors influencing health and disease. MicroRNAs (miRNAs) function as post-transcriptional regulators of gene expression and have emerged as potential biomarkers for various pathological conditions. Moreover, growing evidence suggests that diet and lifestyle can modulate miRNA expression patterns, offering potential pathways for disease prevention and health promotion.

This study examined the relationship between dietary patterns, lifestyle factors, and miRNA expression profiles, with a specific focus on oral health. Oral diseases affect billions of people worldwide and represent a major public health challenge.
Through a comprehensive literature review, six miRNAs linked to oral disease were identified: miR-21, miR-24, miR-142, miR-146a, miR-125b, and miR-30. These miRNAs are thought to play mechanistic roles in disease pathogenesis by modulating immune responses, inflammatory signaling pathways, and tissue remodeling processes. MiRNA expression levels were analyzed in capillary blood and oral mucosa samples from 40 healthy adults following either a vegetarian or omnivorous diet.

Analysis revealed significant differences in miRNA expression between capillary blood and oral mucosa, highlighting tissue-specific miRNA profiles. Moreover, distinct expression patterns were found between dietary groups. Specifically, vegetarians exhibited higher expression of miR-21 and miR-142 in capillary blood, while lower expression of miR-146a was observed in oral mucosa compared to omnivores. These findings suggest that dietary patterns can influence miRNAs involved in regulatory processes with potential consequences for oral health.

Overall, the results support the concept that nutrition may affect oral health through miRNA-mediated mechanisms. Further research in larger and more diverse populations is needed to confirm these results and clarify their relevance for preventive strategies and biomarker development.

3 Zusammenfassung

Ernährung und Lebensstil sind anerkannte Faktoren, die sich auf Gesundheit und Krankheit auswirken. MikroRNAs (miRNAs) fungieren als posttranskriptionelle Regulatoren der Genexpression und haben sich als potenzielle Biomarker für verschiedene pathologische Zustände erwiesen. Außerdem deutet zunehmende Evidenz darauf hin, dass Ernährung und Lebensstil die Expression von miRNAs beeinflussen können und damit neue Ansätze für die Krankheitsprävention und Gesundheitsförderung eröffnen.

Diese Studie untersuchte den Zusammenhang zwischen Ernährungsgewohnheiten, Lebensstilfaktoren und der Expression von miRNAs, mit einem besonderen Fokus auf Mundgesundheit. Orale Erkrankungen betreffen weltweit Milliarden von Menschen und stellen somit eine große Herausforderung für die öffentliche Gesundheit dar.
Durch eine umfassende Literaturrecherche wurden sechs miRNAs, die mit oralen Erkrankungen in Verbindung stehen, identifiziert: miR-21, miR-24, miR-142, miR-146a, miR-125b und miR-30. Es wird angenommen, dass diese miRNAs bei der Pathogenese von Krankheiten eine Rolle spielen, indem sie Immunreaktionen sowie Entzündungs- und Gewebeumbauprozesse beeinflussen. Die Expression dieser miRNAs wurde in Kapillarblut- und Mundschleimhautproben von 40 gesunden Erwachsenen mit vegetarischer oder omnivorer Ernährung analysiert.

Die Analyse zeigte signifikante Unterschiede in der miRNA-Expression zwischen Kapillarblut und Mundschleimhaut, was die gewebespezifische Natur von miRNA-Profilen unterstreicht. Darüber hinaus wurden unterschiedliche Expressionsmuster zwischen den verschiedenen Ernährungsgruppen festgestellt. So zeigten Vegetarier eine höhere Expression von miR-21 und miR-142 im Kapillarblut, während in der Mundschleimhaut eine geringere Expression von miR-146a im Vergleich zu Omnivoren festgestellt wurde. Diese Ergebnisse deuten darauf hin, dass Ernährungsgewohnheiten die Expression von miRNAs beeinflussen können, die an regulatorischen Prozessen mit potenziellen Auswirkungen auf die Mundgesundheit beteiligt sind.

Insgesamt bestätigen die Ergebnisse das Konzept, dass Ernährung die Mundgesundheit über miRNA-vermittelte Mechanismen beeinflussen kann. Weitere Studien mit größeren Kohorten sind erforderlich, um diese Ergebnisse zu belegen und ihre Bedeutung für Präventionsstrategien und die Entwicklung von Biomarkern zu klären.

4 Introduction

4.1 Oral Health

Oral diseases represent a significant health burden worldwide, affecting individuals across all age groups and causing pain, discomfort, disfigurement, and even mortality. The most common conditions include dental caries, periodontal diseases, tooth loss, and oral cancers. It is estimated that nearly 3.5 billion people worldwide are affected by oral diseases and the prevalence of these conditions continues to rise. Interestingly, many oral diseases share modifiable risk factors with other noncommunicable diseases (NCDs), such as high sugar consumption, tobacco and alcohol use, poor hygiene practices, and their wider social and commercial determinants (World Health Organization, 2024).

Oral health plays a crucial role in overall health and well-being. Research has consistently shown that oral health is not only about maintaining healthy teeth but also serves as a foundation for general health. Numerous clinical findings revealed connections between oral diseases and systemic complications such as insulin resistance, cardiovascular disorders, and neurodegenerative conditions. These findings suggest that periodontal disease could have serious systemic effects by spreading pathogenic bacteria through the bloodstream and also through the negative inflammation role, therefore negatively affecting the whole body (Fiorillo, 2019). Periodontitis, for example, is a chronic infection of the tissues supporting the teeth. Studies showed that individuals with chronic periodontitis, as well as those with other chronic conditions, exhibit elevated levels of inflammatory cytokines, underscoring a potential connection between periodontal and systemic health (Fan et al., 2025). Also, the prevalence of periodontitis in diabetic subject is double or triple compared to non-diabetic subjects. Additionally, controlling diabetes is more complicated for patients when they suffer periodontal disease. Patients suffering from periodontitis and diabetes simultaneously are exposed to developing complications of cardiovascular, renal, and retinopathic diseases. Furthermore, periodontal pathogens have been detected in the brain tissue of Alzheimer's patients, suggesting a potential link between oral health and neurodegenerative diseases. Also important to mention are correlations of oral alterations associated with rare diseases. In fact, some authors report

that some pathologies such as Von Willebrand disease, acrodermatitis enteropathica, Chediak-Higashi syndrome, and others have important correlations with oral health (Fiorillo, 2019).

The demonstrated ability of periodontal treatment to reduce both oral and systemic inflammation highlights the vital connection between oral health and overall health (Fan et al., 2025). Enhancing oral health has significant systemic implications, contributing to the prevention of various diseases, improving individual quality of life, and therefore benefiting society (Fiorillo, 2019).

4.2 miRNAs

Epigenetics involves gene expression changes without alterations to the underlying DNA sequence. Mechanisms such as DNA methylation, chromatin remodeling, histone modifications, and non-coding RNAs like miRNAs allow cells to fine-tune gene activation and protein production. Over 2,500 miRNAs have been identified in the human genome, highlighting miRNAs as key modulators of cellular behavior and emphasizing their role in cellular development and disease regulation (Cosín-Villanueva et al., 2024; Micó-Martínez et al., 2021).

MicroRNAs (miRNAs) are small, non-coding RNA molecules approximately 19–24 nucleotides in length that act as epigenetic regulators of gene expression. They achieve this by binding to the 3′-untranslated regions (UTRs) of target mRNAs, leading to either mRNA degradation or translational inhibition. Notably, a single miRNA can influence multiple genes, and each gene can be regulated by various miRNAs (Asa'Ad et al., 2020; Cosín-Villanueva et al., 2024; Palideh et al., 2023).

MiRNAs are transcribed by RNA polymerase II as loop-shaped precursors, known as pri-miRNAs, which then convert into pre-miRNAs. These pre-miRNAs are transported out of the nucleus and are further modified to form mature miRNAs (Palideh et al., 2023).

As post-transcriptional regulators, miRNAs play critical roles in a wide array of biological processes, including cell growth, apoptosis, differentiation, and inflammatory responses.

Dysregulation of miRNAs has been linked to the pathophysiology of numerous diseases, such as cancer, coronary heart disease, diabetes, autoimmune inflammatory conditions (e.g., rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis), and also oral disease. These diseases often share low-grade inflammation, implicating the role of miRNAs in inflammatory processes and systemic health (Asa'Ad et al., 2020; Cosín-Villanueva et al., 2024; Micó-Martínez et al., 2021).

The stability of miRNAs in extracellular biofluids, such as blood, saliva, urine, and cerebrospinal fluid, makes them promising biomarkers for disease diagnosis and prognosis. This, coupled with their involvement in gene expression, positions miRNAs as valuable tools for understanding and managing various conditions. As a result, exploring the role of miRNAs offers critical insights into systemic diseases (Cosín-Villanueva et al., 2024; Micó-Martínez et al., 2021).

4.3 miRNAs and oral health

Recent studies have highlighted the role of microRNAs (miRNAs) in the context of oral health (Asa'Ad et al., 2020). One of the main reasons why miRNAs are so important for oral health is their regulatory function within the immune system. MiRNAs influence a wide range of immunological processes, such as the differentiation of immune progenitor cells, cytokine production, and signaling pathways involved in inflammatory responses. In periodontal tissues, particularly in periodontal ligament cells, miRNAs contribute to the immune responses involved in periodontal disease. They affect both innate and humoral immune responses, shaping the host's defense against bacterial challenges associated with periodontal disease (Micó-Martínez et al., 2021; Palideh et al., 2023). For example, diseases such as periodontitis and peri-implantitis are biofilm-induced conditions, which affect the tissues supporting teeth and implants and are associated with Gram-negative, anaerobic, and microaerophilic bacteria that colonize subgingival tissues. This bacterial colonization triggers an inflammatory host response, influenced by environmental, genetic, and epigenetic factors, including miRNAs. One of the key periodontal pathogens, Porphyromonas gingivalis, interacts with miRNAs to influence endotoxin tolerance through the MAPK signaling pathway. This interaction enhances toll-like receptor

sensitivity to bacterial lipopolysaccharides (LPS) and targets the NF-κB signaling pathway (Asa'Ad et al., 2020).

In addition to their role in immune responses, miRNAs are significant regulators of bone homeostasis. They influence osteoclastogenesis and the osteogenic differentiation of stem cells, suggesting their involvement in alveolar bone resorption, a hallmark of periodontitis and peri-implantitis. Alterations in miRNA expression levels have been observed in these conditions, though their precise contribution to pathogenesis is still being elucidated. Nevertheless, their stability and detectability in oral fluids, such as saliva and gingival crevicular fluid, position miRNAs as promising non-invasive biomarkers for diagnosing, monitoring and managing oral diseases (Asa'Ad et al., 2020; Micó-Martínez et al., 2021).

The following section introduces key microRNAs associated with oral health, each briefly described and selected for deeper analysis in the present study, with an overview summarized in Table 1.

4.3.1 miR-21

MiR-21 has been associated with various oral health conditions, particularly oral squamous cell carcinoma (OSCC) and periodontitis. Research indicates that miR-21 is involved in diverse biological processes, including inflammation, tumor progression, and tissue homeostasis, making it a promising candidate for both diagnostic and therapeutic applications (Aghiorghiesei et al., 2022; Zhou et al., 2018).

Several studies have highlighted the dysregulation of miR-21 in OSCC. Elevated levels of miR-21 have been consistently identified in tumor tissues and body fluids, such as saliva and serum, of OSCC patients. For instance, Yap et al. (2019) developed a predictive test using miR-21 and other miRNAs to identify OSCC with high accuracy from oral swirls. Similarly, Aghiorghiesei et al. (2022) demonstrated that miR-21 was upregulated in OSCC tissues and exhibited strong statistical performance in differentiating tumor tissues from healthy mucosa. Furthermore, high miR-21 expression levels have been linked to lymph node metastasis and tumor depth, emphasizing its role as a potential prognostic marker.

MiR-21 appears to promote OSCC progression by targeting tumor suppressor genes like PTEN, thereby enhancing cell proliferation. Sinha et al. (2023) observed that circulating miR-21 levels were significantly higher in OSCC patients across clinical stages I-IV, further supporting its use as a non-invasive biomarker.

In periodontitis, miR-21 seems to play a role by modulating inflammation. Zhou et al. (2018) demonstrated that miR-21 expression is upregulated in macrophages stimulated by P. gingivalis lipopolysaccharides (LPS). In this context, miR-21 acts as an anti-inflammatory regulator by inhibiting pro-inflammatory cytokine production and suppressing the activation of NF-κB. Animal studies corroborated these findings, as miR-21-deficient mice exhibited exacerbated gingival inflammation and alveolar bone loss in a periodontitis model. Additionally, miR-21 has been proposed as a potential biomarker for both periodontitis and systemic conditions like cardiovascular disease (CVD). Isola et al. (2023) highlighted that miR-21 profile in gingival crevicular fluid (GCF) differs in patients with periodontitis and CVD.

4.3.2 miR-24

MiR-24 seems to have regulatory effects on inflammation (Luan et al., 2018) and cell proliferation (Safari et al., 2023). In periodontal disease, miR-24 is consistently upregulated (Asa'Ad et al., 2020; Luan et al., 2018; Safari et al., 2023). Notably, miR-24 has demonstrated an anti-inflammatory function by suppressing the production of pro-inflammatory cytokines, such as TNF-α, IL-12p40, and IL-6, upon challenge with bacterial lipopolysaccharides (LPS). This highlights the role of miR-24 in counteracting inflammation and limiting inflammation-related tissue destruction (Luan et al., 2018).

In OSCC, the overexpression of miR-24 was associated with enhanced tumor progression, probably through its ability to downregulate PER1, an anti-proliferative protein involved in cell cycle regulation. By suppressing PER1, miR-24 promotes tumor cell proliferation and contributes to the progression of OSCC. Clinical studies have also demonstrated the diagnostic potential of miR-24, with high accuracy in distinguishing OSCC patients from healthy individuals. These findings position miR-24 as a promising biomarker for early detection and monitoring of OSCC (Safari et al., 2023).

4.3.3 miR-142

MiR-142 has been identified as a biomarker in periodontitis-related studies, supported by both microarray and RT-PCR data (Asa'Ad et al., 2020). In smokers with periodontitis and gingivitis, miR-142 expression was notably elevated, suggesting a link between miR-142 and inflammation associated with periodontal diseases (Öngöz Dede et al., 2023).

In peri-implantitis diseases, miR-142 exhibited a different expression pattern, being significantly downregulated in experimental models. Wu et al. (2021) demonstrated that miR-142 influenced the onset, progression, and treatment of peri-implantitis. This was particularly evident in their modulation of target genes involved in the MAPK signaling pathway (Asa'Ad et al., 2020). In OSCC, miR-142 has been implicated in tumorigenesis. Overexpression of miR-142 targeted PTEN which resulted in enhanced proliferation and cell invasion (Sinha et al., 2023)

4.3.4 miR-125b

Studies suggest an association between miR-125b and periodontitis (Xie et al., 2022; Zhu & Zhong, 2022). Data showed a link between overexpression of miR-125b and periodontitis development and progression, indicating diagnostic values (Zhu & Zhong, 2022). Zhu and Zhong (2022) demonstrated that proinflammatory cytokines (IL-1β, IL-6, and TNF-α) found higher in patients with periodontitis correlated positively with miR-125b expression. Luan et al. (2018) confirmed these findings as they also found upregulation of mir-125b in periodontitis patients. They suggest an involvement in periodontal disease through a mechanism where miR-125b sustains proinflammatory macrophage activation.

4.3.5 miR-146a

MiR-146a plays a central role in regulating inflammation and immune responses in oral health. It has been linked to conditions such as periodontitis (Asa'Ad et al., 2020) and pulp inflammation (Muñoz-Carrillo et al., 2021). For example, it has been shown that miR-146a is significantly upregulated in the gingival tissues and saliva of patients with periodontitis, correlating with disease severity (Safari et al., 2023). It regulates inflammation by suppressing the NF-κB signaling pathway, targeting key adaptors like TRAF6 and IRAK1 to reduce pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Its overexpression in

human dental pulp cells (hDPCs) upon bacterial stimulation seems to enhance cell proliferation, odontogenic differentiation, and regeneration. This helps control inflammation and tissue damage (Muñoz-Carrillo et al., 2021).

4.3.6 miR-30

In the context of oral health it has been shown that miR-30 plays a role in modulating inflammation and tissue destruction (Luan et al., 2018). Studies have shown that miR-30 expression is upregulated in periodontal disease, this has been observed in both mouse models and human studies. Its overexpression correlates with levels of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Luan et al., 2018; Zhu & Zhong, 2022) and was associated with the development and progression of periodontitis (Zhu & Zhong, 2022).

Table 1: Overview miRNA expression in disease.

miRNA	Disease	Expression	Tissue	Reference
miR-21	OSCC	↑	Oral swirl	Yap et al.
			tumor tissue	Aghiorghiesei et al.
			not stated	Sinha et al.
	Periodontitis	↑	Periodontal ligament tissues	Zhou et al.
	Periodontitis	↑	gingival crevicular fluid	Isola et al.
miR-24	Periodontal disease	↑	not stated	Asa'ad et al.
	OSCC	↑	saliva	Safari et al.
miR-142	Periodontitis, Gingivitis	↑	Saliva	Öngöz Dede et al.
	Periodontitis	↑	not stated	Asa'ad et al.
	Peri-implantitis	↓	not stated*	Asa'ad et al.
	OSCC	↑	Tumor cells	Sinha et al.
miR-125a	Periodontitis	↑	gingival crevicular fluid	Zhu & Zhong, 2022
		↑	gingival tissue	Luan et al.
miR-146a	Periodontitis	↑	not stated	Asa'ad et al.
		↑	saliva	Safari et al.
	Pulp inflammation	↑	Pulp tissue	Muñoz-Carrillo et al.
miR-30	Periodontitis	↑	gingival tissue	Luan et al.
		↑	gingival crevicular fluid	Zhu & Zhong, 2022

*Note: *Animal study, all other studies used human tissues.*

4.4 Nutrition and Lifestyle

Nutrition and lifestyle factors are increasingly recognized as important modulators of overall health, including oral health. These factors influence biological processes such as inflammation, metabolism, microbiome composition, and immune function. In addition, dietary patterns and lifestyle choices, including physical activity, smoking, and alcohol consumption, can alter miRNA expression, thereby influencing gene regulation and suggesting a potential epigenetic link between behavior and disease risk.

