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The Link Between Diet and Circulating miRNAs with Hepatic Relevance: Identifying Nutritional Risk Profiles for Liver Aging in a Preventive Cohort Study

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Clara Preyer BSc

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMPK	–	*AMP-activated protein kinase*
BH-FDR	–	*Benjamini-Hochberg false discovery rate*
BMI	–	*Body mass index*
cDNA	–	*Complementary DNA*
DBS	–	*Dried blood spots*
df	–	*Degree(s) of freedom*
DHA	–	*Docosahexaenoic acid*
DNA	–	*Deoxyribonucleic acid*
EPA	–	*Eicosapentaenoic acid*
FB	–	*Fragebogen (questionnaire subsample)*
GRB10	–	*Growth factor receptor-bound protein 10*
HDAC	–	*Histone deacetylase*
HNF4α	–	*Hepatocyte nuclear factor 4 alpha*
HSC	–	*Hepatic stellate cell*
IQR	–	*Interquartile range*
IRAK1	–	*IL-1 receptor-associated kinase 1*
MAR	–	*Missing at random*
MASLD	–	*Metabolic dysfunction-associated steatotic liver disease*
MCAR	–	*Missing completely at random*
miRNA	–	*MicroRNA*
MNAR	–	*Missing not at random*
NF-κB	–	*Nuclear factor kappa B*
PDCD4	–	*Programmed cell death protein 4*
PPARα	–	*Peroxisome proliferator-activated receptor alpha*
PTEN	–	*Phosphatase and tensin homolog*
PUFA	–	*Polyunsaturated fatty acid*
qPCR	–	*quantitative polymerase chain reaction*
RNA	–	*Ribonucleic acid*
SASP	–	*Senescence-associated secretory phenotype*
SCFA	–	*Short-chain fatty acids*
SD	–	*Standard deviation*
SEB	–	*Staphylococcal enterotoxin B*

SIRT1	–	*Sirtuin 1*
SMAD	–	*Small mothers against decapentaplegic*
TGF-β	–	*Transforming growth factor beta*
TNF	–	*Tumor necrosis factor*
TRAF6	–	*TNF receptor-associated factor 6*
UPF	–	*Ultra-processed food*
VCAN	–	*Versican*

ABSTRACT

The liver ages silently long before clinical disease becomes visible, and microRNAs (miRNAs) are increasingly recognized as sensitive biomarkers of these early molecular changes. Emerging nutrimiromics research suggests that everyday food choices might leave a detec table indicator in miRNA expression patterns, but human data supporting this link in preventive settings remain scarce.

Against this background, this study explores whether habitual diet and lifestyle are reflected in a panel of six senescence- and inflammation-related circulating miRNAs with hepatic relevance (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p) in adults.

Circulating miRNA with hepatic relevance were quantified from capillary blood collected on filter cards in 81 adults. Dietary and lifestyle information was available for a subset of 48 participants. Multivariable regression models adjusted for age, body mass index (BMI) and sex-linked simplified indicators of diet quality and lifestyle variables to miRNA ΔCt values. Three pre-specified hypothesis-driven model blocks focused on omega-3 intake, plant-based diet quality, and ultra-processed food (UPF) consumption.

Across this panel, one pattern emerged most clearly: participants reporting more diverse vegetable intake tended to show a lower ΔCt values for the hepatoprotective miRNA miR-130a-3p ($\beta = -0.95$, $q = 0.039$, $n = 11$), i.e. higher expression, whereas other dietary and lifestyle factors displayed only weak correspondence with miRNA expression after multiple-testing adjustment. Functionally, these miRNA patterns may mirror shifts in hepatic pathways related to metabolic stress, inflammatory signaling, and cellular senescence, offering an early molecular readout of how diet shapes liver aging. Given the modest sample sizes, technical constraints of the miRNA panel, and the coarse categorization of dietary exposures, these findings are exploratory, but they still point toward vegetable diversity as a promising nutritional lever for molecular markers of liver aging.

Altogether, the study highlights miRNA profiles as a promising tool for studying how diet shapes the molecular trajectory of liver aging, while further research in larger and more heterogeneous samples will be essential to validate these exploratory results and clarify their significance for nutrition-focused prevention and biomarker research.

ZUSAMMENFASSUNG

Die Leber altert unbemerkt, lange bevor klinische Erkrankungen sichtbar werden, und Mikro-RNAs (miRNAs) werden zunehmend als sensible Marker früher molekularer Veränderungen anerkannt. Neue Erkenntnisse aus der Nutrimiromik deuten darauf hin, dass alltägliche Ernährungsgewohnheiten einen nachweisbaren Fingerabdruck in miRNA-Expressionsmustern hinterlassen könnten, doch liegen bislang nur wenige Daten aus humanen Präventionskohorten vor, die diesen Zusammenhang belegen.

Vor diesem Hintergrund untersucht die vorliegende Studie, ob sich die gewohnheitsmäßige Ernährung und der Lebensstil in einem Panel von sechs seneszenz- und entzündungsbezogenen hepatischen miRNAs (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p) bei Erwachsenen widerspiegeln.

Zirkulierende miRNAs mit Leberrelevanz wurden aus Kapillarblut von 81 Erwachsenen quantifiziert, das auf Filterkarten aufgebracht wurde. Für eine Untergruppe von 48 Teilnehmern lagen Informationen zu Ernährung und Lebensstil vor. Multivariable Regressionsmodelle, adjustiert für Alter, Body-Mass-Index (BMI) und Geschlecht, verknüpften vereinfachte Ernährungsqualitäts- und Lebensstilindikatoren mit den miRNA-ΔCt-Werten, ergänzt durch drei vordefinierte hypothesengesteuerte Modellblöcke zu Omega-3-Zufuhr, pflanzenbasierter Ernährungsqualität und Konsum ultra-verarbeiteter Lebensmittel.

Über das Panel hinweg zeigte sich ein Muster am deutlichsten: Teilnehmer mit höherer Gemüsevielfalt im Ernährungsplan wiesen tendenziell niedrigere ΔCt-Werte der leberschützenden miRNA miR-130a-3p (β = -0,95, q = 0,039, n = 11), entsprechend einer höheren Expression, während andere Ernährungs- und Lebensstilfaktoren nach Mehrfachtestkorrektur schwache Assoziationen zeigten. Diese miRNA-Muster könnten frühe, ernährungsbedingte Veränderungen in Stoffwechsel- und Entzündungsprozessen der Leber widerspiegeln. Angesichts der geringen Stichprobengröße, technischer Einschränkungen des Panels und der groben Kategorisierung der Ernährung sind diese Ergebnisse explorativ, deuten aber auf Gemüsevielfalt als potenziellen Hebel für molekulare Marker der Leberalterung hin.

Die Studie unterstreicht das Potenzial der Bestimmung von miRNA-Profilen als vielversprechendes Instrument zur Untersuchung ernährungsbedingter Einflüsse auf den molekularen Verlauf der Leberalterung; weitere Forschung an größeren Stichproben bleibt zur Validierung dieser vorläufigen Befunde unerlässlich.