Nutritional patterns play a role in oral health, particularly in the development and progression of periodontal disease. While the specific mechanisms remain poorly understood, there is evidence that plant-based diets positively affect oral health by modulating inflammation and the oral microbiome. For example, vegetarians have been found to have a healthier subgingival microbiota, positively correlating with anti-inflammatory cytokines like IL-10. A predominantly plant-based diet reduces periodontitis risk through its anti-inflammatory properties and ability to balance the oral microbial environment. In contrast, a pro-inflammatory diet increases the risk of periodontitis, highlighting the importance of anti-inflammatory dietary patterns. Diets such as the Mediterranean diet, which includes legumes, fruits, vegetables, and fish, have demonstrated antioxidant and anti-inflammatory effects that reduce the risk of periodontal disease and promote oral hygiene. In addition, dairy products such as milk and yogurt have been shown to improve oral health by preventing tooth decay, reducing periodontitis risk and influencing microbial abundance due to their probiotic properties and potential fluoride content (Fan et al., 2025).

Beyond oral health, plant-based diets are gaining significant attention worldwide due to their possible health benefits. Research has increasingly highlighted the potential of plant-based diets to improve health and prevent chronic diseases (Rizzo & Baroni, 2024). These diets emphasize nutrient-rich plant foods such as fruits, vegetables, legumes, whole grains, nuts and seeds, and are often low in saturated fat and cholesterol (Guler Senturk et al., 2025). Large-scale clinical studies have shown that vegetarian and vegan diets can reduce the risk for metabolic syndrome, insulin resistance, chronic kidney disease, and liver disorders such as non-alcoholic fatty liver disease (NAFLD). These health benefits are

primarily attributed to the nutrient composition of plant-based diets, which are rich in fiber, antioxidants, and phytochemicals that support improved body composition, glucose metabolism, and lipid profiles while reducing inflammation and oxidative stress. For example, incorporating plant-based proteins into the diets of patients with chronic kidney disease has been associated with slower disease progression and lower mortality (Guler Senturk et al., 2025). Furthermore, plant-based diets have been associated with reduced GERD (Gastroesophageal reflux disease) symptoms and improved cardiovascular parameters when compared to high-fat diets (El Shikieri et al., 2024).

Nutrition also affects the expression of various miRNAs involved in both systemic and oral health. This emerging area of research suggests that specific dietary components may regulate miRNAs that modulate inflammation, immune responses, and microbial balance.

Beyond dietary influences, other lifestyle factors such as physical activity, smoking, and alcohol consumption appear to modulate miRNA expression patterns. Regular physical activity induces specific changes in circulating miRNA profiles that affect inflammation, metabolism, and cardiovascular function. Garai et al. (2021) identified miRNAs in exosomes that were altered by both short and long-term exercise, suggesting that miRNAs may mediate some of the beneficial effects of physical activity. These exercise-regulated miRNAs are implicated in the pathogenesis of cancer and chronic diseases, including diabetes and Parkinson's disease.

Smoking and alcohol consumption also impact miRNA regulation. Pointner et al. (2024) demonstrated that smokers exhibit downregulation of miRNA-142, previously linked to lung cancer pathogenesis. Interestingly, moderate alcohol consumption was linked to upregulation of miR-150 and miR-139, miRNAs proposed to have protective roles in cardiovascular health and cancer regulation. Smoking has also been shown to alter salivary miRNA profiles relevant to oral health. Öngöz Dede et al. (2023) found elevated levels of miR-142 and miR-146 in smokers, with both miRNAs implicated in periodontal disease.

Overall, nutrition and lifestyle choices may contribute to miRNA-mediated pathways with potential implications for both systemic and oral health. Understanding the interplay between these modifiable factors and specific miRNAs could offer valuable insights into maintaining oral health and preventing related diseases.

5 Objectives

Oral diseases represent a significant global health challenge, affecting billions of people worldwide and contributing to significant social and economic costs (Bernabe et al., 2020). miRNAs have recently gained attention as valuable biomarkers and potential therapeutic targets in oral health due to their central role in gene regulation and involvement in disease processes (Cuevas-González et al., 2021). At the same time, growing evidence suggests that nutrition and lifestyle not only influence general health but also shape miRNA expression patterns (Pointner et al., 2024). This intersection points to a promising opportunity: targeting oral health through nutrition- and lifestyle-driven modulation of miRNAs.

The primary objective of this study is to explore whether and how different dietary patterns affect the expression of miRNAs associated with oral health. It is hypothesized that vegetarian dietary patterns can induce distinct miRNA expression profiles associated with improved oral health outcomes compared to omnivorous diets.
Furthermore, the study aims to determine whether lifestyle factors independently or synergistically also influence these miRNA patterns.
Additionally, a comparison is conducted to investigate whether dietary and lifestyle-induced miRNA changes observed in capillary blood are similarly reflected in oral mucosa tissues.

The findings of this research may yield valuable insights into the potential utility of miRNAs as targeted biomarkers or therapeutic candidates, offering novel approaches to the prevention and treatment of oral diseases.

5.1 Overview Hypotheses and Objectives

5.1.1 Hypotheses

1. Vegetarian and omnivorous diets lead to distinct expression profiles of oral health-related miRNAs, reflecting dietary modulation of inflammatory and regulatory pathways.
2. Lifestyle factors such as physical activity, stress, and smoking independently influence miRNA expression, and may interact synergistically with diet.
3. Changes in miRNA profiles observed in capillary blood mirror those in oral mucosa, suggesting systemic biomarkers can reflect localized oral health processes.

5.1.2 Objectives

This study aims to investigate the impact of dietary patterns and lifestyle factors on the expression of miRNAs related to oral health.

1. Main Objective:
   To examine whether vegetarian and omnivorous diets cause distinct miRNA expression profiles associated with oral health status.

2. Secondary Objectives:
   To assess the independent and combined effects of lifestyle factors (e.g., physical activity, smoking, stress) on the modulation of oral health-related miRNAs.
   To compare miRNA expression in capillary blood and oral mucosa, evaluating whether nutrition- and lifestyle-driven changes are systemically and locally reflected.

These objectives aim to advance understanding of miRNAs as potential biomarkers or therapeutic targets in the context of oral disease prevention through nutrition and lifestyle.

6 Methods

The present work is carried out as part of a larger study conducted at HealthBioCare GmbH (Nußdorfer Straße 67, 1090 Vienna). The aim of this study is to determine biological age and inflammation in the oral mucosa and capillary blood using epigenetic markers, miRNAs and methylation.

For the purpose of this master's thesis, only the microRNA (miRNA) data relevant to the research context were analyzed and discussed. In this pilot study, the identification of potential oral health markers was based exclusively on data obtained from a group of healthy individuals.

6.1 Marker selection

A literature review was conducted to identify relevant miRNAs. The primary database used for searching articles related to miRNAs and oral health was PubMed. Key search terms included 'miRNAs,' 'oral health,' 'oral inflammation,' 'mucosa inflammation,' and 'periodontitis.' The search in yielded over 500 results. This was then further narrowed down using abstracts. From the 14 remaining studies, 70 relevant miRNAs were identified. In the end, the following 6 miRNAs were selected: miR-21-5p, miR-24-3p, miR-142-3p, miR-125b-3p, miR-146a-5p and miR-30b-3p.

6.2 Subjects and Study Design

This study includes data from 40 participants. Participants had to be healthy adults aged between 18 and 65 years. All participants completed a questionnaire regarding oral and dental care, diet, and lifestyle. The detailed questionnaire can be found in Appendix 12.1. The participants also provided samples of oral mucosa and capillary blood.

6.3 Sample Collection

A Safety Lancet Extra 18G was used for capillary blood sampling on a Protein Saver Card (Whatman). For the collection of oral mucosa samples, sterile gynecological cervical brushes (Tequler, Article Number: T133685) were used. Detailed protocols for sample collection are available in the attached documents (12.2).

6.4 miRNAs

6.4.1 RNA Extraction

The RNA extraction method used is based on binding RNA molecules to magnetic beads, followed by washing steps to remove other components. The process takes two days: samples are incubated overnight in a protease solution on day one using a ThermoMixer. On day two, extraction is performed with the KingFisher™ Duo Prime Magnetic Particle Processor (96-well deep setting) and the MagMAX FFPE DNA/RNA Ultra Kit (Applied Biosystems™ by Thermo Scientific™). The detailed protocol is provided in Appendix 12.3.

6.4.2 cDNA-Synthesis

For the detection and quantification of miRNAs, the extracted RNA is first transcribed into complementary DNA (cDNA), which has a higher stability. A pre-amplification is then performed to increase the amount of cDNA, making it easier to detect in subsequent analyses. The cDNA synthesis was performed using the TaqMan™ Advanced miRNA cDNA Synthesis Kit and the SimpliAmp™ Thermal Cycler (both from Applied Biosystems™ by Thermo Scientific™), according to the protocol (see Appendix 12.4).

6.4.3 qPCR

After cDNA synthesis, a probe-based qPCR was performed. For each miRNA, a mixture of TaqMan™ Fast Advanced Master Mix, the specific miRNA assay and nuclease-free water (NFW) was prepared. The qPCR reaction mixture was then transferred to a 96-well plate and the samples were added. The reaction was performed using the QuantStudio™ 3 (QS3) Real-Time PCR System according to the protocol provided by Thermo Fisher Scientific (see Appendix 12.5).

The data obtained were then analyzed using qPCR analysis modules from Applied Biosystems® (Thermo Fisher Scientific). The ΔCt method was applied for statistical calculations, using two housekeeping genes, miR-103a-3p and miR-93-5p, as endogenous controls.

$$\Delta Ct = \text{mean Ct (sample)} - [\text{mean Ct (housekeeper 1)} + \text{mean Ct (housekeeper 2)}]$$

6.5 Statistical Analysis

All evaluations of miRNAs were performed using the ΔCt value. Statistical analyses were performed using RStudio (version 2024.04.2+764). P-values < 0.05 were considered as statistically significant.

The overall goal of this analysis was to determine whether nutrition influences the selected miRNAs, with a specific focus on comparing vegetarian and omnivorous diets.

The data was analyzed for normal distribution and outliers using the Shapiro-Wilk test and the Rosner test. All identified outliers were removed.

To compare differences in food and lifestyle characteristics between the two diet groups for metric variables, a t-test was performed for normally distributed data. For non-normally distributed data a Wilcoxon test was applied. A Chi-square test was used to assess differences for ordinal variables. Boxplots and bar charts were generated for visual representation.

If significant differences in ΔCt values were found between dietary groups, further analysis was performed to determine whether specific dietary or lifestyle characteristics influenced these effects. For this purpose, an ANCOVA was performed. The food and lifestyle characteristics that showed significant differences between diet groups in earlier analyses were included as covariates. This method was used to adjust for confounding variables, ensuring that the effect of diet on ΔCt values was assessed without interference from other factors.

To check the ANCOVA assumptions Shapiro-Wilk test was applied for normal distribution, and Levene test for homoscedasticity. In cases where significant interactions were observed between the independent variable and the covariate, post-hoc analysis was performed to better understand these interactions. Tukey's HSD was used for post-hoc analysis.

7 Results

7.1 Characteristics of the Study Population

The study included a total of 40 participants, 20 females and 20 males. The average age of the participants was 26.28 years with a mean body mass index (BMI) of 22.44 kg/m². Table 2 provides an overview of the demographic characteristics of the study population.

Table 2: Descriptive of the 40 study participants.

	Study Population
Total	40
Males	20 (50%)
Females	20 (50%)
Age [in years]	26.28 ± 4.26
BMI [kg/m²]	22.44 ± 2.66

Note: Data are presented in n (%) or mean ± SD. BMI: body mass index.

7.2 Overview miRNAs

Table 3 shows the mean ΔCt values of miRNAs analyzed in both capillary blood and oral mucosa samples. miR-125b and miR-30 were undetectable in both sample types, and miR-142 had more than 50% missing data (NA) in oral mucosa. Therefore, these miRNAs were excluded from further analyses.

Table 3: ΔCt values of all miRNAs in capillary blood and oral mucosa.

miRNA	ΔCT [mean ± SD]	Range	NA's
Capillary blood			
miR-21	3.22 ± 1.02	0.12 - 4.51	-
miR-24	2.34 ± 0.32	1.47 - 2.95	-
miR-142	2.83 ± 1.2	0.27 - 4.39	-
miR-125	NA	NA	40
miR-146a	4.41 ± 0.8	1.44 - 5.50	-
miR-30	NA	NA	40
Oral mucosa			
miR-21	-1.95 ± 1.36	-4.99 - 0.38	-
miR-24	-4.42 ± 0.57	-5.57 - -3.38	-
miR-142	3.36 ± 1.98	-0.12 - 6.90	24
miR-125	NA	NA	40
miR-146a	3.61 ± 1.84	0.05 - 6.47	11
miR-30	NA	NA	39

Note: Data are presented as mean ± SD and range. NA's represent the number of missing data points.

7.3 Gender differences

To examine potential differences in ΔCt values between males and females, a t-test was performed for normally distributed data (miR-24 in capillary blood and oral mucosa, miR-21 and miR-146a in oral mucosa, Table 4). For non-normally distributed data (miR-21, miR-142, and miR-146a in capillary blood), a Wilcoxon test was applied, with the median values shown in Table 4. The statistical tests yielded p-values greater than 0.05 for all miRNAs, indicating no significant gender differences. The box plots in Figure 1 visualize the result and show no significant differences between the sexes. Gender was therefore not taken into account in subsequent analyses.

Table 4: Analysis of miRNA ΔCt values in capillary blood and oral mucosa by gender.

miRNA	Females	Males	p-value
Capillary blood			
miR-21[a]	3.55	3.55	0.87
miR-24[b]	2.27	2.41	0.18
miR-142[a]	2.96	3.51	0.48
miR-146a[a]	4.48	4.73	0.10
Oral mucosa			
miR-21[b]	-1.90	-2.01	0.81
miR-24[b]	-4.35	-4.48	0.49
miR-146a[b]	3.63	3.57	0.93

Note: [a]Non-normally distributed data, median is presented and Wilcoxon test was applied;
[b]Normally distributed data, mean is presented, and t-test was applied.

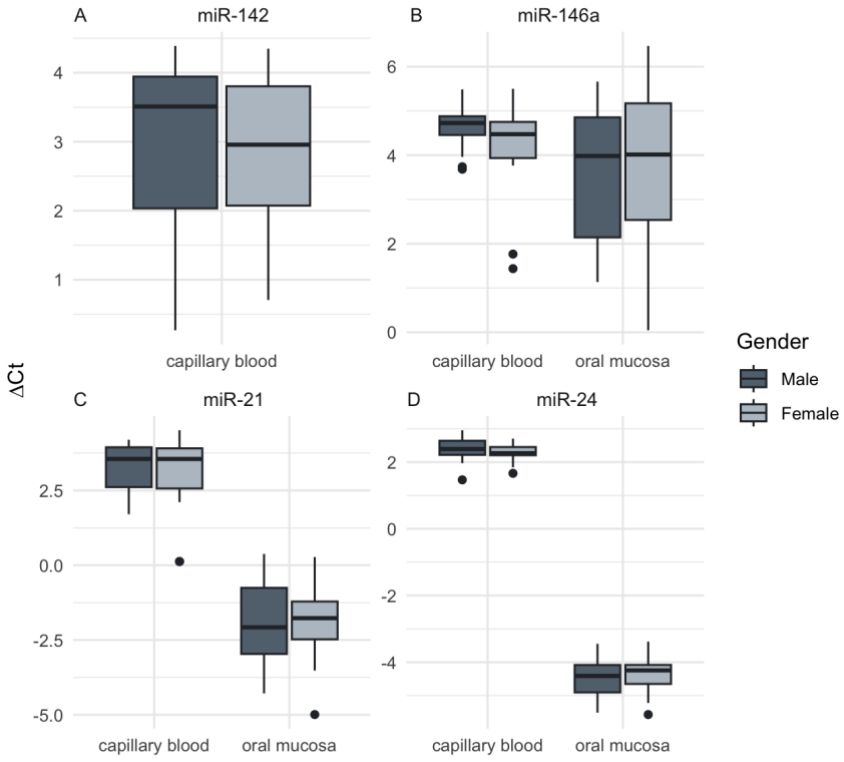

Figure 1: Boxplot representation of ΔCt values for miR-142 (A), miR-146a (B), miR-21 (C) and miR-24 (D) in capillary blood and oral mucosa by gender. The box covers the interquartile range (IQR), representing the middle 50% of the data, and the whiskers extend to the minimum and maximum values, excluding outliers, shown as dots. The median is shown as the horizontal line within the box.

7.4 Capillary blood vs. oral mucosa

To assess whether the ΔCt values of the miRNAs in oral mucosa differ from those in capillary blood, a t-test was performed for normally distributed data (miR-24 and miR-146a), while a Wilcoxon test was applied for non-normally distributed data (miR-21). All three miRNAs showed significant differences in ΔCt values between capillary blood and oral mucosa, with p-values less than 0.05 (Table 5). Overall, the ΔCt values were higher in capillary blood compared to oral mucosa (Figure 2).

Table 5: Comparison of miRNA ΔCt values in capillary blood vs oral mucosa.

miRNA	Capillary blood	Oral mucosa	p-value
miR-21[a]	3.604	-1.809	<0.001 *
miR-24[b]	2.34145	-4.41535	<0.001 *
miR-146a[b]	4.561237	3.605483	0.01 *

Note: [a]Non-normally distributed data, median is presented and Wilcoxon test was applied;
*[b]Normally distributed data, mean is presented, and t-test was applied. *p-value <0.05*

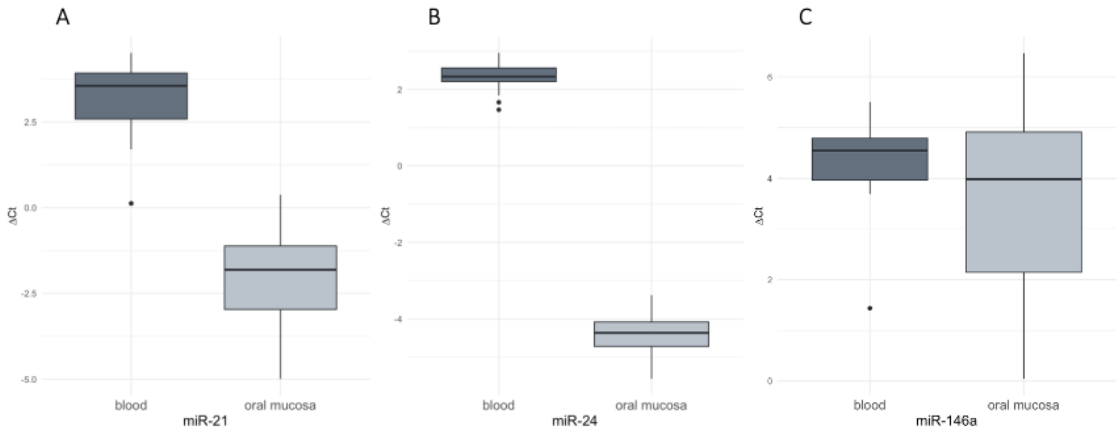

Figure 2: Boxplot illustrating distribution of ΔCt values for miR-21 (A), miR-24 (B) and miR-146a (C) in capillary blood and oral mucosa. The box covers the interquartile range (IQR), representing the middle 50% of the data, and the whiskers extend to the minimum and maximum values, excluding outliers, shown as dots. The median is shown as the horizontal line within the box.

7.5 Vegetarian vs. Omnivore

A t-test was performed to compare the ΔCt values of each miRNA between vegetarians and omnivores. Significant differences were found for miR-21 in capillary blood, miR-142

in capillary blood and miR-146a in oral mucosa. For all other miRNAs, no significant differences were observed (Table 6).

Table 6: Comparison of miRNA ΔCt values between diet groups (vegetarian vs omnivore).

miRNA	Vegetarian	Omnivore	p-value
Capillary blood			
miR-21	2.92	3.97	<0.001 *
miR-24	2.34	2.34	0.10
miR-142	2.15	3.91	<0.001 *
miR-146a	4.45	4.69	0.11
Oral mucosa			
miR-21	-1.62	-2.28	0.13
miR-24	-4.24	-4.59	0.05
miR-146a	4.18	2.67	0.04 *

*Note: Mean is presented, and t-test was applied. *p-value <0.05*

On average, the ΔCt values of miR-21 in capillary blood were lower in vegetarians compared to omnivores. Similarly, miR-142 in capillary blood also showed lower ΔCt values for vegetarians than for omnivores. In contrast, miR-146a in the oral mucosa showed higher ΔCt values in vegetarians than in omnivores. Figure 3 illustrates the differences in ΔCt values between diet groups, presented as boxplots.

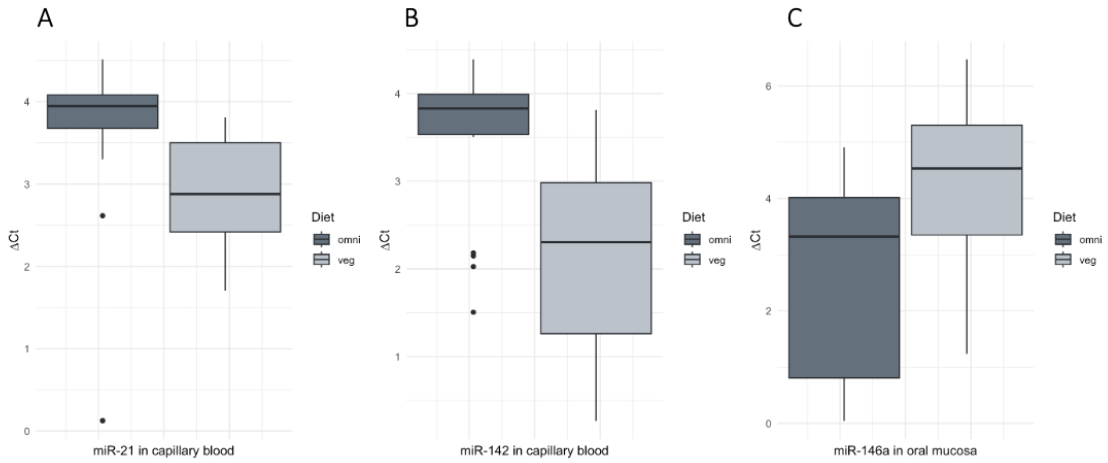

Figure 3: Boxplot of miR-21 ΔCt values in capillary blood (A), miR-142 ΔCt values in capillary blood (B) and miR-146a ΔCt values in oral mucosa (C): veg vs omni. The box covers the interquartile range (IQR), representing the middle 50% of the data, and the whiskers extend to the minimum and maximum values, excluding outliers, shown as dots. The median is shown as the horizontal line within the box.

7.6 Nutrition: vegetarian vs. omnivore

To determine whether the frequency of consumption for certain food groups differs between omnivores and vegetarians, a chi-square test was performed. Significant differences were observed for the following food groups (Table 7): vegetables, fruit, wholegrain products, animal dairy products, plant-based dairy products, fermented products, fish, and meat. Vegetarians consumed significantly more vegetables, fruit, whole grains, plant-based dairy products, and fermented products, while omnivores consumed more animal-based dairy products, fish, and meat (Figure 4). No significant differences were found for processed products, sweets, or liquid intake.

Table 7: Differences in consumption of certain food groups between vegetarians and omnivores.

Food groups	p-value
Vegetable	0.001 *
Fruit	0.022 *
Whole grains	0.025 *
Animal milk(products)	0.002 *
Plant-based milk(products)	0.010 *
Fermented products	0.001 *
Fish	0.005 *
Meat	<0.001 *
Processed foods	0.324
Sweets	0.320
Liquid intake	0.133

*Note: All variables are non-metric and chi-square test was applied; *p-value <0.05*

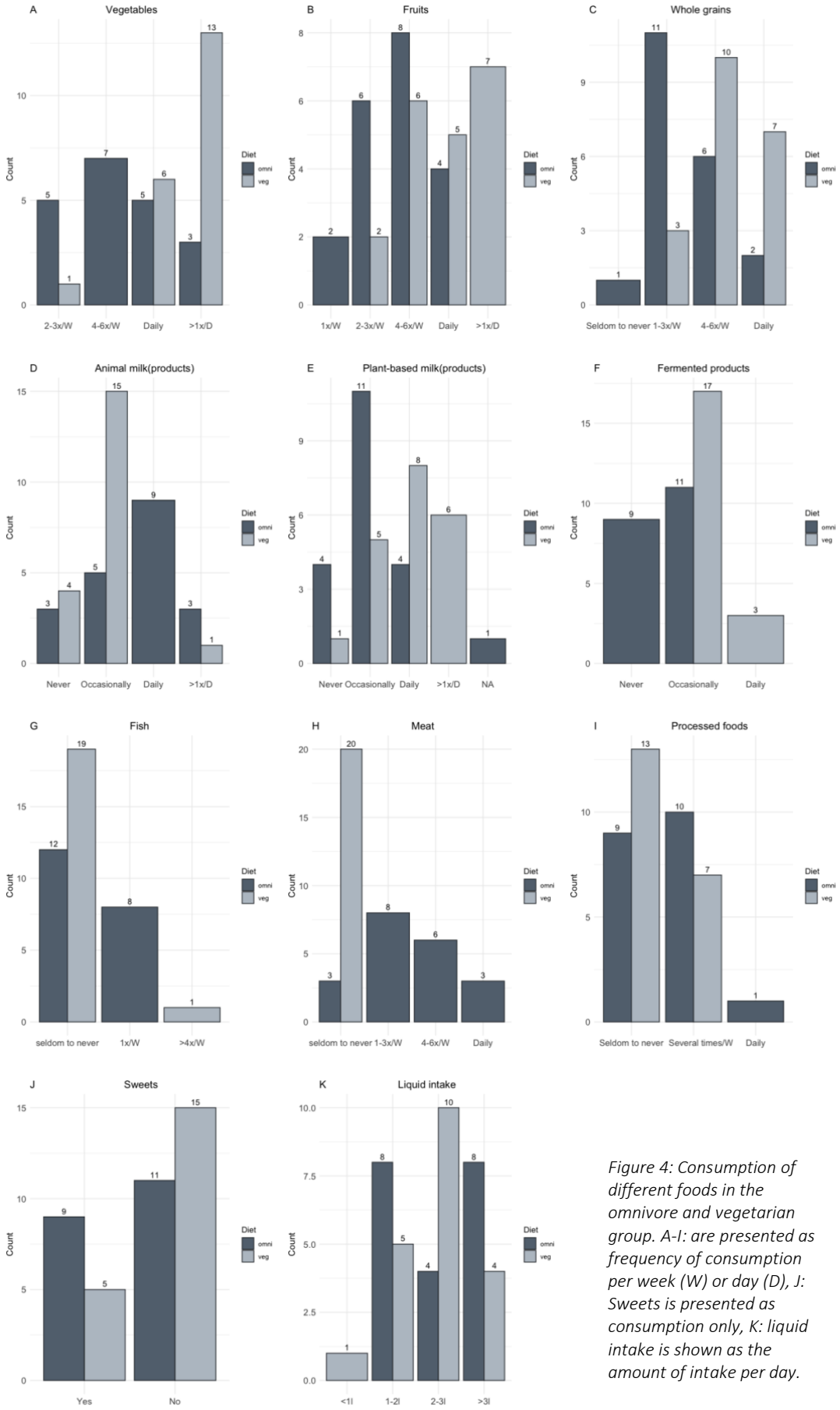

Figure 4: Consumption of different foods in the omnivore and vegetarian group. A-I: are presented as frequency of consumption per week (W) or day (D), J: Sweets is presented as consumption only, K: liquid intake is shown as the amount of intake per day.

7.7 Lifestyle: vegetarian vs. omnivore

The chi-square test was used to assess differences in non-metric lifestyle characteristics between vegetarians and omnivores, while the Wilcoxon test was applied for endurance and strength training, which were measured in training hours per week and are thus metric variables. A significant difference was found only for physical activity, with vegetarians reporting more frequent physical activity per week (Figure 5). No further significant differences were observed between the diet groups for other lifestyle characteristics, as shown in Table 8.

Table 8: Lifestyle differences between vegetarians and omnivores.

Lifestyle characteristics	p-value
Inflammation[a]	0.73
Metabolic disease[a]	0.47
General disease[a]	0.95
Allergies[a]	0.75
Physical activity[a]	0.02 *
Exercise[a]	0.80
Stress[a]	0.53
Smoking[a]	1.00
Alcohol[a]	0.20
Endurance[b]	0.66
Strength[b]	0.46

*Note: [a]Non-metric variables and chi-square test was applied; [b]Metric variables, non-normally distributed data and Wilcoxon test was applied. *p-value <0.05*

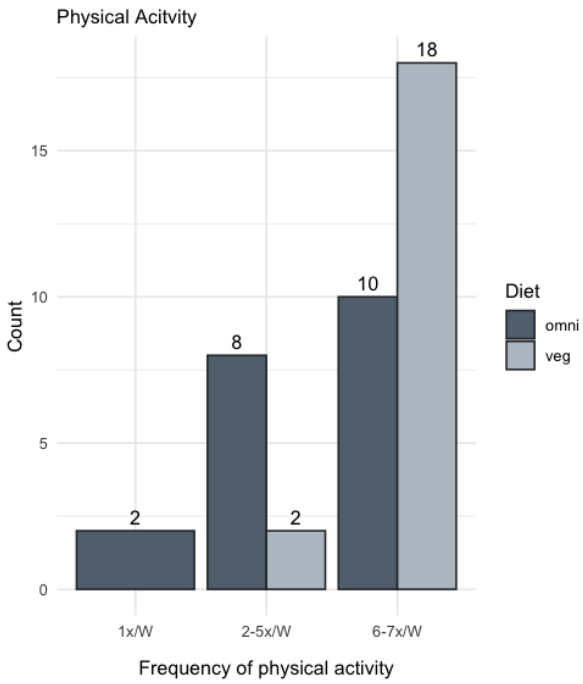

Figure 5: Physical activity across diet groups (omni vs veg).

7.8 ANCOVA

To determine whether the observed differences in ΔCt values for miRNAs between dietary groups were influenced by specific dietary or lifestyle characteristics, an ANCOVA was performed. This method was used to adjust for confounding variables, ensuring that the effect of diet on ΔCt values was assessed without interference from other factors. The ANCOVA was performed only for miRNAs where previous analyses indicated significant differences between the two diet groups- specifically miR-21 and miR-142 in capillary blood, and miR-146a in oral mucosa. The food and lifestyle characteristics that showed significant differences between diet groups in earlier analyses (Table 7, Table 8) were included as covariates. These covariates were: vegetables, fruit, whole grain products, animal dairy products, plant-based dairy products, fermented products, fish, meat, and physical activity. The aim of the ANCOVA was to determine whether diet influences miRNA ΔCt levels independently of these covariates.

7.8.1 miR-21 (capillary blood)

After removing two identified outliers from the data set, an ANOVA confirmed a significant effect of diet on miR-21 levels in capillary blood (p-value = $6.35e^{-06}$). The Shapiro-Wilk test (p-value = 0.368) showed that the data met the assumption of normality. However, the Levene test (p-value = 0.002347) showed that the assumption of homogeneity of variance was violated, which should be considered when interpreting the results. A TukeyHSD post-hoc analysis revealed that the mean ΔCt value of miR-21 in capillary blood was 0.97 higher in the omnivorous group compared to the vegetarian group.

When individual covariates (vegetables, fruits, whole grains, animal dairy products, plant dairy products, fermented products, fish, meat and physical activity) were included in the model, the main effect of diet on miR-21 remained significant. None of the covariates showed a significant main effect, nor did they show significant interactions with diet. This suggests that the influence of diet on miR-21 is not driven by a single food group but is likely due to a combination of several dietary factors.

7.8.2 miR-142 (capillary blood)

An ANOVA confirmed a significant effect of diet on the ΔCt values of miR-142 in capillary blood (p-value = $9.42e^{-05}$). The Shapiro-Wilk test (p-value = 0.01192) indicated that the assumption of normal distribution was violated, which should be taken into account when interpreting the results. The Levene test (p-value = 0.05688) showed that the assumption for homogeneity of variance was met. A TukeyHSD post-hoc analysis showed that the mean ΔCt value of miR-142 in capillary blood was 1.3663 higher in the omnivorous group than in the vegetarian group.

When the individual covariates (vegetables, fruits, whole grains, animal dairy products, plant dairy products, fermented products, fish, meat, and physical activity) were included in the model, the main effect of diet on miR-142 remained significant. None of the covariates showed a significant main effect. However, a significant interaction between diet and physical activity was observed (p-value = 0.0345), suggesting that physical activity partially explains the difference in miR-142 expression between the vegetarian and omnivorous groups. No significant interactions were found for the other variables.

To better understand the interaction between diet and physical activity, a post-hoc analysis (Tukey HSD) was performed. The comparison between vegetarians and omnivores who are physically active 6-7 times per week showed a significant difference (p-value = 0.0003386), with vegetarians having a 1.828 lower ΔCt value than omnivores. No significant differences were found for the other activity groups (1x/week and 2-5x/week). It should be noted that no vegetarians reported being physically active 1x/week, and only 3 vegetarians reported being active 2-5x/week- most vegetarians were in the 6-7x/week group.

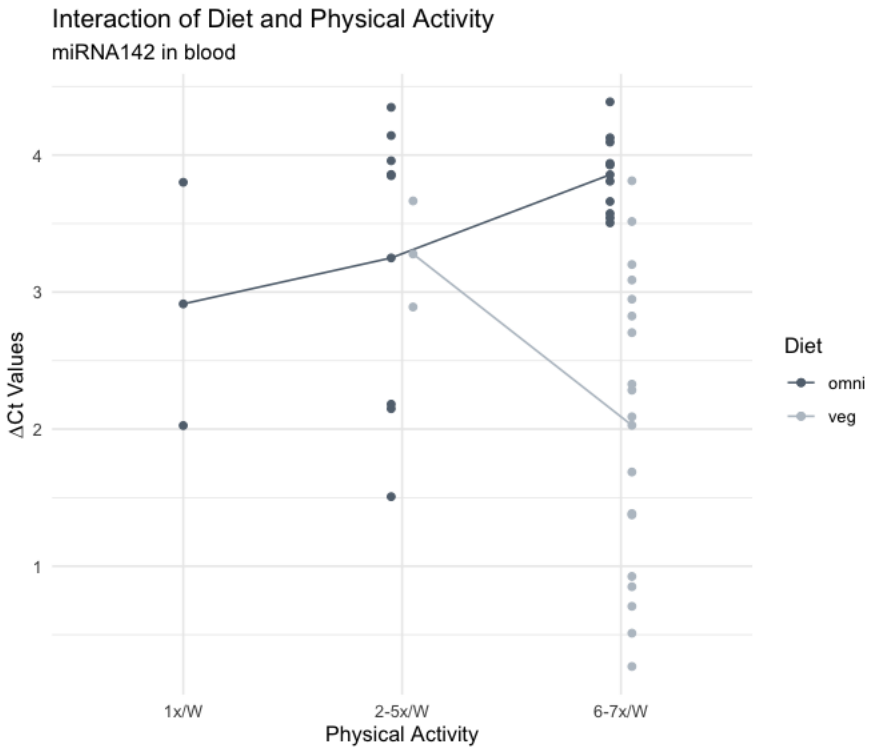

Figure 6: Interaction plot: miRNA142, diet and physical activity.