1. INTRODUCTION

Healthy aging is increasingly understood as a process that begins long before clinical manifestations become apparent and is strongly shaped by chronic low-grade inflammation ("inflammaging"). In this work, the liver is considered a central organ in this trajectory because age-related inflammatory remodeling in hepatic tissue can influence systemic aging even before standard laboratory markers change (Ekiz et al., 2020; Xing & Zhu, 2025). This makes hepatic aging a particularly relevant entry point for preventive, nutrition-focused strategies.

Capturing these early molecular shifts requires markers that respond earlier and more specifically than conventional clinical chemistry. MiRNAs are well suited to this purpose, because they can be quantified from a few drops of capillary blood, respond to habitual diet and lifestyle, and reflect epigenetic processes that nutrition is able to modulate (Hochreuter et al., 2022). The detailed regulatory biology of these molecules is introduced in Section 3.2.

The six miRNAs investigated in the present study were selected to represent the inflammatory balance of the aging liver. MiR-21, miR-34a, and miR-155-5p act as pro-senescent and pro-inflammatory drivers, while miR-146a, miR-126a-5p and miR-130a-3p exert regulatory and hepatoprotective effects. The relative weight between these two opposing groups defines the molecular phenotype that this work explores in the context of habitual diet and lifestyle (Figure 1).

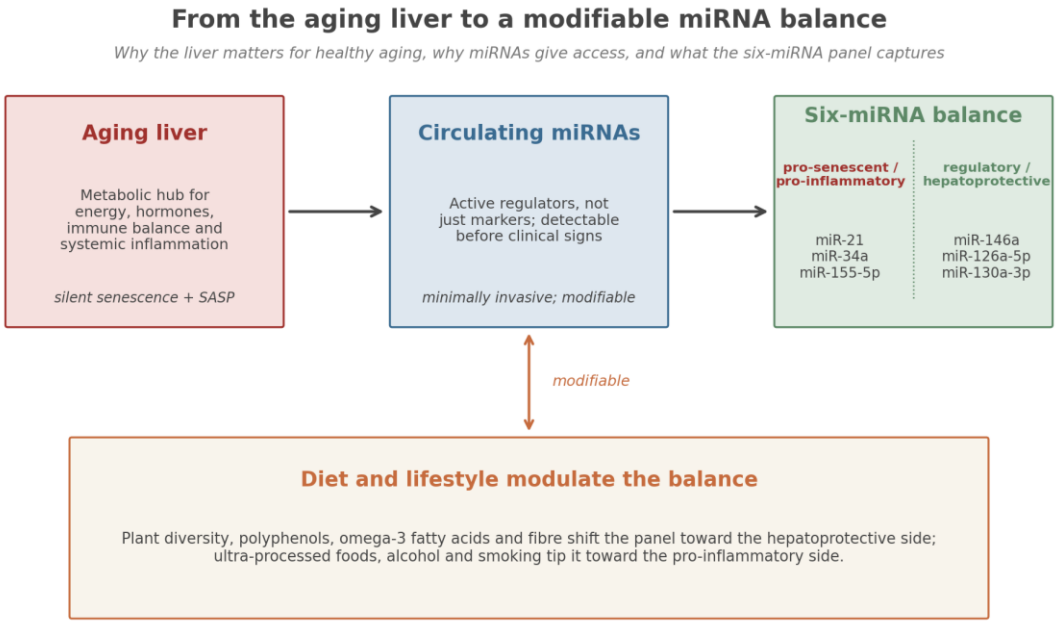

Figure 1: Conceptual overview of the aging liver, circulating miRNAs as a modifiable access point, and the six-miRNA balance investigated in the present study

1.1. Liver Aging and Metabolic Diseases

The liver is the key organ for metabolic homeostasis, performing essential functions in lipid and glucose metabolism, detoxification, and immune regulation. With advancing age, the liver undergoes structural and functional changes, including reduced regenerative capacity, increased oxidative stress, and accumulation of senescent cells (Lin et al., 2025). Cellular senescence, characterized by irreversible cell cycle arrest and the secretion of pro-inflammatory factors known as the senescence-associated secretory phenotype (SASP), is now recognized as a hallmark of liver aging that contributes to chronic inflammation, fibrosis, and impaired tissue repair (Xing & Zhu, 2025; Figure 2).

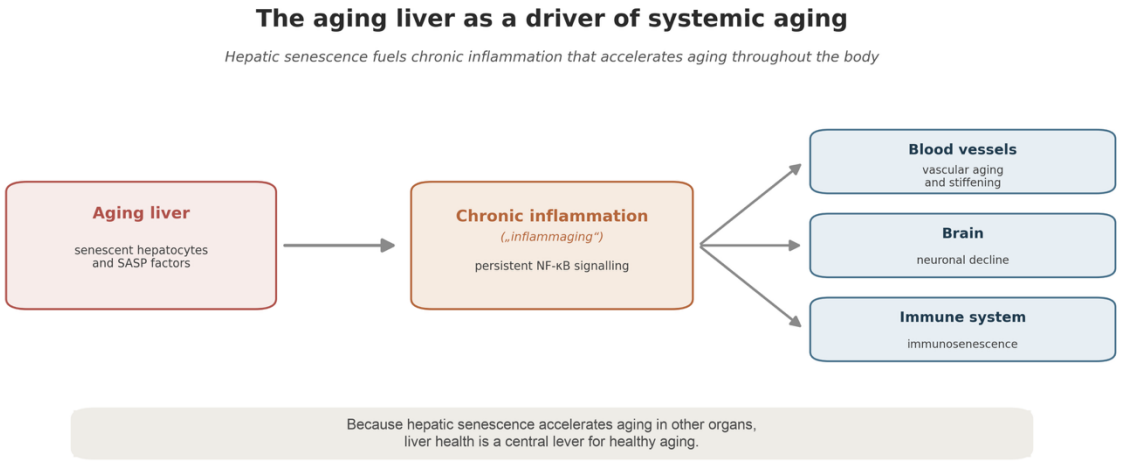

Figure 2: The aging liver as a driver of systemic aging via inflammaging (adapted from Xing & Zhu, 2025; Ekiz et al., 2020).

These age-related changes are closely linked to the growing global burden of metabolic dysfunction-associated steatotic liver disease (MASLD), formerly also known as non-alcoholic fatty liver disease (NAFLD). A recent meta-analysis estimated the worldwide prevalence of MASLD at 38% of all adults (2016-2019), representing a 50% increase since 1990-2006, with projections suggesting a rise to over 55% by 2040 (Younossi et al., 2025; Figure 3). MASLD encompasses a spectrum from simple steatosis to steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma, and has emerged as one of the leading indications for liver transplantation, particularly in the United States (Le et al., 2025). The rising prevalence of obesity and type 2 diabetes as primary risk factors underlines the need for preventive strategies that target the molecular mechanisms driving hepatic aging and disease progression (Michalopoulou et al., 2025).