Figure 6 provides a visual representation of the relationship between diet, physical activity and the ΔCt values of miR-142 in capillary blood. The lines in the interaction plot showed opposite trends for the vegetarian and omnivore groups, suggesting that the relationship between physical activity and ΔCt values differs between the two dietary patterns. In the omnivorous group, ΔCt values of miR-142 increased with higher levels of physical activity, while in the vegetarian group, ΔCt values decreased as physical activity increases.

7.8.3 miR-146a (oral mucosa)

The ANOVA revealed a significant effect of diet on the ΔCt values of miR-146a in the oral mucosa (p-value = 0.0292). The Shapiro-Wilk test (p-value = 0.02242) indicated that the assumption of normal distribution was not met, which should be considered when interpreting the results. The Levene test (p-value = 0.6428) confirmed that the assumption of homogeneity of variance was met. A TukeyHSD post-hoc analysis showed that the mean ΔCt value of miR-146a in the oral mucosa was 1.5098 lower in the omnivorous group compared to the vegetarian group.

When individual covariates (vegetables, fruits, whole grains, animal dairy products, plant-based dairy products, fermented products, fish, meat, and physical activity) were included in the model, the main effect of diet on miR-146a remained significant. None of the covariates had a significant main effect. A significant interaction was found between diet and plant-based milk(-products) (p-value = 0.00298), indicating that consumption of plant-based milk(-products) partially explains the difference in ΔCt values of miR-146a between vegetarians and omnivores. No significant interactions were observed for the other variables.

To further investigate this interaction, a post-hoc Tukey HSD analysis was performed. A significant difference was found between vegetarians who consume plant-based milk(-products) daily and omnivores who never consume plant-based milk(-products) (p-value = 0.0072), with vegetarians having higher ΔCt values than omnivores. A significant difference was also found between vegetarians and omnivores who both consume plant-based dairy products daily (p-value = 0.0096), with vegetarians having a higher ΔCt value. No significant differences were found in the other group comparisons.

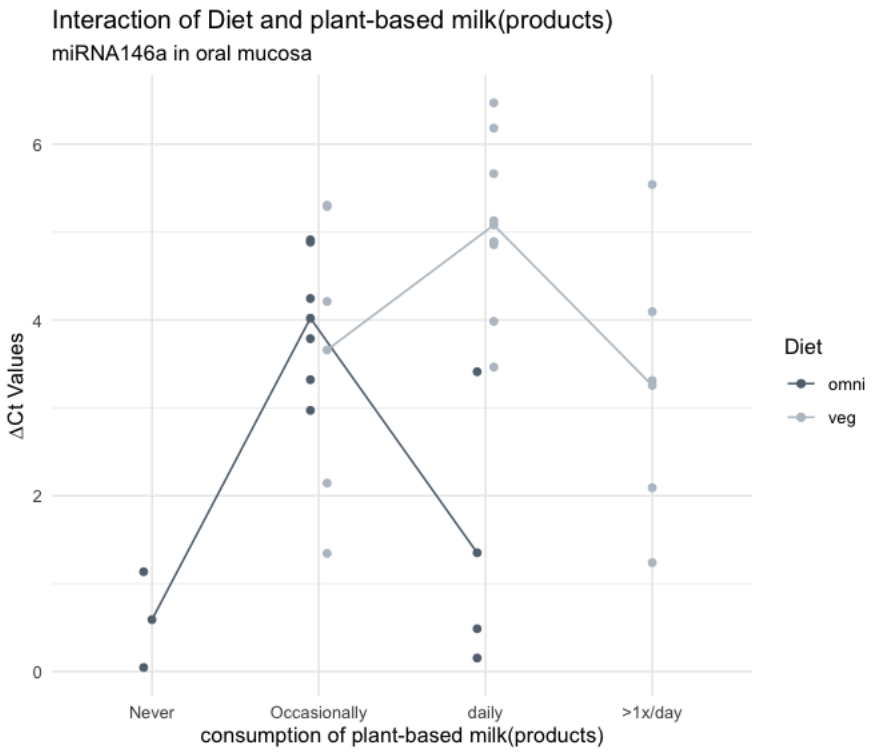

Figure 7: Interaction plot: miRNA146a, diet and plant-based milk(-products).

An interaction plot (Figure 7) illustrates the relationship between diet, consumption of plant-based milk(-products), and the ΔCt values of miR-146a in the oral mucosa. In the omnivore group, ΔCt values increased from the 'never' to the 'occasional' consumption group, but decreased again with more frequent consumption. In the vegetarian group, ΔCt values rose from 'occasional' to 'daily' consumption, then declined when plant-based milk (-products) were consumed multiple times per day. This indicates that the relationship between diet and miR-146a expression in the oral mucosa is moderated by the consumption of plant-based milk(-products). In other words, the effect of diet on the ΔCt values of miR-146a varies depending on the intake frequency of plant-based milk(-products).

These patterns suggest that the relationship between plant-based dairy products and miR-142 expression may follow a non-linear trend, potentially influenced by both dietary context and intake frequency.

8 Discussion

The present study investigated the influence of diet and lifestyle on miRNA expression in two distinct tissues, capillary blood and oral mucosa, with a specific focus on miRNAs associated with oral health. The findings revealed notable variations between dietary groups, as well as significant differences in miRNA expression between capillary blood and oral mucosa samples.

The observed differences in the expression levels of miR-21, miR-24, and miR-146a between capillary blood and oral mucosa (Table 5), with a higher expression for the latter, underscore the tissue-specific nature of miRNAs. The concept of tissue-specific miRNA expression is well-established in the literature, as studies have repeatedly demonstrated that miRNAs exhibit distinct expression patterns across various tissues (Ludwig et al., 2016). A possible explanation for the observed differences in this study, beyond tissue specificity, is the variation in sampling methods. Buccal swabs were used for oral mucosa collection and yielded more RNA material compared to the blood cards. Although the same RNA volume (2 µL) was used for cDNA synthesis in all samples, the higher RNA concentration in buccal swab extracts likely resulted in a higher total RNA input, thus generating greater cDNA yield. Since normalization was only performed using two housekeeping genes without adjusting for total RNA input or using an external reference, this difference in RNA quantity may have contributed to the higher expression levels observed in buccal samples.

To my knowledge, this is the first study to directly compare miRNA profiles between capillary blood and oral mucosa samples. There is a study which used saliva and plasma samples but did not directly compare these two, but rather evaluated expression-differences between patients affected by OSCC and OPMDs and healthy people (Rocchetti et al., 2024). Since circulating and tissue-resident miRNAs exhibit distinct regulatory roles they may serve as different types of biomarkers for systemic and localized health conditions. Recognizing this distinction is critical when selecting appropriate sample types for evaluating miRNA biomarkers related to oral health. Furthermore, investigating the relationships between cellular and circulating miRNA concentrations, and determining

how they relate to each other, would be essential and could significantly enhance our understanding.

Some miRNAs previously linked to oral health, specifically miR-125 and miR-30 respectively, could not be detected in our capillary blood and oral mucosa samples. This may be due to our cohort consisting solely of healthy individuals, as previous studies have identified a high expression of these miRNAs predominantly under pathological conditions (Zhu & Zhong, 2022). This suggests these miRNAs could be disease-specific biomarkers rather than indicators of general oral health.
In addition, methodological differences between our study and others may have contributed to the divergent findings. These miRNAs might be expressed at very low levels, and our approach may not have yielded sufficient RNA material for their detection. In studies using different sampling methods or tissues, the RNA yield might have been higher, allowing for the detection of low-abundance miRNAs.

MiR-142 was detected in the capillary blood of all samples, while in the oral mucosa it was below the detection limit in more than half of the samples. This disparity may indicate that miR-142 circulates primarily in blood, which seems reasonable since it is associated with hematopoietic stem cells, resulting in generally lower or variable expression in oral mucosa-again underlining the tissue-specific expression of miRNAs. MiR-142 has been shown to play various roles in various organs/tissues but seems to be especially important for hematopoietic physiology and hematological malignancies. It was shown to be notably more expressed in hematopoietic cells and dysregulations were especially evident in blood abnormalities and immune system defects (Huang et al., 2023).

Our analysis revealed that dietary patterns significantly influenced miRNA expression (Table 6). Vegetarians had lower ΔCt values for miR-21 and miR-142 in capillary blood samples, indicating higher expression of these miRNAs compared to omnivores. MiR-146a showed higher ΔCt values in oral mucosa samples from vegetarians, indicating lower expression compared to omnivores.
Among the miRNAs analyzed, diet showed a significant effect only for miR-146a expression in oral mucosa. One possible explanation is that the local impact of diet in the

oral cavity may be limited due to the short exposure time of dietary components, as nutrients typically need to undergo digestion and absorption before exerting systemic effects. However, the lack of significant results for other miRNAs could also be attributed to the small cohort size. Further studies with larger sample sizes are needed to determine whether diet truly has no effect on other miRNAs or whether such effects were simply not detectable in this study.

The observed effect on miR-146a expression in oral mucosa suggests that a vegetarian diet may be beneficial for oral health. In our study, miR-146a levels were lower in vegetarians compared to omnivores. While the direct influence of a vegetarian diet on miR-146a has not been previously reported, existing literature has associated elevated expression of this miRNA with oral diseases such as periodontitis (Safari et al., 2023; Wu et al., 2021). Therefore, the lower expression observed in vegetarians could indicate a more favorable oral health profile in this group. Further research is needed to explore whether dietary patterns directly influence miR-146a expression and to confirm the potential beneficial role of a vegetarian diet in oral health.

The literature reviewed primarily focused on oral health using saliva or oral tissues, making direct comparisons with our capillary blood-derived miRNA data challenging. However, research investigating blood miRNAs in relation to inflammation and metabolic conditions indicates that dietary influences observed here may have broader systemic health effects (Backes et al., 2016).

Another notable finding is that dietary influence on miRNA expression appears to result from the interplay of multiple dietary factors rather than being driven by a single food group. One plausible explanation for this observation is that miRNA expression is regulated by complex biological pathways influenced by multiple nutrients and bioactive compounds. Different foods provide different combinations of macronutrients, micronutrients, phytonutrients, prebiotics, probiotics, and other bioactive constituents and their combined effects might be necessary to induce meaningful changes in miRNA profiles. These nutrients do not exist in isolation but rather in a complex matrix that interacts with multiple enzymatic and metabolic targets. Moreover, synergistic effects

among dietary components are already well documented (Jacobs et al., 2009; Townsend et al., 2023). Therefore, it is plausible that the overall dietary pattern, the combination and variety of foods consumed, is critical in shaping miRNA expression, rather than the influence of a single food or food group, as observed in this study.

The observed interaction between diet, physical activity, and miR-142 expression (Figure 6) in capillary blood should be interpreted with caution. It is important to note that none of the vegetarians in our cohort reported being physically active only once per week, and only three reported activity levels of 2-5 times per week. Most vegetarians belonged to the 6-7 times per week group. This uneven distribution and the resulting small subgroup sizes may have limited the robustness of the statistical analysis, increasing the likelihood that the observed significance occurred by chance –a possibility also suggested by the graphical data. Previous research has demonstrated that physical activity can influence miRNA expression (Garai et al., 2021) , supporting the relevance of including lifestyle factors in such analyses. However, further studies with larger and more balanced cohorts are needed to better understand the interaction between diet, physical activity, and miR-142 expression, and to confirm whether physical activity may partially account for the differences observed between diet groups.

An additional interaction between miRNA-146a expression in the oral mucosa and diet was observed. The effect of diet on the ΔCt values of miR-146a appeared to vary depending on the frequency of plant-based milk(-product) consumption (Figure 7). Among omnivores, the highest ΔCt values were observed with occasional consumption, while vegetarians showed peak values with daily intake of plant-based milk(-products). However, interpretation is limited by the absence of omnivores in the '>1x/day' group and vegetarians in the 'never' group. These findings should therefore be interpreted cautiously and confirmed in larger, controlled studies.

Although statistically significant differences were limited to miR-146a, observed trends suggest that a diet rich in vegetables, whole grains, and fermented foods may positively influence miRNA expression in oral mucosa. Supporting visualizations of these trends are provided in Appendix 12.6. Specifically, a higher intake of vegetables, whole grains, and

fermented products appeared to be associated with lower expression levels of certain oral miRNAs, while greater consumption of meat and processed foods seemed to increase expression. Given that elevated oral miRNA expression has been associated with a higher risk of disease in previous studies, these trends may point toward a potential protective effect of plant-based, fiber-rich diets on oral health. However, results for other food groups, such as fruit, animal and plant-based milk products, fish, and sweets, were inconsistent, making it difficult to propose a clear theory. It is important to emphasize that these are merely observational findings without statistical significance, and no definitive conclusions can be drawn from the current data.

Nevertheless, the influence of diet on miRNA regulation has already been well-documented in other contexts. Studies have shown that plant-based foods and their bioactive compounds, such as curcumin and resveratrol, can beneficially modulate the expression of miRNAs involved in inflammation, metabolism, and cardiovascular function (Delucas et al., 2024). These effects are hypothesized to involve anti-inflammatory and antioxidant mechanisms, as well as potential influences on the microbiome. While these pathways have not been directly studied in the context of oral health, it is plausible that similar mechanisms could also affect miRNA expression in oral tissues.

In the present study, gender did not significantly influence miRNA expression (Table 4), which is consistent with findings reported by Meder et al. (2014). However, other studies have identified sex-specific differences in miRNA expression and proposed biological mechanisms to explain these variations, including the modulatory effects of sex hormones such as estrogen, progesterone, and testosterone (Pointner et al., 2024). Given these potential influences, gender remains an important variable and should continue to be considered in future miRNA research.

9 Limitations

While this study provides valuable insights into the relationship between dietary patterns, lifestyle factors, and miRNA expression relevant to oral health, several limitations should be acknowledged.

First, the relatively small sample size (n = 40) limits the statistical power of the analyses and restricts the generalizability of the findings to broader populations. In addition, the study population consisted exclusively of young and healthy individuals, and no separate control group was included. This homogeneity reduces variability and may mask effects that could be more pronounced in populations with different health statuses, age groups, or pre-existing conditions.

Furthermore, although miRNA expression can reflect regulatory processes associated with inflammation and oral health, the lack of direct measurements of inflammatory biomarkers constrains the interpretation of functional outcomes. The absence of such physiological markers makes it difficult to draw firm conclusions about the biological relevance of observed miRNA changes.

To strengthen future research, studies should aim to include larger and more diverse samples, incorporate control groups, and integrate direct measurements of inflammatory and clinical biomarkers. Additionally, interventional study designs would be valuable for validating findings and better understanding the temporal dynamics and causative links between diet, lifestyle, and miRNA expression.

10 Conclusion

This study demonstrated that dietary patterns can influence the expression of miRNAs associated with oral health. Vegetarian and omnivorous diets showed different miRNA expressions, particularly affecting miR-21, miR-142 in capillary blood and miR-146a in oral mucosa, suggesting a potential role in both oral and systemic health. Diets rich in vegetables, whole grains, and fermented products may offer promising strategies for positively modulating miRNA expression and improving oral health outcomes.
Significant differences in miRNA profiles between capillary blood and oral mucosa highlight the importance of sample selection when investigating miRNA biomarkers.

Despite limitations such as the small, homogeneous study population and the absence of a diseased control group, the results provide initial insights into the complex relationship between nutrition, lifestyle, and miRNA expression related to oral health.

Future research should aim to include larger, more diverse populations, incorporate clinical biomarkers and a diseased control group, and consider interventional study designs to better understand how diet-driven miRNA modulation may contribute to oral health maintenance and disease prevention.

Together, these results highlight the potential of diet and lifestyle as modifiable factors influencing oral health through epigenetic regulation.

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AI tools used in this work:

This thesis represents the author's independent and original work. ChatGPT was used to improve language clarity throughout the text, and DeepL assisted with the translation of the abstract.

12 Appendix

12.1 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: _____

12.2 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 passende 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

12.3 Protocol: RNA 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

- 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

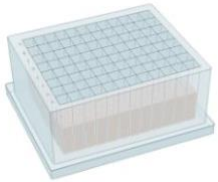
- Prepare **RNA Binding Buffer**

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.

12.4 Protocol: cDNA Synthesis

SOP-LAB-003-01 10.08.22
cDNA Synthesis

Instruments

- SimpliAmp Endpoint PCR
- Centrifuge
- Tube adaptor plate

Consumables

- 1.5 ml tubes
- 0.1/0.2 ml tubes
- pipette tips

Kits

- TaqMan® Advanced miRNA cDNA Synthesis Kit (A28007)
- 1xTE buffer
- NFW or RFW

Procedure

Before start

- Which samples need miRNA analysis? (check Probenliste Panels: Metabo, Kombi, Stress, Sport, EBV, Virepair, Virmune, Prevention, Intervention… For all panels except aging panel)
- Label the 0.1/0.2 pcr tubes: **3 tubes per sample**: one for **RT** (Poly(A), Adaptor ligation and RT), one for **miR-Amp** reaction and one for **1:10 dilution** of miR-Amp (mark with a dot on the lid; sample Nr +RT/miR-Amp/1:10)
- Prepare a 1:10 dilution of 1xTE buffer with NFW for the dilution of the miR-Amp products

Poly(A) tailing reaction

1.) Thaw samples and cDNA synthesis reagents on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.
Note: Keep the assays in storage until ready for use.
IMPORTANT! The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (next section).

2.) In a 1.5-mL microcentrifuge tube, prepare sufficient Poly(A) Reaction Mix for the required number of reactions according to the following table.

Component	**1 Rxn**	**12 Rxns**[1]
10X Poly(A) Buffer	0.5 µl	6.6 µl
ATP	0.5 µl	6.6 µl
Poly(A) Enzyme	0.3 µl	3.96 µl
RNase-free water	1.7 µl	22.44 µl
Total Poly(A) Reaction Mix volume	3.0 µl	39.6 µl

[1] Volumes include 10% overage

3.) Vortex the Poly(A) Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

4.) Add 3 µL of Poly(A) Reaction Mix to each pcr tube. Add 2µl sample (RNA) and mix by pipetting up and down. The total volume should be 5 µL per tube.

5.) Seal the reaction tubes.