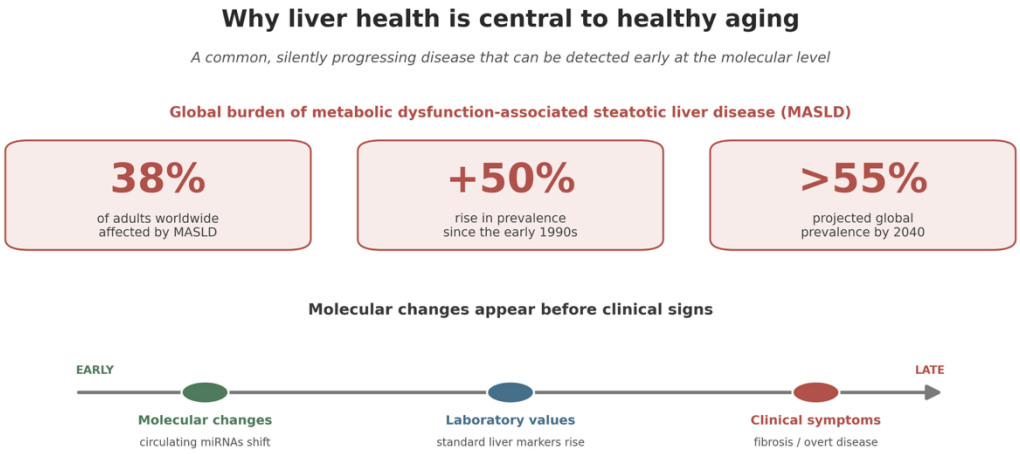

Figure 3: Global burden of MASLD and conceptual sequence of molecular, laboratory and clinical changes (schematic; based on Younossi et al., 2025).

Most importantly, preclinical evidence suggests that dietary intervention can reverse the expression of molecular markers of hepatocellular senescence. In a mouse model of diet-induced steatohepatitis, dietary modification led to substantial improvements in metabolic and histological hallmarks of non-alcoholic steatohepatitis (NASH), accompanied by progressive clearance of senescent hepatocellular cells and suppression of SASP-related gene expression (Flensted-Jensen et al., 2024). These findings highlight the potential for nutrition-based approaches to modulate liver aging at the molecular level.

1.2. MiRNAs as Regulators of Hepatic Function

MiRNAs are small, non-coding ribonucleic acid (RNA) molecules of approximately 21-23 nucleotides that regulate gene expression post-transcriptionally by binding to the 3' untranslated region of target messenger RNAs, leading to mRNA (messenger ribonucleic acid) degradation or translational repression. A single miRNA can regulate hundreds of target genes, and miRNAs are involved in virtually all major biological processes, including cell proliferation, apoptosis, differentiation, and immune regulation (DeLucas et al., 2024; Flensted-Jensen et al., 2024). In the liver, miRNAs play critical roles in lipid metabolism, inflammatory signaling, fibrogenesis, and the regulation of cellular senescence (Hochreuter et al., 2022).

Beyond their intracellular regulatory roles, miRNAs are also secreted into the circulation. Once exported, they are protected from rapid enzymatic degradation by being packaged into small vesicles or bound to carrier molecules, which gives them remarkable stability in plasma and serum (Mori et al., 2019). This stability underpins their use as biomarkers of hepatic injury and

senescence, particularly during the long preclinical phase of MASLD progression (Hochreuter et al., 2022).

The emerging field of nutrimiromics investigates how habitual diet and specific food components modulate miRNA expression and thereby influence metabolic health and chronic disease risk (Chodur & Steinberg, 2024; DeLucas et al., 2024). Dysregulation of hepatic miRNAs has been consistently observed across the spectrum of MASLD, from early steatosis to advanced fibrosis and hepatocellular carcinoma, and several miRNAs have been identified as key mediators of liver pathology and potential biomarkers for disease staging (Hochreuter et al., 2022). The present study focuses on a panel of six miRNAs with established roles in hepatic senescence, inflammation and metabolic regulation:

miR-21 is one of the most consistently upregulated miRNAs in hepatic fibrosis and steatosis. It promotes pro-fibrotic signaling by targeting phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) and contributes to hepatic stellate cell (HSC) activation. MiR-21 expression increases with the progression of MASLD and has been implicated in lipogenesis and inflammatory pathways (Hochreuter et al., 2022).

miR-34a is a p53-regulated miRNA that is elevated in MASLD and increases with disease severity. It promotes hepatocyte apoptosis, inhibits fatty acid oxidation by repressing PPARα (peroxisome proliferator-activated receptor alpha), and disrupts cholesterol homeostasis by targeting Sirtuin 1 (SIRT1) and hepatocyte nuclear factor 4 alpha (HNF4α); miR-34a is considered one of the most reliable diagnostic miRNA markers for MASLD (Hochreuter et al., 2022).

miR-155-5p is a major regulator of inflammatory responses in the liver. It is transcriptionally induced by nuclear factor kappa B (NF-κB) signaling and promotes M1 macrophage polarization in Kupffer cells, contributing to steatohepatitis and fibrosis. miR-155 knockout mice are protected from alcohol-induced liver injury and show attenuated fibrosis markers (Bala et al., 2016).

miR-146a functions as a negative feedback regulator of the NF-κB inflammatory cascade by targeting the signaling intermediates IL-1 receptor-associated kinase 1 (IRAK1) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). It acts in opposition to miR-155: while miR-155 amplifies inflammatory responses, miR-146a restrains them. The balance between miR-155 and miR-146a is critical for immune homeostasis during aging, as

demonstrated in models where loss of miR-146a leads to chronic inflammation and reduced lifespan (Ekiz et al., 2020).

miR-126a-5p is downregulated in the aging liver and plays a protective role against hepatic senescence. It regulates hepatic metabolic function by targeting versican (VCAN), and its deletion promotes telomere attrition, NF-κB pathway activation, and hepatocyte senescence. miR-126a-5p has been proposed as a potential drug target for the treatment of hepatic failure (Yan et al., 2019).

miR-130a-3p has well-characterized roles in hepatic lipid metabolism and anti-fibrotic signaling. It suppresses HSC activation by targeting components of the transforming growth factor beta (TGF-β)/small mothers against decapentaplegic (SMAD) pathway and improves insulin sensitivity by targeting growth factor receptor-bound protein 10 (GRB10). Higher miR-130a-3p expression is associated with lower hepatic lipid accumulation and a more favorable metabolic liver phenotype (Y. Wang et al., 2017; Xiao et al., 2014).

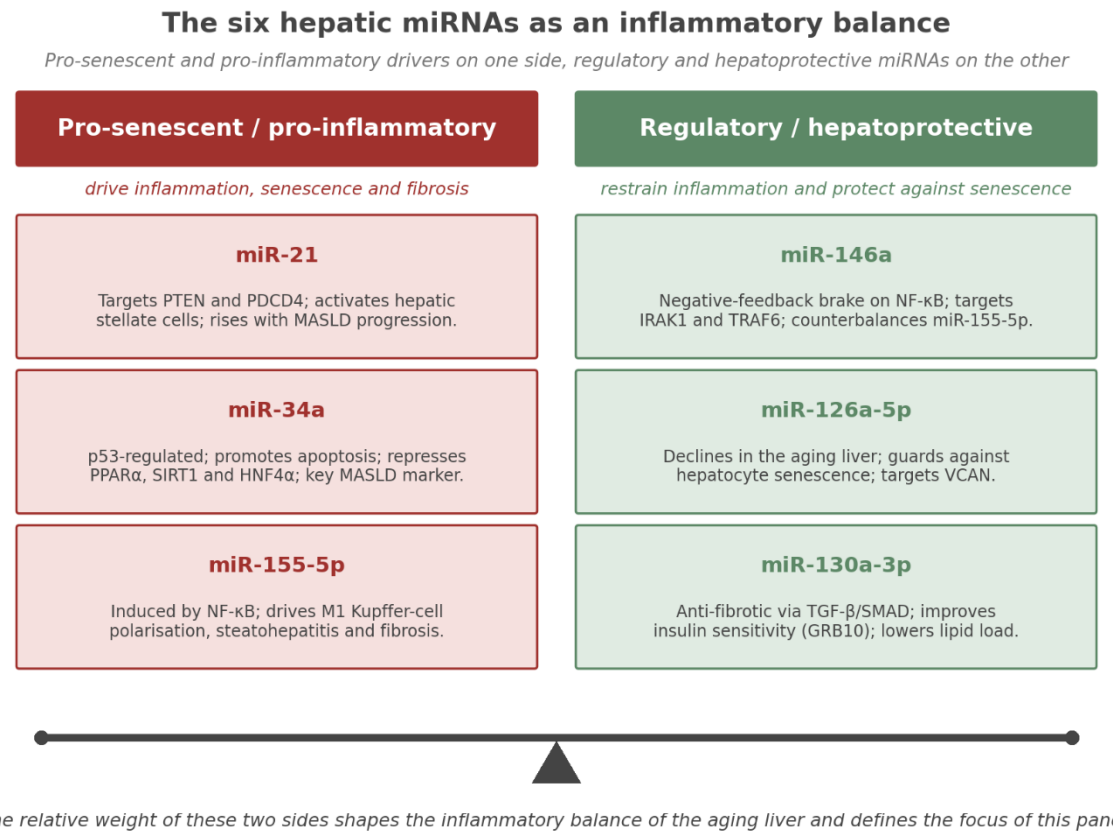

Figure 4: The six liver-associated circulating miRNAs investigated in this study, grouped by regulatory function (compiled from sources cited in Section 3.2).

Together, these six miRNAs span predominantly pro-inflammatory (miR-21, miR-34a, miR-155-5p), regulatory (miR-146a), and hepatoprotective (miR-126a-5p, miR-130a-3p) functions, providing a coherent framework for investigating the molecular basis of liver aging (Figure 4).

1.3. Dietary and Lifestyle Modulation of miRNAs

As outlined above, the emerging field of nutrimiromics investigates how dietary components and nutritional patterns influence miRNA expression and thereby modulate gene regulation networks and disease risk. Evidence from cell culture, animal models, and human trials demonstrates that miRNAs respond to a wide range of dietary exposures, including polyphenols, fatty acids, dietary fiber, and overall dietary patterns, and that these responses are highly context-dependent (Chodur & Steinberg, 2024).

Polyphenols, a structurally diverse group of bioactive plant compounds found in fruits, vegetables, and whole grains, have received particular attention for their ability to modulate miRNA expression relevant to hepatic health. In vitro and animal studies demonstrate that polyphenol-rich treatments can downregulate pro-inflammatory miRNAs such as miR-155 and miR-34a while upregulating hepatoprotective miRNAs, illustrating how a single dietary exposure class can shift the balance between pro- and anti-inflammatory nodes of the hepatic miRNA network (Quintanilha et al., 2017). Plant-derived polyphenols have also been shown to regulate the expression of miRNAs involved in hepatic lipid metabolism and to prevent diet-induced fatty liver disease in experimental models (Bayram et al., 2021). More broadly, polyphenol-rich and Mediterranean dietary patterns have been linked to modulation of miRNAs involved in NF-κB pathway signaling, oxidative stress response and autophagy pathways (DeLucas et al., 2024).

Omega-3 polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), represent another dietary exposure with relevance to hepatic miRNA regulation. EPA- and DHA-derived lipid mediators have been shown to reduce NF-κB activity in liver macrophages and to promote anti-inflammatory macrophage phenotypes in chronic liver disease models (Videla et al., 2023). Given that miR-155-5p and miR-146a both operate within the NF-κB signaling axis, the former amplifying and the latter restraining inflammatory responses, omega-3 PUFAs may selectively modulate the inflammatory tone of this miRNA axis rather than acting on the full panel uniformly. Direct evidence for omega-3-mediated modulation of the specific miRNAs investigated in this study remains limited, but the mechanistic plausibility supports their inclusion in hypothesis-driven analyses.

In contrast, dietary patterns characterized by high UPF consumption have been associated with increased systemic inflammation and metabolic dysfunction (Asensi et al., 2023). Whether these associations extend to the modulation of hepatic miRNAs remains largely unexplored. A scoping review of human studies confirmed that miRNAs are modulated following a variety of dietary interventions, although considerable heterogeneity exists across study populations, intervention types, and miRNA assessment methods. Notably, the intensity of miRNA response appears to be greater in metabolically healthy individuals (Chodur & Steinberg, 2024). This suggests that preventive cohorts may exhibit different miRNA response patterns compared to clinical populations with established metabolic disease.

Lifestyle factors beyond diet also influence miRNA expression. Alcohol consumption is a well-established inducer of hepatic miR-155 expression through NF-κB activation in Kupffer cells (Bala et al., 2011), and smoking has been linked to systemic miRNA dysregulation affecting inflammation-related pathways (Karabegović et al., 2023). These exposures are therefore relevant covariates in any investigation of diet–miRNA associations.

1.4. Knowledge Gap and Study Rationale

Despite growing evidence that dietary components can modulate miRNA expression, several important gaps remain in the current literature. First, most nutrimiromics research has been conducted in cell culture or animal models, and human data remain scarce, particularly from preventive cohorts of generally healthy individuals (Chodur & Steinberg, 2024). Second, existing human studies have predominantly focused on single nutrients or isolated bioactive compounds rather than dietary patterns or diversity measures, which may better capture the cumulative effects of multiple bioactive exposures. Third, few studies have simultaneously investigated multiple miRNAs spanning both pro-inflammatory and hepatoprotective functions, limiting the ability to characterize the selectivity of dietary effects on the miRNA landscape.

Research from the same research group as the present study has begun to address some of these gaps. Pointner et al. (2024) demonstrated that lifestyle-driven variations in nutrimiromic miRNA expression are detectable in human cohorts, with dietary patterns characterized by high vegetable, fruit, and legume intake associated with decreased expression of miR-21 and distinct miRNA profiles. Building on this, Schneider et al. (2025) developed the MiRNA-3Age model, a miRNA-based estimator of biological age in which miR-21 and miR-155-5p were identified as key components, further underscoring the relevance of these miRNAs to biological aging processes.