6.) Centrifuge the reaction tubes briefly to spin down the contents and eliminate air bubbles.
7.) Place the reaction tubes into a thermal cycler (use the black tube adaptor plate) then incubate using the following settings and standard cycling:

Step	Temp	Time
Polyadenylation	37°C	45 min
Stop reaction	65°C	10 min
Hold	4°C	Hold

8.) Proceed immediately to the adaptor ligation reaction (next section).

Adaptor ligation reaction

1.) In a 1.5-mL microcentrifuge tube, prepare sufficient Ligation Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	12 Rxns[1]
5X DNA Ligase Buffer	3 µl	39.6 µl
50% PEG 8000	4.5 µl	59.4 µl
25X Ligation Adaptor	0.6 µl	7.92 µl
RNA Ligase	1.5 µl	19.8 µl
RNase-free water	0.4 µl	5.28 µl
Total Ligation Reaction Mix volume	10 µl	132 µl

[1] Volumes include 10% overage

IMPORTANT! For accurate pipetting of 50% PEG 8000:
· Use 50% PEG 8000 at room temperature. Sometimes PEG solution can crystalize. Incubate at 32°C on a thermomixer for 5-10 minutes (or until crystals are dissolved). Bring to room temperature before use.

· Aspirate and dispense solution slowly.

a. Hold the pipette tip in the solution for ~10 seconds after releasing the plunger during aspiration. This action allows the solution to be fully drawn into the pipette tip.

b. Keep the plunger depressed for ~10 seconds to allow the solution to be fully dispensed into the Ligation Reaction Mix.

2.) Vortex the Ligation Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
3.) Transfer 10 µL of the Ligation Reaction Mix to each reaction tube containing the poly(A) tailing reaction product and mix by pipetting up and down. The total volume should be 15 µL per well or tube
4.) Seal the tubes.
5.) Centrifuge the tubes briefly to spin down the contents.

6.) Place the tubes into a thermal cycler (black adaptor plate), then incubate using the following settings and standard cycling:

Step	Temp	Time
Ligation	16°C	60 min
Hold	4°C	Hold

7.) Proceed immediately to the reverse transcription (RT) reaction (next section).

Reverse transcription (RT) reaction

1.) In a 1.5-mL microcentrifuge tube, prepare sufficient RT Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	12 Rxns[1]
5X RT Buffer	6 µl	79.2 µl
dNTP Mix (25 mM each)	1.2 µl	15.84 µl
20X Universal RT Primer	1.5 µl	19.8 µl
10X RT Enzyme Mix	3 µl	39.6 µl
RNase-free water	3.3 µl	43.56 µl
Total RT Reaction Mix volume	15 µl	198 µl

2.) Vortex the RT Reaction Mix, then centrifuge briefly.
3.) Transfer 15 µL of the RT Reaction Mix to each reaction tube containing the adaptor ligation reaction product and mix by pipetting up and down. The total volume should be 30 µL per tube.
4.) Seal the reaction tubes.
5.) Centrifuge the tubes briefly to spin down the contents.

6.) Place the reaction tubes into a thermal cycler (black adaptor plate), then incubate using the following settings and standard cycling:

Step	Temp	Time
Reverse transcription	42°C	15 min
Stop reaction	85°C	5 min
Hold	4°C	Hold

7.) Proceed to the miR-Amp reaction (next section). Store the RT reaction product at –20°C for up to 2 months

miR-Amp reaction

1.) In a 1.5-mL microcentrifuge tube, prepare sufficient miR-Amp Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	12 Rxns[1]
2X miR-Amp Master Mix	25 µl	330 µl
20X miR-Amp Primer Mix	2.5 µl	33 µl
RNase-free water	17.5 µl	231 µl
Total miR-Amp Reaction Mix volume	45 µl	594 µl

2.) Vortex the miR-Amp Reaction Mix, then centrifuge briefly.
3.) Transfer 45 µL of the miR-Amp Reaction Mix to a **new reaction tube each**.
4.) Vortex and centrifuge each RT reaction product before adding 5 µL of the RT reaction product to each reaction tube and mix by pipetting up and down. The total volume should be 50 µL per tube.
5.) Seal the tubes.
6.) Centrifuge the reaction tubes briefly to spin down the contents.

7.) Place the reaction tubes into a thermal cycler (black adaptor plate), then incubate using the following settings MAX ramp speed, and standard cycling:

Step	Temp	Time	Cycles
Enzyme activation	95°C	5 min	1
Denature	95°C	3 sec	14
Anneal/Extend	60°C	30 sec	
Stop reaction	99°C	10 min	1
Hold	4°C	Hold	1

8.) Dilute the miR-Amp products 1:10 with 0.1xTE buffer in a new reaction tube for each sample in a total volume of 50 to 100 µl (depending on analysis panel).
E.g. 5 µl miR-Amp product + 45 µl 0.1xTE buffer

9.) Proceed to performing the real-time PCR.
Store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

12.5 Protocol: qPCR

SOP-miRNA Analysis Lab

Instruments

- QS3
- centrifuge
- plate rack, tube rack
- pipettes

Consumables

- 96 well qPCR plate + sealing film
- pipette tips
- 1.5 ml tubes

Reagents

- TaqMan Fast Advanced MasterMix
- TaqMan miRNA assays
- Nfw
- miR-Amp reactions 1:10 (samples)

Before starting

- **Design the plate/run** in QuantStudio Design Software"

Important: all miRNAs for one sample have to be on the same plate.

- Write down your **pipetting layout**
- **Turn on the QS3** instrument

Procedure

1. **Thaw** your samples and miRNA assays
2. Prepare sufficient amount of your **Master Mixes** according to the table in labeled 1.5 ml tubes

	1 Rxn	6.5 Rxns
TagMan Fast Advanced MM	5 µl	32.5 µl
miRNA assay	0.5 µl	3.2 µl
NFW	2 µl	13 µl

Note: Prepare **1 Master Mix per miRNA**. If you have 12 different miRNAs on the same plate, you need 12 different Master Mixes. Total Rxns per Master Mix can differ!

- Take a clean 96 well plate and put it on a plate rack
- Pipette **7.5 µl of Master Mix** on a 96 well plate according to your pipetting layout. Use reverse pipetting and the same pipette tip per Master Mix
- Take a new pipette tip box. Add **2.5 µl diluted 1:10 miR-Amp sample** to the 96 well plate according to your pipetting layout. Use new pipette tip each time.
- After adding all the samples and controls to the plate, **seal the plate** with a sealing film. Make sure that the film sticks properly outside the wells and between each of the wells
- Load plate into **QS3** (without well rack)
- **Load Experiment**: Network drive: 3 QS3/miRNAs: choose your run template: Start Run
- After **Runs is completed:** Export Run to network drive: 3 QS3/miRNAs

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12.6 miRNA Expression according to consumption of specific food groups

12.6.1 miR-21 (capillary blood)

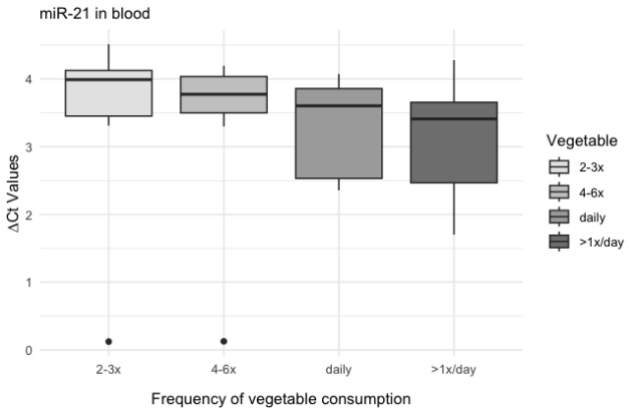

Figure 8: ΔCt values of miR-21 in blood according to vegetable consumption per week

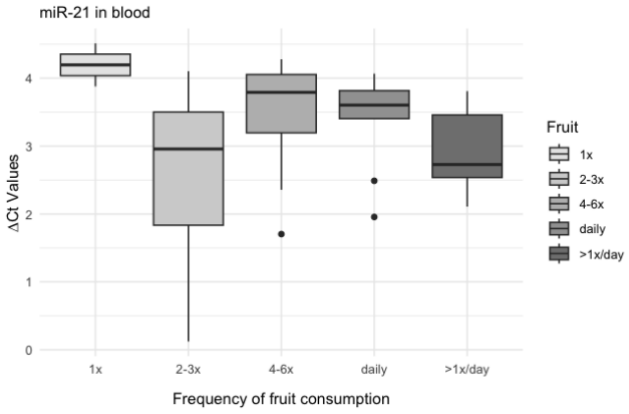

Figure 9: ΔCt values of miR-21 in blood according to fruit consumption per week

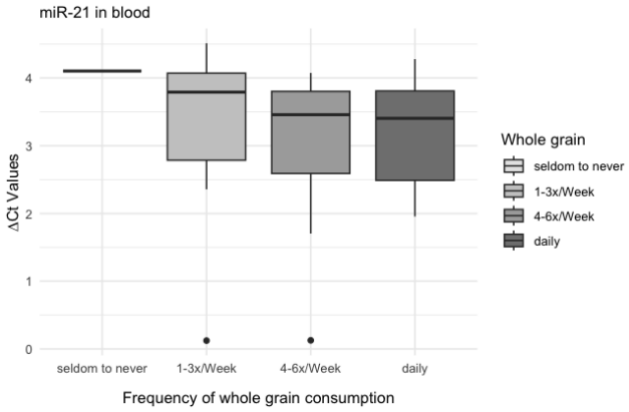

Figure 10: ΔCt values of miR-21 in blood according to whole grain consumption per week

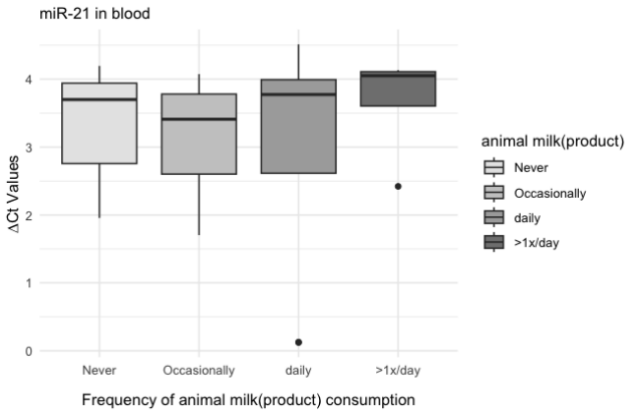

Figure 11: ΔCt values of miR-21 in blood according to animal milk(product) consumption per week

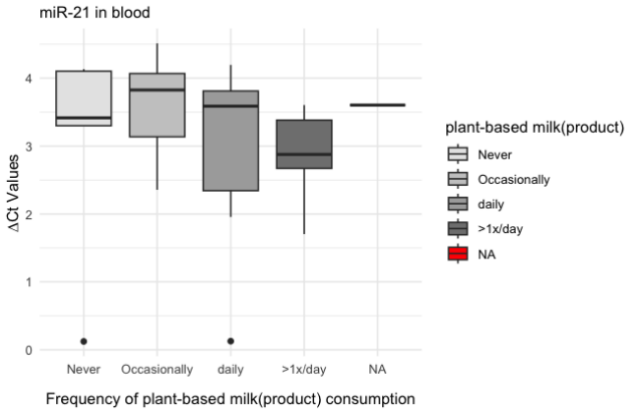

Figure 12: ΔCt values of miR-21 in blood according to plant-based milk(product) consumption per week

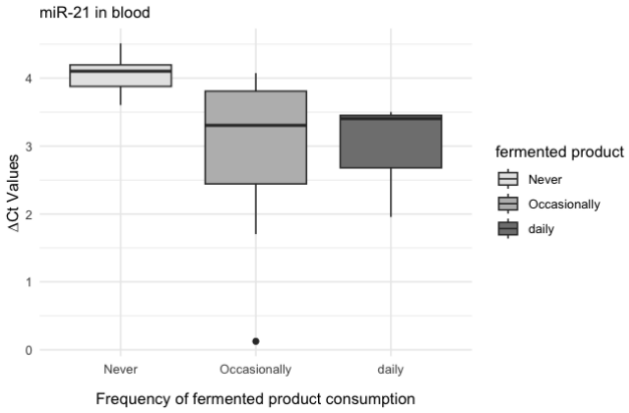

Figure 13: ΔCt values of miR-21 in blood according to fermented product consumption

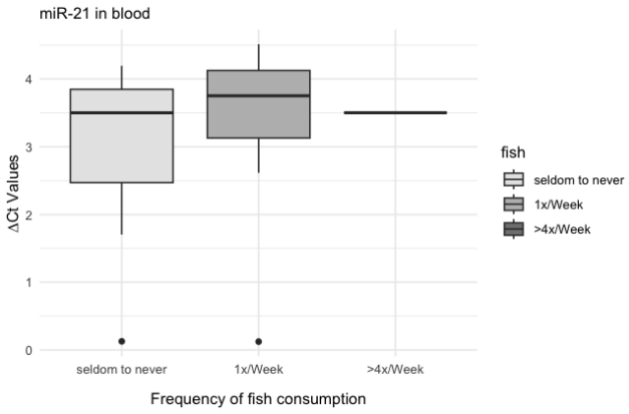

Figure 14: ΔCt values of miR-21 in blood according to fish consumption

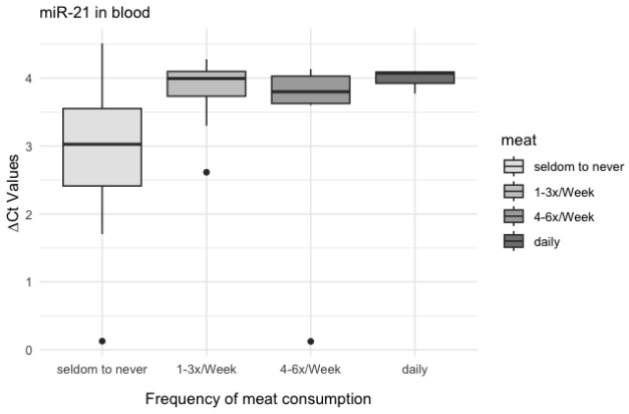

Figure 15: ΔCt values of miR-21 in blood according to meat consumption

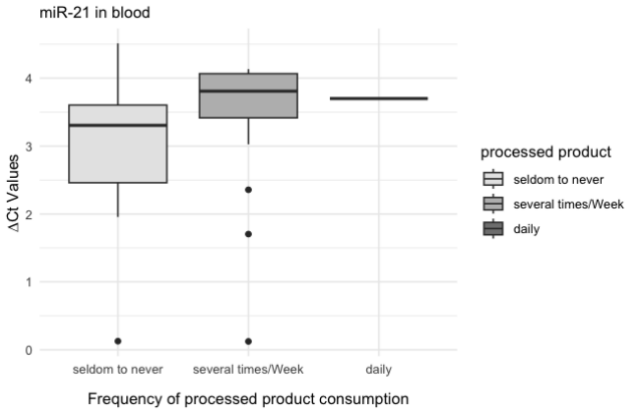

Figure 16: ΔCt values of miR-21 in blood according to processed product consumption

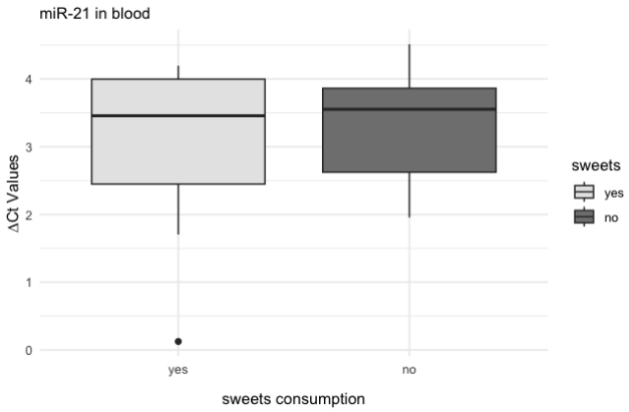

Figure 17: ΔCt values of miR-21 in blood according to sweets consumption

12.6.2 miR-21 (oral mucosa)

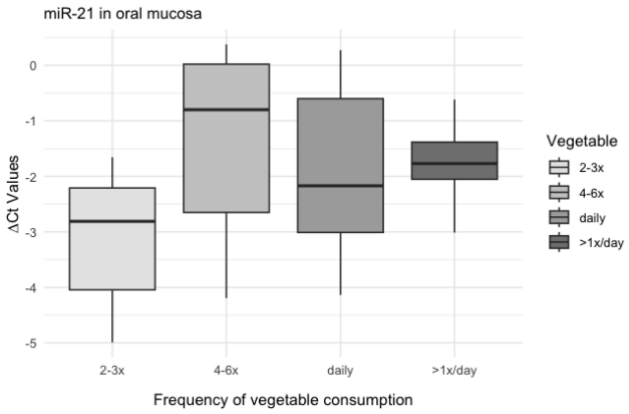

Figure 18: ΔCt values of miR-21 in oral mucosa according to vegetable consumption per week

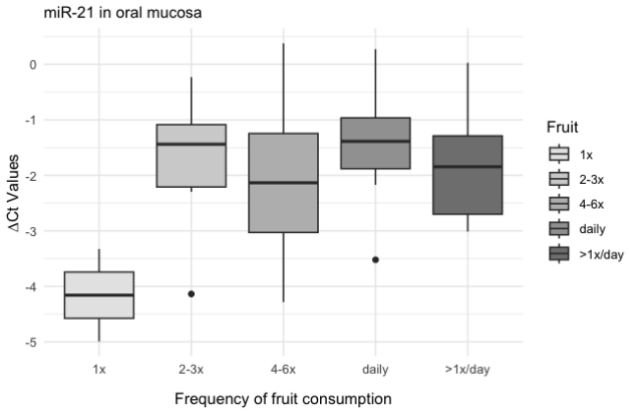

Figure 19: ΔCt values of miR-21 in oral mucosa according to fruit consumption per week