The present study extends this line of research by investigating whether dietary and lifestyle exposures are associated with the expression of six senescence- and inflammation-related hepatic miRNAs (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p) in a preventive health cohort. MiRNA expression was measured from capillary whole blood collected on Whatman filter cards in 81 adults; of these, 48 had available dietary questionnaire data and formed the analytic subsample for diet–miRNA analyses (n=48). The analytical approach combines exploratory multivariable regression with three pre-specified hypothesis-driven models targeting omega-3 fatty acid intake, plant-based diet quality, and UPF consumption. The aim is to identify selective diet–miRNA associations that may serve as hypothesis-generating evidence for the role of nutrition in modulating molecular markers of liver aging.

2. AIMS AND HYPOTHESES

2.1. Hypothesis

The aim of this study is to investigate the association between specific dietary and lifestyle exposures and the expression of liver-associated miRNAs involved in cellular senescence in humans.

Dietary and lifestyle exposures are expected to be associated with the expression of liver-related miRNAs linked to cellular senescence. In particular, higher exposure to UPF is associated with an upregulation of pro-senescent and inflammation-related, liver-associated circulating miRNAs (e.g., miR-21, miR-34a, miR-155), while diets rich in secondary plant compounds are associated with a downregulation of these miRNAs and an upregulation of protective miRNAs (e.g., miR-126a-5p, miR-130a-3p) (Chodur & Steinberg, 2024). These miRNA profiles may serve as early molecular indicators of liver health and may be modifiable through targeted nutritional interventions.

2.2. Objectives

1. **Polyphenol Intake:** Lower dietary polyphenol intake within participants is expected to be associated with higher expression of pro-senescent, liver-associated circulating miRNAs (miR-21, miR-34a, miR-155) compared with higher polyphenol intake.
2. **Processed Food:** Higher consumption of UPF is expected to be associated with higher expression of inflammation-related, liver-associated circulating miRNAs, particularly miR-155.
3. **Protective Nutrients**: Higher intake of omega-3 fatty acids, antioxidant-rich foods, and dietary fiber is expected to be linked with an increased expression of protective liver-associated miRNAs (miR-126a-5p, miR-130a-3p).

Together, these objectives aim to clarify the role of diet in modulating aging-related pathways in the liver and pave the way for miRNA-based nutritional diagnostics.

3. METHODS

The present work is a pilot study conducted at HealthBioCare GmbH in a preventive health context, using data from adult participants who underwent routine testing and completed a dietary and lifestyle questionnaire. Samples and questionnaire data were collected in collaboration with Dr. Ursula Jacob, and participants provided written informed consent via the order form, which included permission for anonymized use of their data and samples for scientific research purposes.

3.1. MiRNA Panel

The miRNA panel included miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p and miR-130a-3p. These liver-related miRNAs were selected based on previous evidence linking them to hepatic senescence, inflammation and metabolic regulation, as summarized in the Introduction. Together, they cover predominantly pro-senescent and pro-inflammatory (miR-21, miR-34a, miR-155-5p), regulatory (miR-146a) and hepatoprotective (miR-126a-5p, miR-130a-3p) functions, providing a focused set of markers for investigating nutrimiromic signatures of liver aging.

3.2. Study Population and Design

This study is a cross-sectional, exploratory analysis nested within a preventive health setting; it does not include follow-up data or direct measures of liver aging, and miRNA ΔCt values serve as a molecular surrogate rather than clinical endpoints. Accordingly, the analysis cannot establish prospective risk and is hypothesis-generating in nature.

In total, the study was based on 81 adults with available profiling of circulating miRNA with hepatic relevance (ALL cohort), who underwent routine testing at HealthBioCare GmbH. Capillary blood samples were collected on filter cards for miRNA analysis. Of these 81 participants, 48 completed a self-administered questionnaire on diet and lifestyle and were included in the main analyses. The full questionnaire is provided in Appendix 9.1. Within this full dataset, age ranged from 28 to 95 years, whereas in the questionnaire subsample age ranged from 28 to 77 years.

3.3. Sample Collection

Capillary blood samples were self-collected by participants via finger prick using Extra 18G Safety Lancets and applied to Whatman Protein Saver Cards. The dried blood spots (DBS) cards were returned to HealthBioCare GmbH by mail as part of the routine workflow and stored at -20°C prior to miRNA ΔCt analysis.

It should be noted that circulating miRNAs measured from capillary blood originate from multiple tissues, including immune cells, vascular endothelium, adipose tissue and hepatocytes, so the hepatic contribution to the signal cannot be isolated (Mori et al., 2019). This is taken up again in the Limitations.

3.4. MiRNA

3.4.1. RNA Extraction

Total nucleic acids were isolated from DBS using a magnetic bead-based workflow (MagMAX™ FFPE DNA/RNA Ultra Kit) on a KingFisher™ Duo. For each participant, four 4-mm punches from their DBS were extracted and incubated in protease solution on a ThermoMixer at 55°C overnight. The solution was then processed on the KingFisher™ Duo using the DBS RNA and DNA extraction program. The detailed protocol is provided in Appendix 9.2.

3.4.2. cDNA-Synthesis

For the detection and quantification of miRNA, extracted RNA was converted into complementary DNA (cDNA), which is more stable than RNA. Afterwards, a pre-amplification step was performed to increase the amount of cDNA for improved detection in subsequent analyses. cDNA synthesis and pre-amplification were carried out using the TaqMan® Advanced miRNA cDNA Synthesis Kit and a SimpliAmp™ Thermal Cycler (Applied Biosystems™, Thermo Fisher Scientific), following the manufacturer's multi-step protocol consisting of poly(A) tailing, adaptor ligation, reverse transcription and pre-amplification (miR-Amp) (see Appendix 9.3).

3.4.3. qPCR

Afterwards, real-time quantitative polymerase chain reaction (qPCR) was performed on a QuantStudio™ 3 system using TaqMan® Fast Advanced Master Mix and TaqMan® Advanced

miRNA assays. Reactions were run in a 96-well format with a total volume of 10 µL per well, consisting of 7.5 µL assay/master mix and 2.5 µL of 1:10 diluted miR-Amp product. Real-time PCR was performed following the Thermo Fisher Scientific protocol (see Appendix 9.4).

qPCR data were processed using the Applied Biosystems® QuantStudio™ Design & Analysis Software and Thermo Fisher Cloud, applying a fixed fluorescence threshold of 0.100 across all targets. For statistical analyses, miRNA expression was expressed as ΔCt values, calculated relative to two endogenous control miRNAs (miR-103a-3p and miR-24-3p):

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

Lower ΔCt values correspond to higher relative miRNA expression.

MiR-103a-3p and miR-24-3p were chosen as endogenous controls because both have been reported as stably expressed and frequently used reference miRNAs in human serum and plasma (Gharbi et al., 2015; Sriram et al., 2021). To further reduce sample-specific variability, the geometric mean of both reference miRNAs was used for normalization, in accordance with recommendations to combine multiple references genes for more robust qPCR normalization (Schwarzenbach et al., 2015).

3.5. Statistical Analysis

All statistical analyses were conducted in RStudio (version 2024.09.1+394). A two-sided p-value < 0.05 was considered statistically significant. Continuous variables were summarized as mean ± standard deviation (SD), and categorical variables as counts and percentages. MiRNA expression was analyzed using ΔCt values throughout; primary outcomes were ΔCt values for six senescence- and inflammation-related miRNAs (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p), with lower ΔCt indicating higher relative expression.

The primary aim of the analysis was to investigate whether dietary and lifestyle factors are associated with the expression of these liver-associated miRNAs in the questionnaire subsample (n=48).

To describe data availability, non-missing counts and distributions of ΔCt values were summarized for each miRNA in the full dataset and in the questionnaire subsample. Not all miRNAs were detectable in every participant. For miRNAs with few or no missing values (miR-21, miR-146a, miR-155-5p: n=48; miR-34a: n=36), all available observations were

included in the respective models. For miR-126a-5p and miR-130a-3p, ΔCt values were available for 19 participants each due to a panel update; non-detection in the remaining participants was considered a technical limitation of the qPCR assay rather than random data loss, as ΔCt values likely fell below the detection threshold. Missing values were therefore not imputed, and results for miR-126a-5p and miR-130a-3p were interpreted with caution given the small effective sample size. Outliers were identified using the interquartile range (IQR) rule (values < Q1 − 1.5 × IQR or > Q3 + 1.5 × IQR). To evaluate the robustness of findings, all regression analyses were conducted on three parallel datasets: (i) unmodified raw data, (ii) a winsorized dataset in which outlier values were capped at the respective IQR boundary, and (iii) an excluded-outlier dataset in which participants with outlying values on any primary miRNA were removed. Unless otherwise stated, inferential interpretation in the Results and Discussion sections is based primarily on the winsorized models, as they balance robustness to extreme values with retention of sample size. Normality of model residuals was assessed visually using Q–Q plots; no systematic departures from normality were identified, and formal tests of the marginal ΔCt distributions were therefore not performed.

Associations between dietary and lifestyle variables and miRNA expression were analyzed using multivariable linear regression, For the exploratory dietary analyses, a single multivariable model was fitted for each miRNA outcome, including simplified dietary and lifestyle variables simultaneously (vegetable and fruit intake frequency, vegetable variety, fast food, sweets, fish intake, and green tea/capsules), together with age, BMI, and sex; effect estimates for each dietary variable are therefore mutually adjusted for the other dietary exposures in the panel. Exploratory lifestyle models (alcohol intake, smoking status, perceived stress) were fitted separately, adjusted for age, BMI, and sex. Vegetable variety was defined as the number of vegetable categories selected by each participant (ranging from 1 to 5): leafy greens, cruciferous, legumes, starchy vegetables, and other vegetables; the specific types consumed within each category were not assessed. For regression analyses, vegetable variety was dichotomized at the median (score ≥ median = high variety; < median = low variety). Hypothesis-driven models were pre-specified for three exposure domains: (i) proxies of omega-3 fatty acid intake, (ii) plant-based diet quality (vegetable and fruit intake, whole grains, fiber supplementation, vegetable variety), and (iii) ultra-processed food consumption (fast food and sweets). These models focused on miRNAs with prior mechanistic relevance: miR-126a-5p and miR-130a-3p for protective effects, and miR-21, miR-34a, miR-155-5p, and miR-146a for pro-inflammatory or regulatory functions. Because the exploratory models adjust for all dietary variables simultaneously, whereas the hypothesis-driven models test each exposure

individually, effect estimates for nominally identical predictors (e.g., vegetable intake) can differ between the two analytical approaches. For each hypothesis block, p-values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure, and $q < 0.05$ was considered statistically significant. Exploratory analyses report unadjusted p-values alongside FDR-adjusted q-values, which were interpreted descriptively.

4. RESULTS

4.1. Study Population

Of the 81 participants initially included, 48 completed the questionnaire and were therefore considered for further analysis. This subsample consisted of 52.1% male (n=25) and 47.9% female (n=23) participants, with a mean age of 55.2 ± 13.7 years and a mean BMI of 24.8 ± 4.8 kg/m². Comparison with the full dataset (n=81) showed descriptively distributions across age, BMI, and sex, indicating no marked selection bias in the questionnaire subsample. A comprehensive overview of the demographic characteristics of both cohorts is provided in Table 1.

Table 1: Characteristics of the study population (FB) and comparison with the full dataset (ALL).

Variable	All (n=81)	FB = 1 (n=48)
Total participants	81	48
Age (years)	57.1 ± 15.2	55.2 ± 13.7
BMI (kg/m²)	24.9 ± 4.8	24.8 ± 4.8
Height (m)	1.7 ± 0.1	1.7 ± 0.1
Weight (kg)	71.4 ± 17.1	71.4 ± 17.4
Sex, n (%)		
Male, n (%)	44 (54.3%)	25 (52.1%)
Female, n (%)	37 (45.7%)	23 (47.9%)

Data are presented as mean ± SD or n (%). FB, questionnaire subsample; ALL, full dataset; BMI, body mass index.

4.2. MiRNA Data Availability and Descriptive Overview

To provide context for the following analyses, miRNA data availability was summarized for the full dataset (ALL) and the questionnaire subsample (FB). MiRNA availability varies across targets due to a panel update during the study period: MiR-33a and miR-379 were discontinued, whereas miR-126a-5p, miR-130a-3p, miR-217-5p and miR-181b-5p were introduced at a later stage. Consequently, not all miRNAs are available for the same number of participants, and differences in n reflect both subsample restriction and panel-related availability. An overview of non-missing ΔCt values for each miRNA in ALL and FB is shown in Table 2.

Table 2: MiRNA availability in the full dataset (ALL) and the questionnaire subsample (FB).

miRNA	n_ALL	n_FB	comment
miR-107	81	48	
miR-122	48	31	
miR-126a-5p	26	19	added later
miR-130a-3p	26	19	added later
miR-132	81	48	
miR-146a	81	48	
miR-155-5p	81	48	
miR-181b-5p	26	19	added later
miR-185	81	48	
miR-199a	81	48	
miR-20b	81	48	
miR-21	81	48	
miR-210	81	48	
miR-217-5p	18	12	added later
miR-223	81	48	
miR-26a	81	48	
miR-27b	81	48	
miR-33a	29	14	removed at one point from panel
miR-34a	58	36	
miR-379	16	9	removed at one point from panel
miR-660	81	48	

4.2.1. MiRNA Overview and Missingness in the Questionnaire Subsample

Note: In the ΔCt method, a lower ΔCt value corresponds to higher relative expression. This inverse relationship should be kept in mind when interpreting all subsequent results.