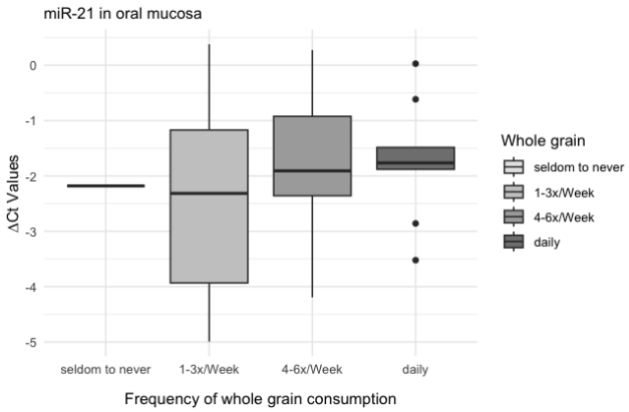

Figure 20: ΔCt values of miR-21 in oral mucosa according to whole grain consumption per week

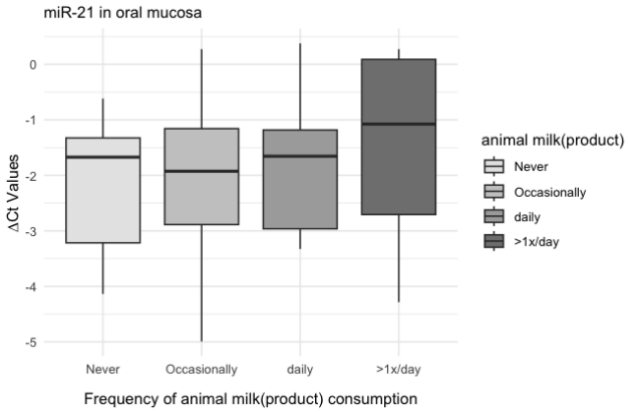

Figure 21: ΔCt values of miR-21 in oral mucosa according to animal milk (product) consumption

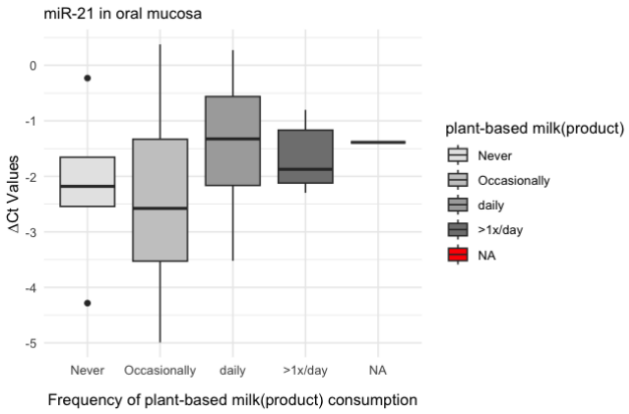

Figure 22: ΔCt values of miR-21 in oral mucosa according to plant-based milk(product) consumption

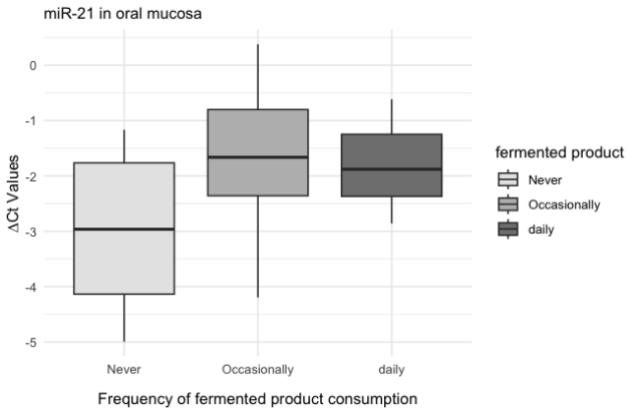

Figure 23: ΔCt values of miR-21 in oral mucosa according to fermented product consumption

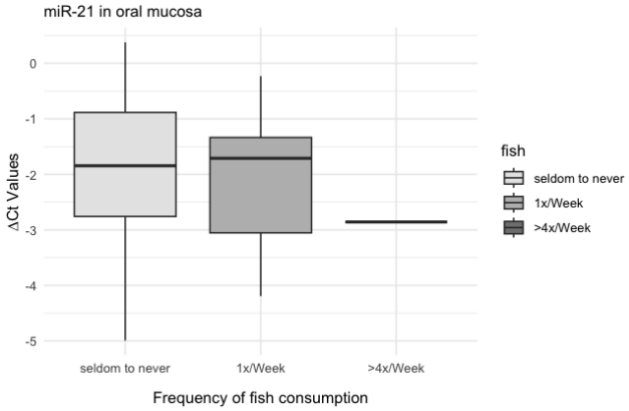

Figure 24: ΔCt values of miR-21 in oral mucosa according to fish consumption

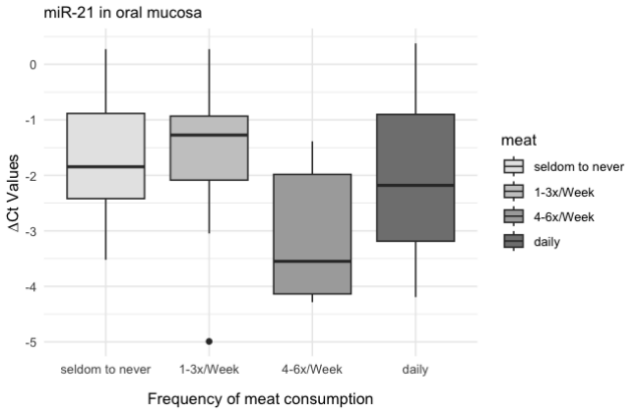

Figure 25: ΔCt values of miR-21 in oral mucosa according to meat consumption

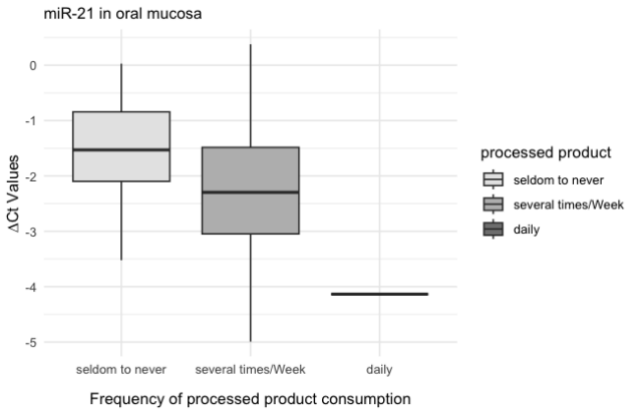

Figure 26: ΔCt values of miR-21 in oral mucosa according to fish consumption

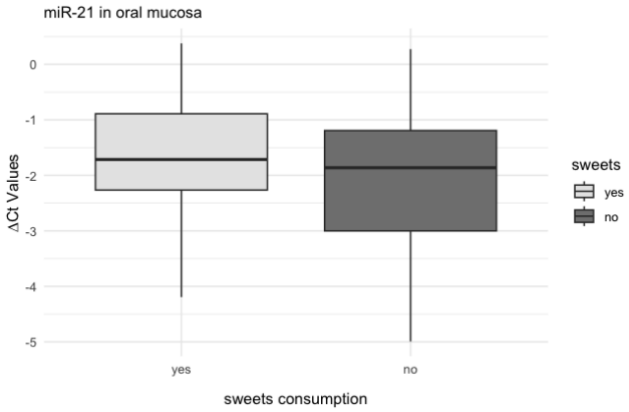

Figure 27: ΔCt values of miR-21 in oral mucosa according to sweets consumption

12.6.3 miR-24 (capillary blood)

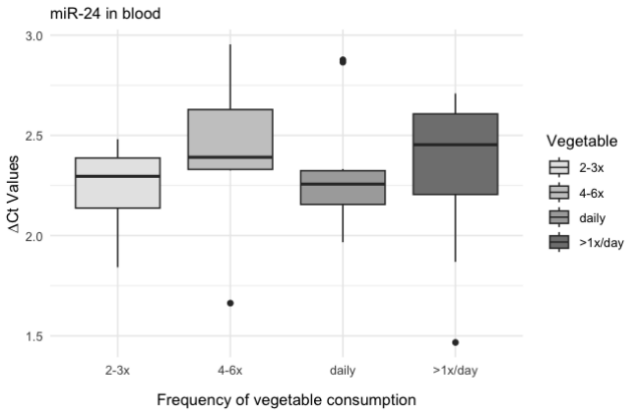

Figure 28: ΔCt values of miR-24 in blood according to vegetable consumption per week

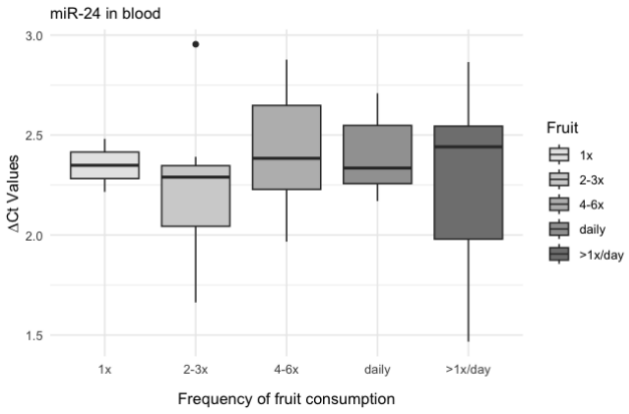

Figure 29: ΔCt values of miR-24 in blood according to fruit consumption per week

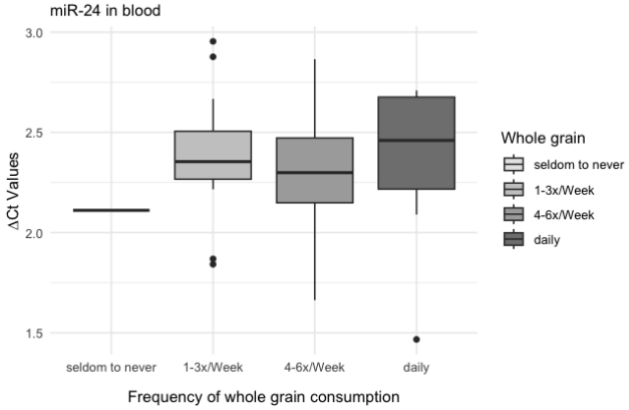

Figure 30: ΔCt values of miR-24 in blood according to vegetable consumption

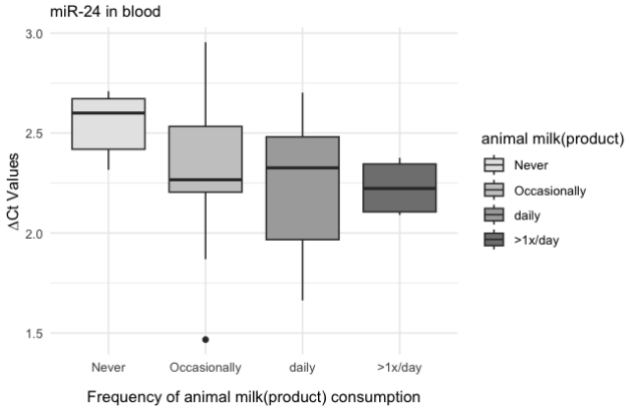

Figure 31: ΔCt values of miR-24 in blood according to animal milk(product) consumption

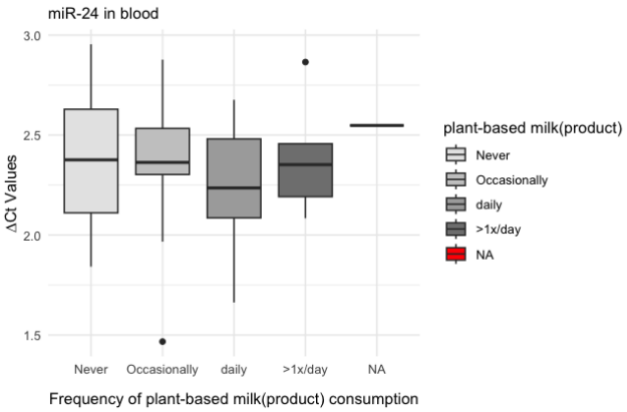

Figure 32: ΔCt values of miR-24 in blood according to plant-based milk(product) consumption

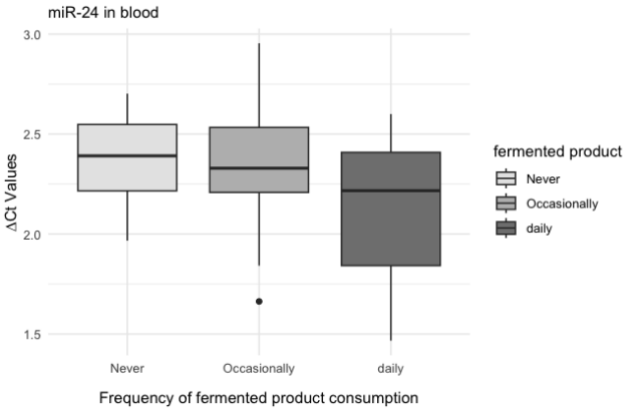

Figure 33: ΔCt values of miR-24 in blood according to fermented product consumption

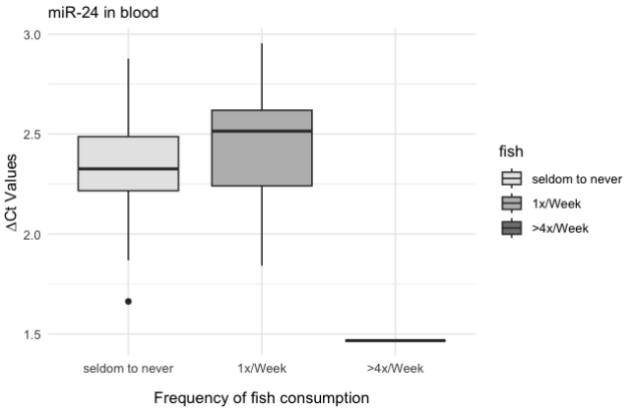

Figure 34: ΔCt values of miR-24 in blood according to fish consumption

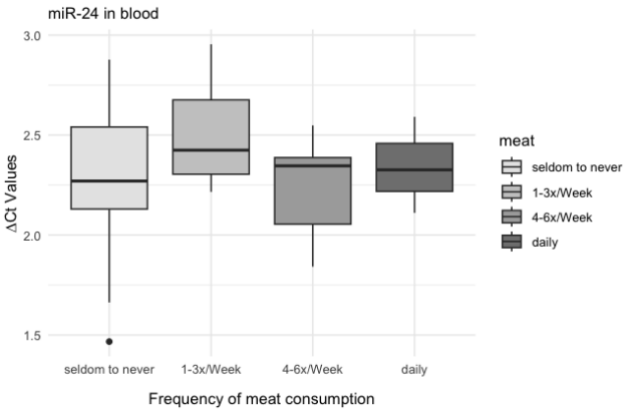

Figure 35: ΔCt values of miR-24 in blood according to meat consumption

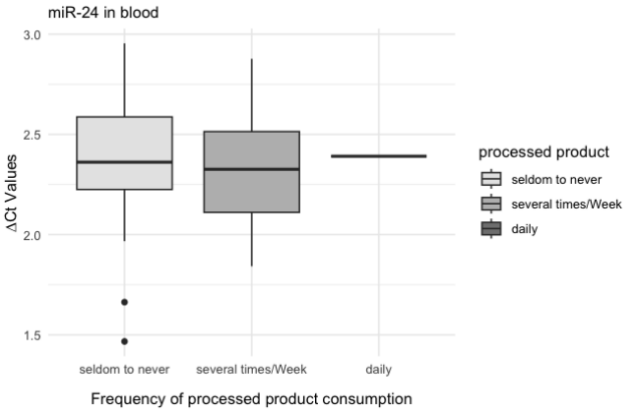

Figure 36: ΔCt values of miR-24 in blood according to processed product consumption

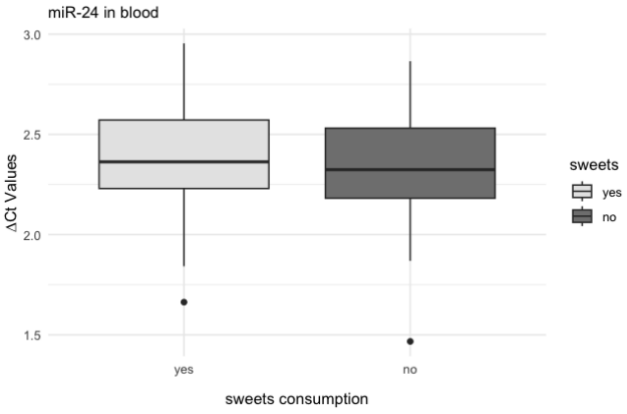

Figure 37: ΔCt values of miR-24 in blood according to sweets consumption

12.6.4 miR-24 (oral mucosa)

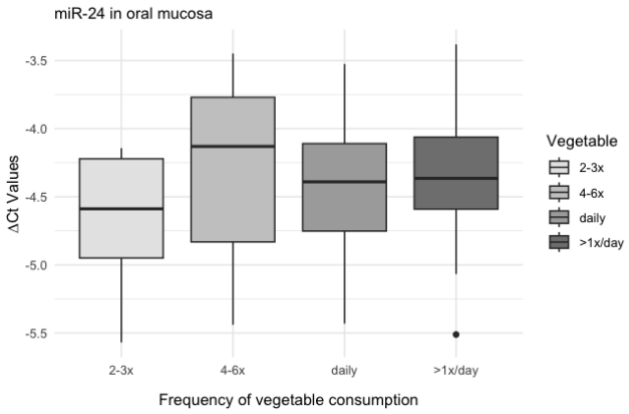

Figure 38: ΔCt values of miR-24 in oral mucosa according to vegetable consumption per week