Within the questionnaire subsample (n=48), ΔCt distributions and the number of non-missing observations were summarized for the primary miRNA outcomes (Table 3). Data availability was high for miR-21, miR-155-5p and miR-146a, whereas fewer observations were available for miR-34a and particularly for miR-126a-5p and miR-130a-3p. This pattern is important for

the interpretation of multivariable regression models (adjusted for age, BMI and sex), as complete-case analyses reduce the effective sample size (n_used) for miRNAs with substantial missingness.

Missingness in miR-126a-5p and miR-130a-3p was attributable to a panel update and is therefore considered missing completely at random (MCAR). For miR-34a, a formal missingness analysis revealed two statistically significant associations: participants with missing miR-34a values had a lower mean BMI compared to those with available values (21.85 vs. 25.47 kg/m²; $p = 0.013$), and missingness was associated with vegetable variety (Fisher exact test, $p = 0.043$). No significant associations were observed for age, sex, or other dietary and lifestyle variables. These findings suggest that missingness in miR-34a may not be entirely random and should be interpreted with caution; results from miR-34a models may disproportionately reflect participants with higher BMI and greater dietary variety.

Table 3: Summary of ΔCt values and missingness for primary miRNAs in the questionnaire subsample.

miRNA	n (non-missing)	ΔCt [mean ± SD]	Range	NA's
miR-107	48	0.87 ± 0.52	-0.42 – 2.15	0
miR-122	31	13.10 ± 2.99	8.04 – 18.25	17
miR-126a-5p	19	2.29 ± 0.60	1.30 – 3.33	29
miR-130a-3p	19	3.38 ± 0.51	2.23 – 4.14	29
miR-132	48	5.82 ± 0.64	3.06 – 7.27	0
miR-146a	48	2.74 ± 0.75	1.56 – 5.95	0
miR-155-5p	48	5.37 ± 1.03	2.23 – 8.17	0
miR-181b-5p	19	6.93 ± 0.88	5.36 – 8.35	29
miR-185	48	-1.60 ± 0.56	-3.61 – -0.73	0
miR-199a	48	7.51 ± 1.47	4.05 – 12.39	0
miR-20b	48	0.27 ± 0.43	-0.69 – 1.54	0
miR-21	48	1.13 ± 1.03	-3.73 – 2.80	0
miR-210	48	4.09 ± 0.80	2.65 – 7.01	0
miR-217-5p	12	8.10 ± 2.03	4.03 – 10.18	36
miR-223	48	-2.40 ± 0.72	-4.18 – -0.91	0
miR-26a	48	-1.71 ± 0.48	-3.01 – -0.78	0
miR-27b	48	5.71 ± 0.93	4.16 – 10.73	0

miRNA	n (non-missing)	ΔCt [mean ± SD]	Range	NA's
miR-33a	14	13.55 ± 2.55	9.67 – 18.33	34
miR-34a	36	13.47 ± 1.44	8.84 – 16.92	12
miR-379	9	14.82 ± 1.73	12.49 – 17.59	39
miR-660	48	3.78 ± 0.49	2.76 – 5.37	0

Note: Values are ΔCt (mean ± SD), range, n (non-missing), and number of missing values (NA's).

4.3. Sex Differences in miRNA ΔCt

To assess potential sex-related differences in miRNA expression, Welch's t-tests were conducted for each miRNA, comparing male ($n = 25$) and female ($n = 23$) participants. Three miRNAs showed nominally significant differences: miR-132 ($p = 0.010$; males: 5.60 ± 0.68, females: 6.06 ± 0.52), miR-223 ($p = 0.016$; males: -2.17 ± 0.66, females: -2.66 ± 0.70), and miR-107 ($p = 0.020$; males: 0.71 ± 0.51, females: 1.05 ± 0.47). However, after Benjamini-Hochberg FDR correction for multiple testing across all miRNAs, none of these associations remained statistically significant (all $q \geq 0.142$). Sex was therefore not identified as a robust source of systematic variation in miRNA ΔCt values in this sample but was retained as a covariate in all subsequent regression models to account for potential residual confounding. As miR-132, miR-223, and miR-107 are not part of the primary six-miRNA panel central to this thesis, these nominal findings were not investigated further.

Table 4: ΔCt values of primary miRNAs by sex in the questionnaire subsample.

miRNA	n ♂ / ♀	ΔCt ♂ (mean± SD)	ΔCt ♀ (mean± SD)	p (Welch)	q (FDR)
miR-21	25 / 23	1.25 ± 0.64	1.00 ± 1.33	0.418	0.675
miR-34a	20 / 16	13.10 ± 1.31	13.93 ± 1.50	0.092	0.384
miR-155-5p	25 / 23	5.22 ± 1.14	5.53 ± 0.90	0.301	0.631
miR-146a	25 / 23	2.91 ± 0.88	2.57 ± 0.55	0.112	0.385
miR-126a-5p	10 / 9	2.18 ± 0.47	2.40 ± 0.73	0.454	0.682
miR-130a-3p	10 / 9	3.37 ± 0.59	3.40 ± 0.45	0.892	0.936

Note: Values are mean±SD. p: Welch t-test; q: Benjamini-Hochberg FDR correction. No comparison reached statistical significance after FDR correction.

4.4. Exploratory Dietary Associations

To explore potential associations between simplified dietary variables and primary miRNA expression, multivariable linear regression models were fitted for each miRNA-predictor combination, adjusted for age, BMI, and sex. For miR-126a-5p and miR-130a-3p, the effective sample sizes were insufficient for model estimation ($n = 9$, $df = 0$); results for these two miRNAs are therefore not reported for the exploratory dietary analyses.

Among the four remaining primary miRNAs ($n = 17–22$), in the exploratory dietary models, daily vegetable and fruit consumption was nominally associated with higher miR-21 ΔCt values ($\beta = 1.11$, $p = 0.047$), indicating lower miR-21 expression in participants who consumed fruit or vegetables every day compared to less frequent consumers (see Figure 5). This estimate stems from a multivariable model in which vegetable and fruit intake was entered simultaneously with seven other dietary variables, plus age, BMI, and sex. In the pre-specified plant-based hypothesis block (Table A2: $\beta = 0.66$, $p = 0.38$; $n = 26$), the same vegetable and fruit intake variable was tested as the sole dietary exposure, adjusted only for age, BMI, and sex. The difference in effect estimates reflects this difference in covariate adjustment and the resulting difference in complete-case sample size, rather than a difference in how vegetable and fruit intake itself was coded; both specifications are reported in Appendix Table A2. A trend in the same direction was observed for high vegetable variety and miR-155-5p ($\beta = 1.58$, $p = 0.058$). Neither association survived Benjamini-Hochberg FDR correction (both $q = 0.666$), and no other dietary predictor reached nominal significance (all $p > 0.09$). Sensitivity analyses using winsorized data yielded consistent estimates for both trends, suggesting they are not attributable to single outlying values. Overall, the exploratory dietary analysis provided no evidence of robust associations between simplified dietary exposures and primary miRNA expression in this sample.