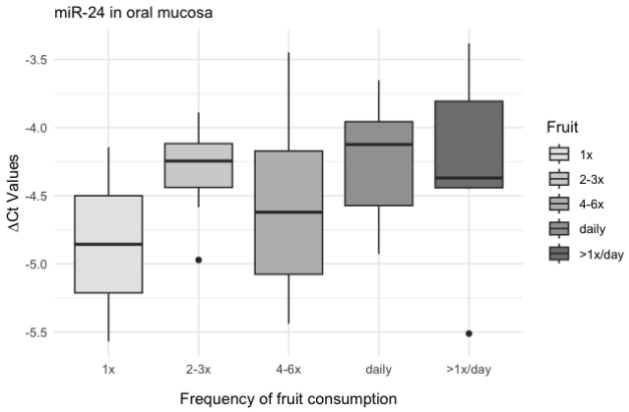

Figure 39: ΔCt values of miR-24 in oral mucosa according to fruit consumption per week

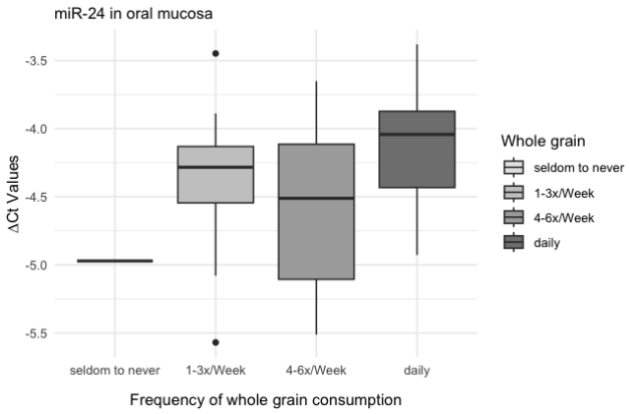

Figure 40: ΔCt values of miR-24 in oral mucosa according to whole grain consumption

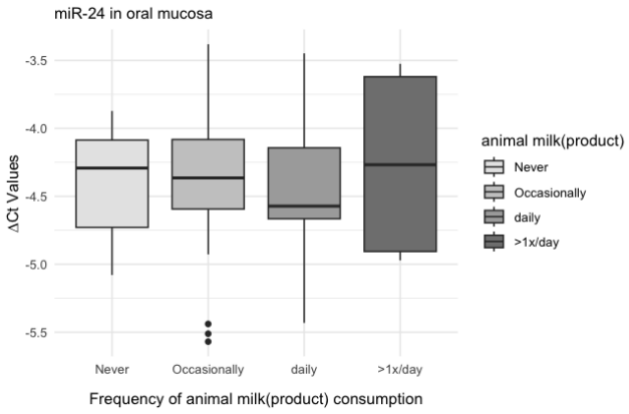

Figure 41: ΔCt values of miR-24 in oral mucosa according to animal milk(product) consumption

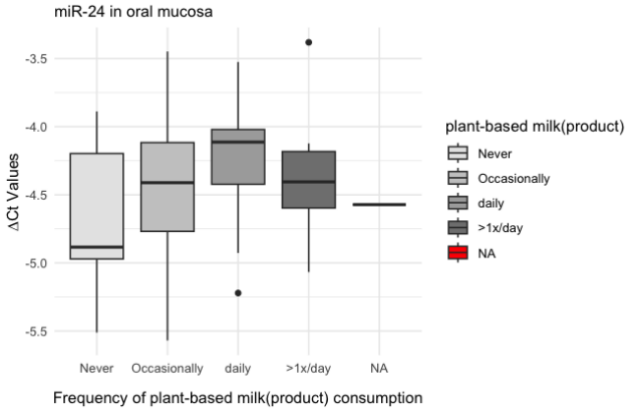

Figure 42: ΔCt values of miR-24 in oral mucosa according to plant-based milk(product) consumption

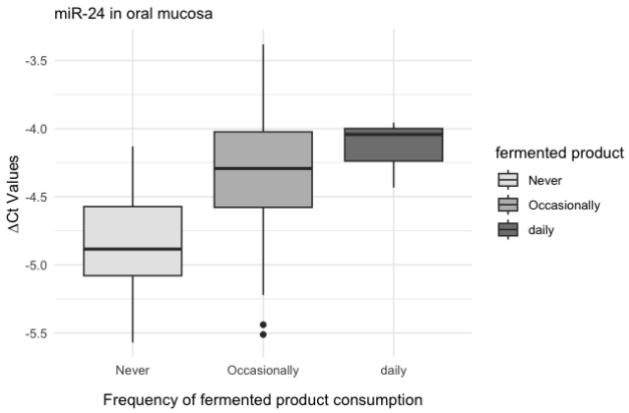

Figure 43: ΔCt values of miR-24 in oral mucosa according to fermented product consumption

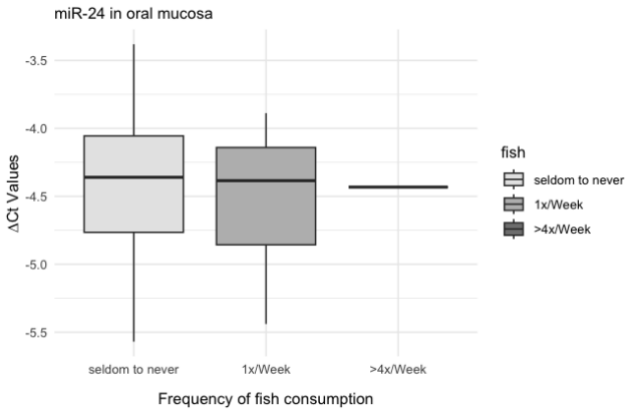

Figure 44: ΔCt values of miR-24 in oral mucosa according to fish consumption

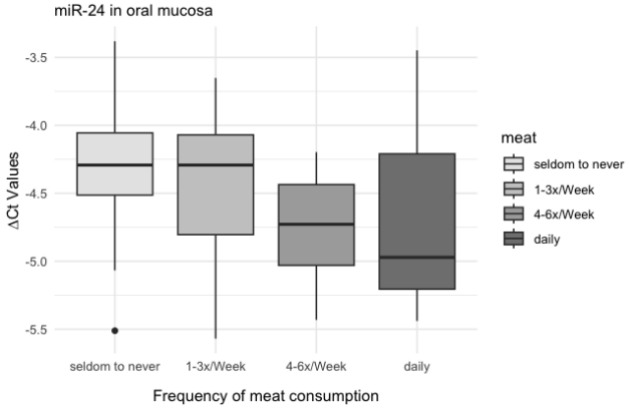

Figure 45: ΔCt values of miR-24 in oral mucosa according to meat consumption

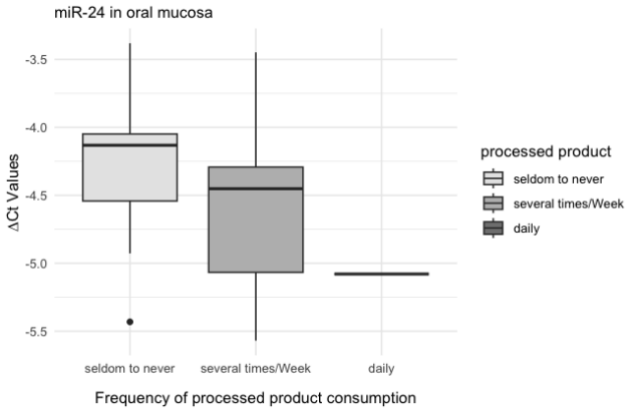

Figure 46: ΔCt values of miR-24 in oral mucosa according to processed product consumption

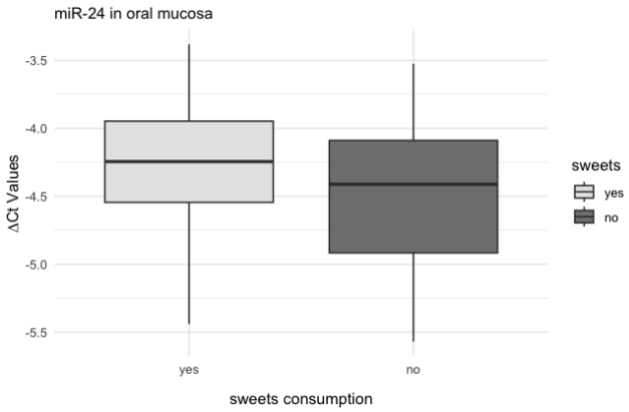

Figure 47: ΔCt values of miR-24 in oral mucosa according to sweets consumption

12.6.5 miR-142 (capillary blood)

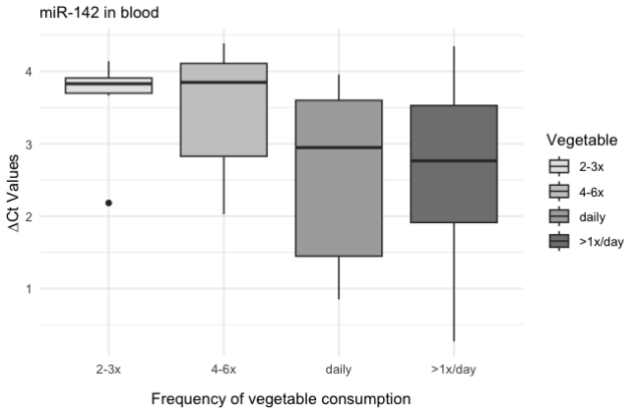

Figure 48: ΔCt values of miR-142 in blood according to vegetable consumption per week

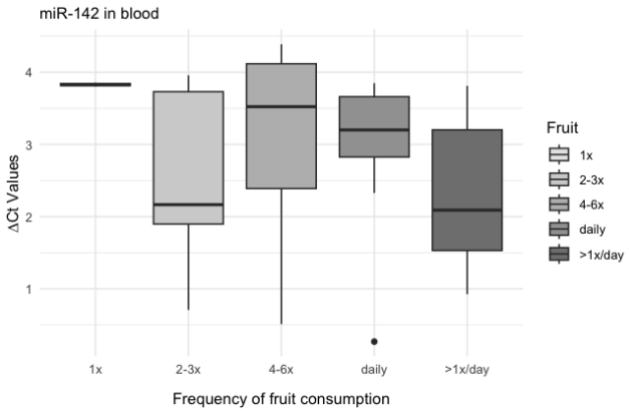

Figure 49: ΔCt values of miR-142 in blood according to fruit consumption per week

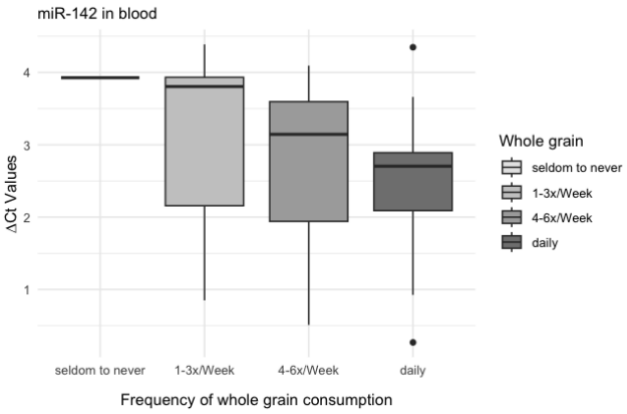

Figure 50: ΔCt values of miR-142 in blood according to whole grain consumption

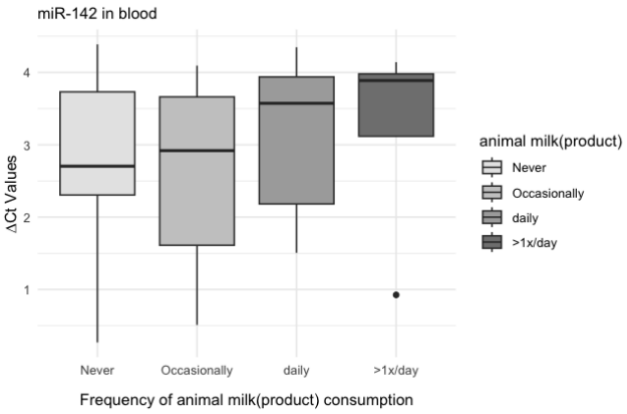

Figure 51: ΔCt values of miR-142 in blood according to animal milk(product) consumption

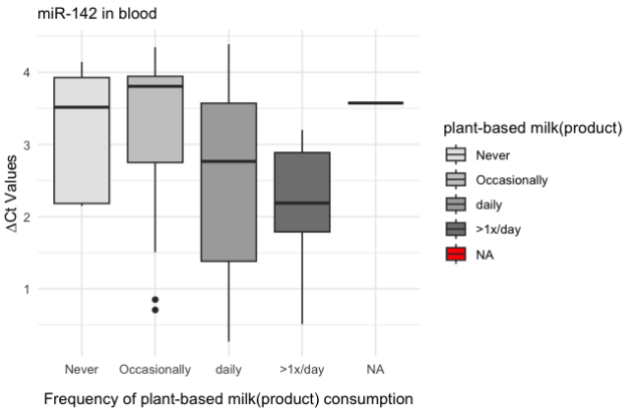

Figure 52: ΔCt values of miR-142 in blood according to plant-based milk(product) consumption

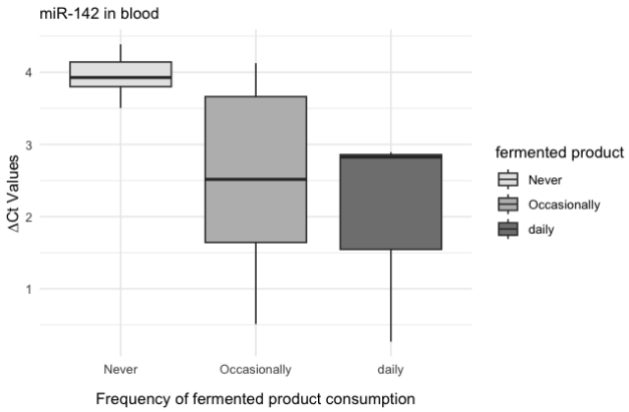

Figure 53: ΔCt values of miR-142 in blood according to fermented product consumption

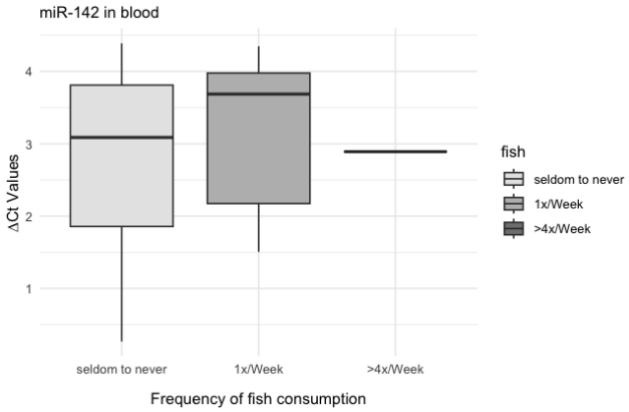

Figure 54: ΔCt values of miR-142 in blood according to fish consumption

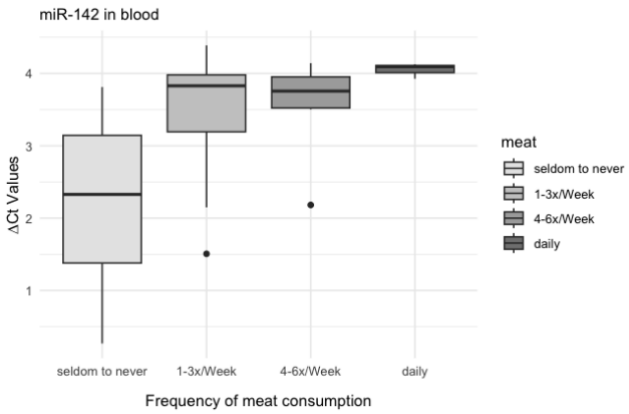

Figure 55: ΔCt values of miR-142 in blood according to meat consumption

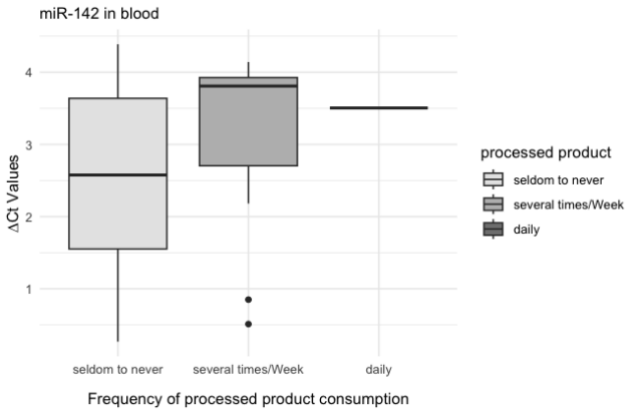

Figure 56: ΔCt values of miR-142 in blood according to processed product consumption

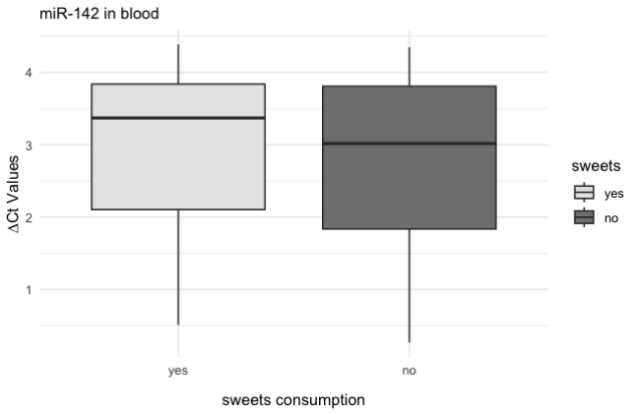

Figure 57: ΔCt values of miR-142 in blood according to sweets consumption

12.6.6 miR-146a (capillary blood)

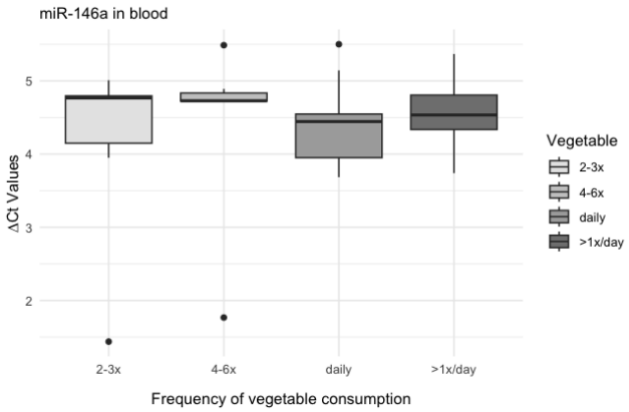

Figure 58: ΔCt values of miR-146a in blood according to vegetable consumption per week

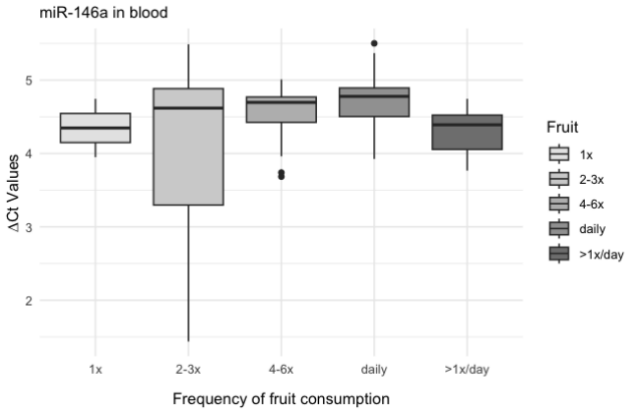

Figure 59: ΔCt values of miR-146a in blood according to fruit consumption per week

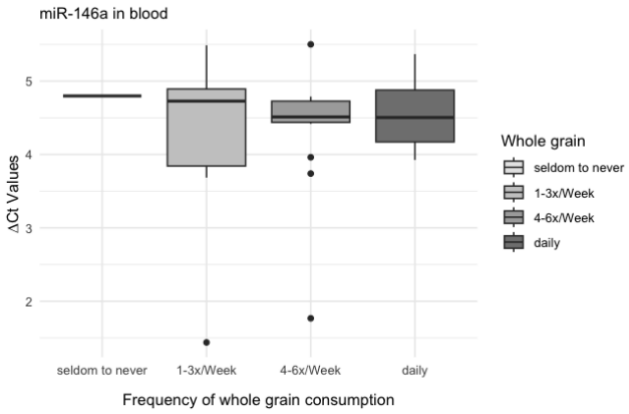

Figure 60: ΔCt values of miR-146a in blood according to whole grain consumption

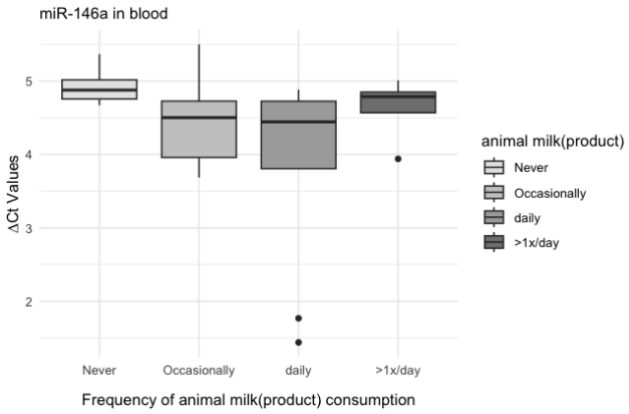

Figure 61: ΔCt values *of miR-146a in blood according to animal milk(product) consumption*

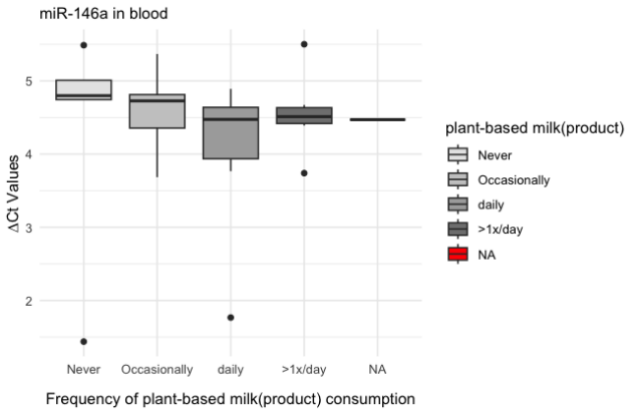

Figure 62: ΔCt values of miR-146a in blood according to plant-based milk(product) consumption

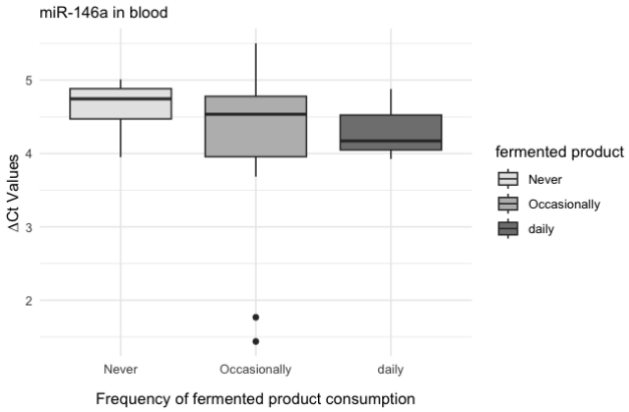

Figure 63: ΔCt values of miR-146a in blood according to fermented product consumption

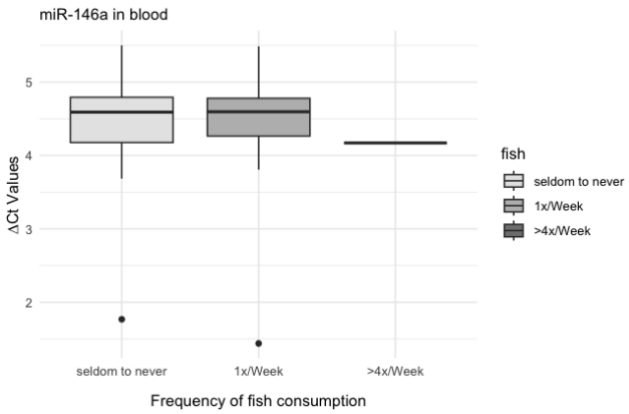

Figure 64: ΔCt values of miR-146a in blood according to fish consumption

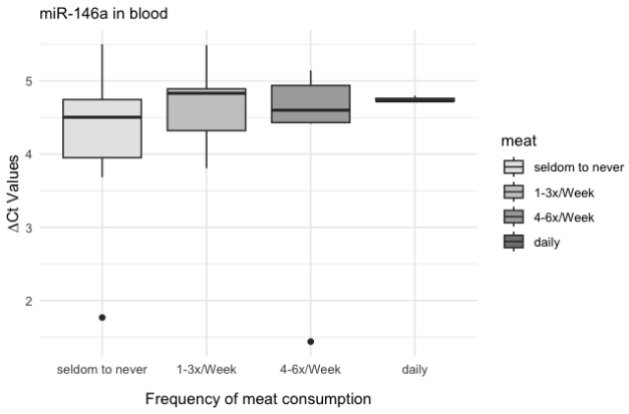

Figure 65: ΔCt values of miR-146a in blood according to meat consumption

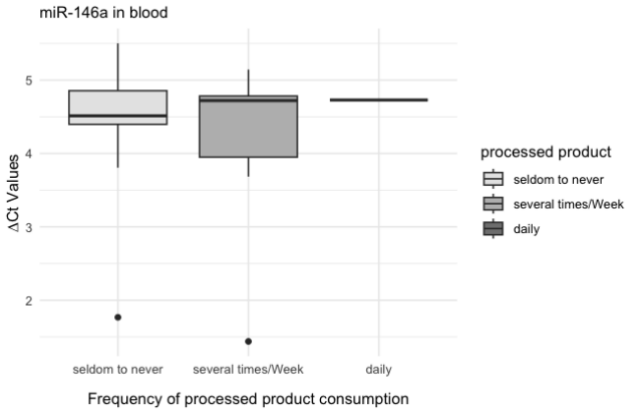

Figure 66: ΔCt values of miR-146a in blood according to processed product consumption

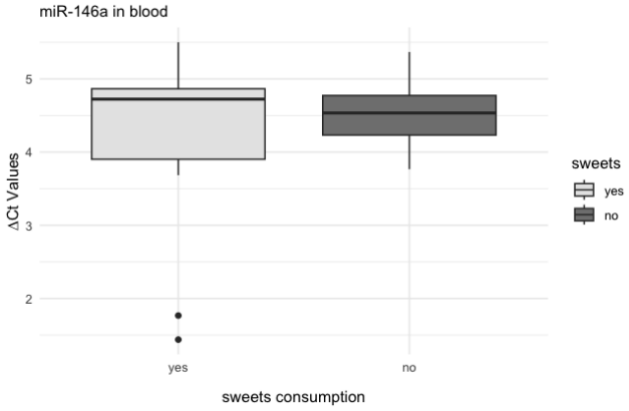

Figure 67: ΔCt values of miR-146a in blood according to sweets consumption

12.6.7 miR-146a (oral mucosa)

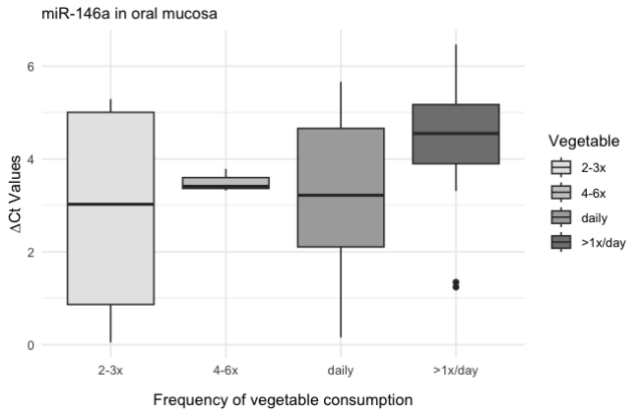

Figure 68: ΔCt values of miR-146a in oral mucosa according to vegetable consumption per week

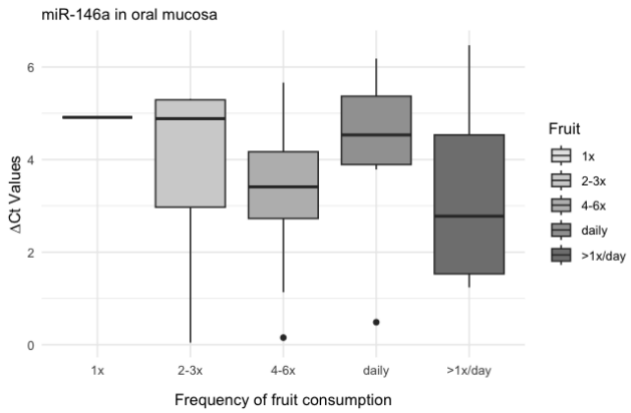

Figure 69: ΔCt values of miR-146a in oral mucosa according to fruit consumption per week

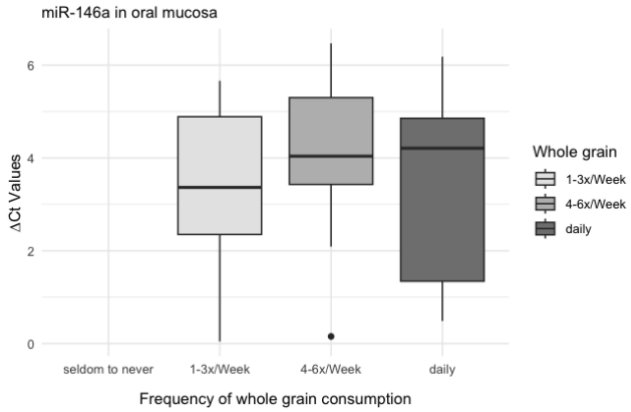

Figure 70: ΔCt values of miR-146a in oral mucosa according to whole grain consumption

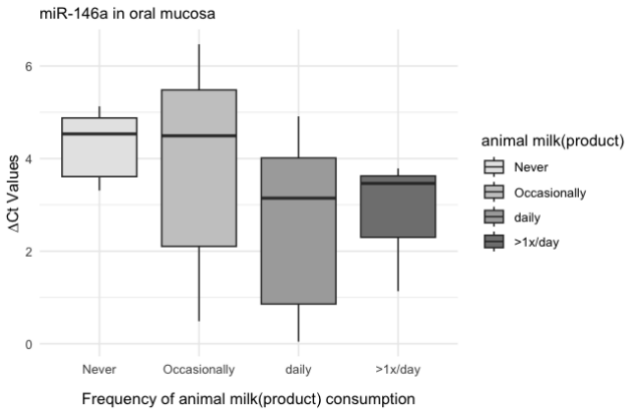

Figure 71: ΔCt values of miR-146a in oral mucosa according to animal milk(product) consumption

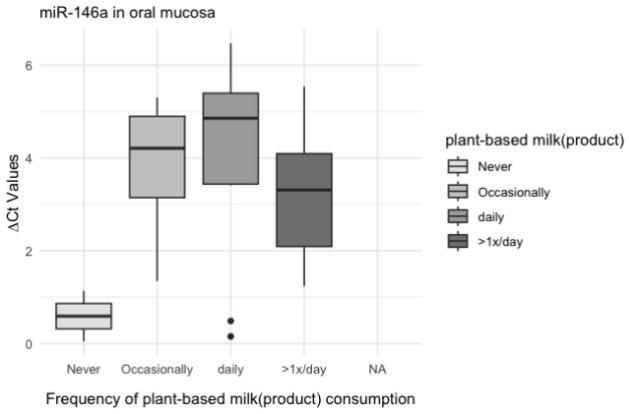

Figure 72: ΔCt values of miR-146a in oral mucosa according to plant-based milk(product) consumption

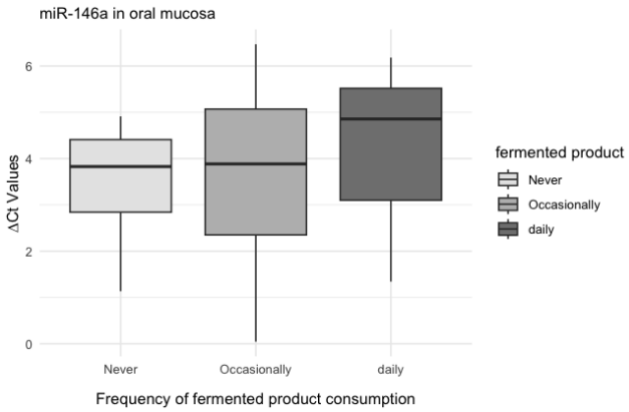

Figure 73: ΔCt values of miR-146a in oral mucosa according to fermented product consumption

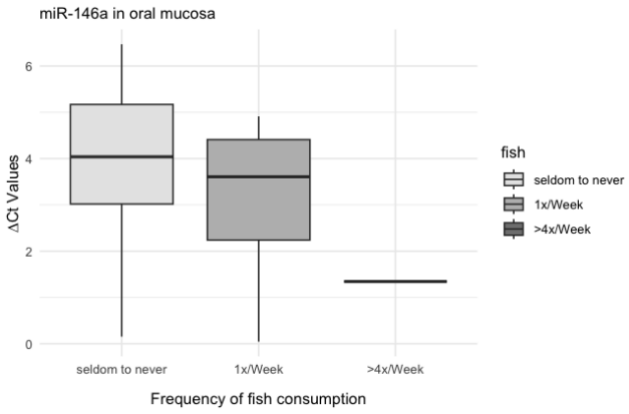

Figure 74: ΔCt values of miR-146a in oral mucosa according to fish consumption

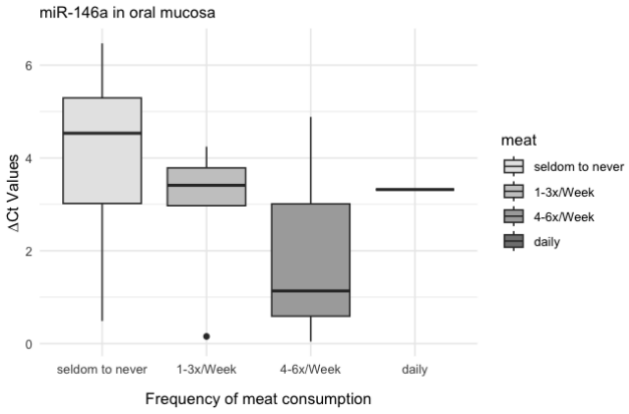

Figure 75: ΔCt values of miR-146a in oral mucosa according to meat consumption

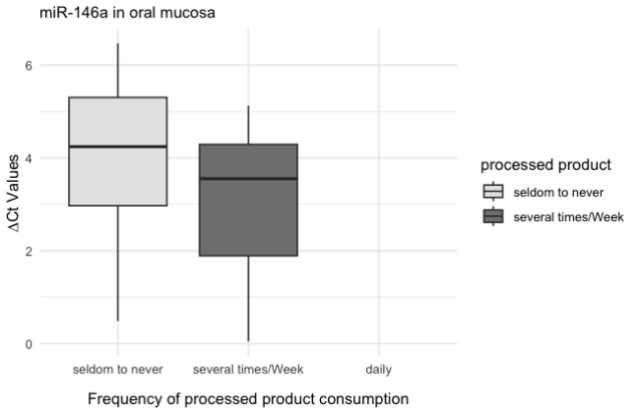

Figure 76: ΔCt values of miR-146a in oral mucosa according to processed product consumption

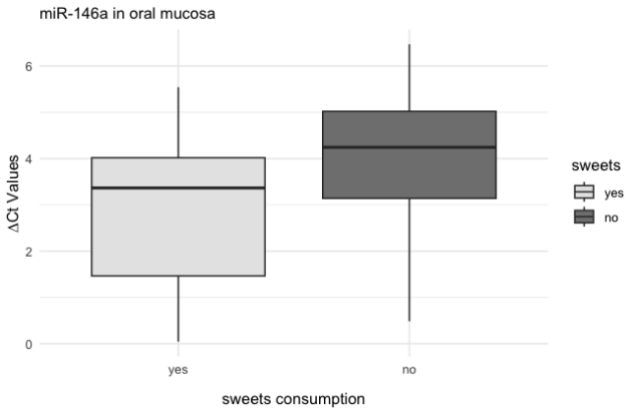

Figure 77: ΔCt values of miR-146a in oral mucosa according to sweets consumption