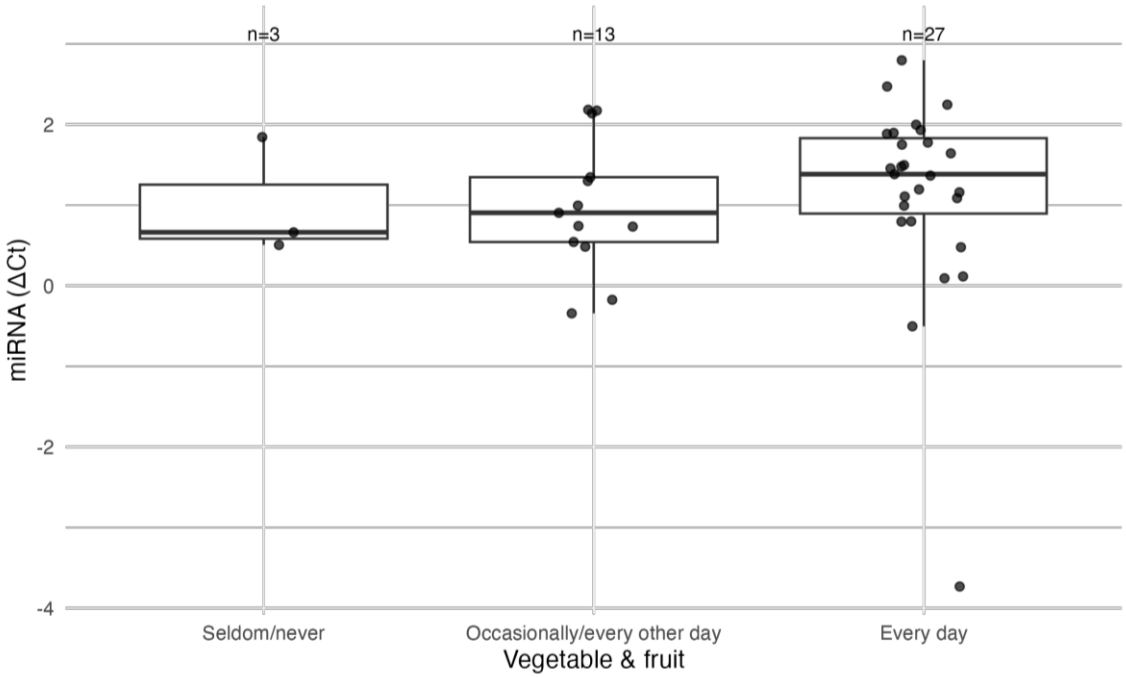

Figure 5: miR-21 ΔCt by frequency of vegetable and fruit intake (n = 43 in plot; n = 22 in adjusted model).

Note: Higher frequency was nominally associated with higher ΔCt values (lower expression; β = 1.11, p = 0.047, q = 0.666). Model adjusted for age, BMI, sex. Individual data points shown; box indicates median and IQR.

4.5. Exploratory Lifestyle Associations

Lifestyle predictors (perceived stress, alcohol consumption, and smoking status) were examined in the same modeling framework (n = 21–26, adjusted for age, BMI, and sex). For miR-126a-5p and miR-130a-3p, degrees of freedom were again insufficient for model estimation (df = 0).

The most notable finding was a nominally significant positive association between daily alcohol consumption and miR-155-5p ΔCt (RAW: β = 4.07, p = 0.017; WINSORIZED: β = 3.73, p = 0.017), indicating lower miR-155-5p expression in daily drinkers relative to non-drinkers (Figure 6). However, this inverse pattern must be interpreted with caution, as the "daily" category comprised only three participants, making the group mean highly sensitive to individual high ΔCt values driven by age, medication, or random biological variability. The consistency of this finding across both sensitivity datasets suggests it is not driven by extreme values. Additionally, a non-significant trend toward lower miR-155-5p ΔCt was observed for current smoking (<1 pack/day; β = − 1.63, p = 0.066), consistent with potential upregulation of miR-155-5p in current smokers. No associations were observed between stress, alcohol, or smoking and miR-21, miR-34a, or miR-146a (all p > 0.14). After FDR correction, no lifestyle predictor reached statistical significance (all q ≥ 0.528).

To assess whether the nominal association between daily alcohol consumption and miR-155-5p was driven by the small group of daily drinkers (n=3), a sensitivity analysis recoded alcohol intake as binary (seldom/never vs. regular [weekly or daily]). After recoding, no significant association with miR-155-5p was observed ($\beta = 0.72$, $p = 0.377$, $q = 0.747$), suggesting that the nominal finding in the main analysis reflects the extreme daily consumption group rather than a broader dose-response pattern (Figure 7).

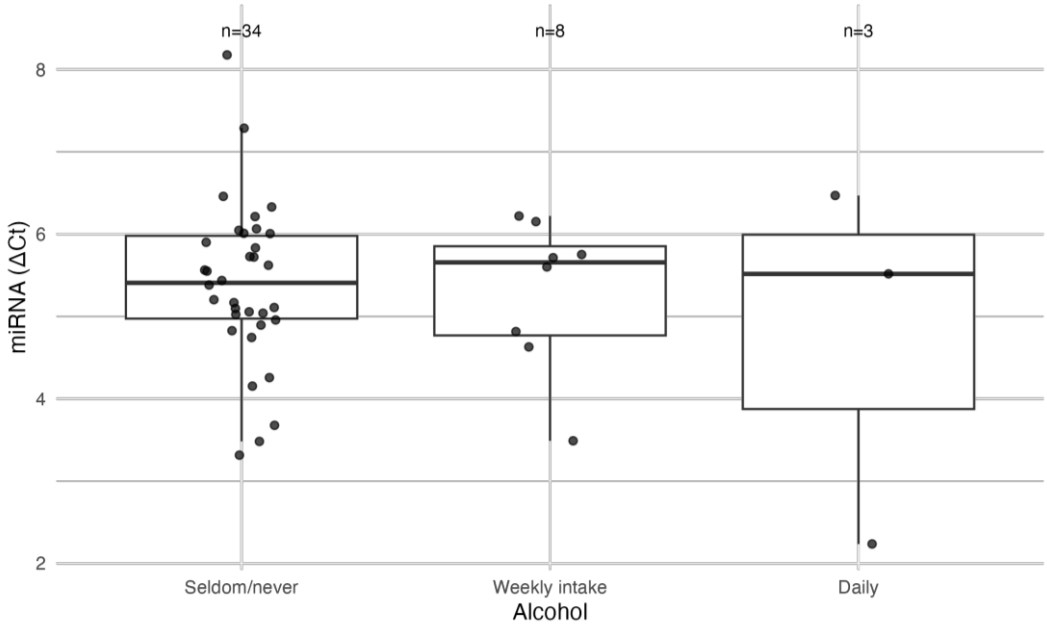

Figure 6: miR-155-5p ΔCt by alcohol intake category (n=45 in plot; n=26 in adjusted model).

Note: Daily consumption was nominally associated with higher ΔCt values (lower expression; β = 3.73, p = 0.017, q = 0.551). Model adjusted for age, BMI, sex. Individual data points shown; box indicates median and IQR.

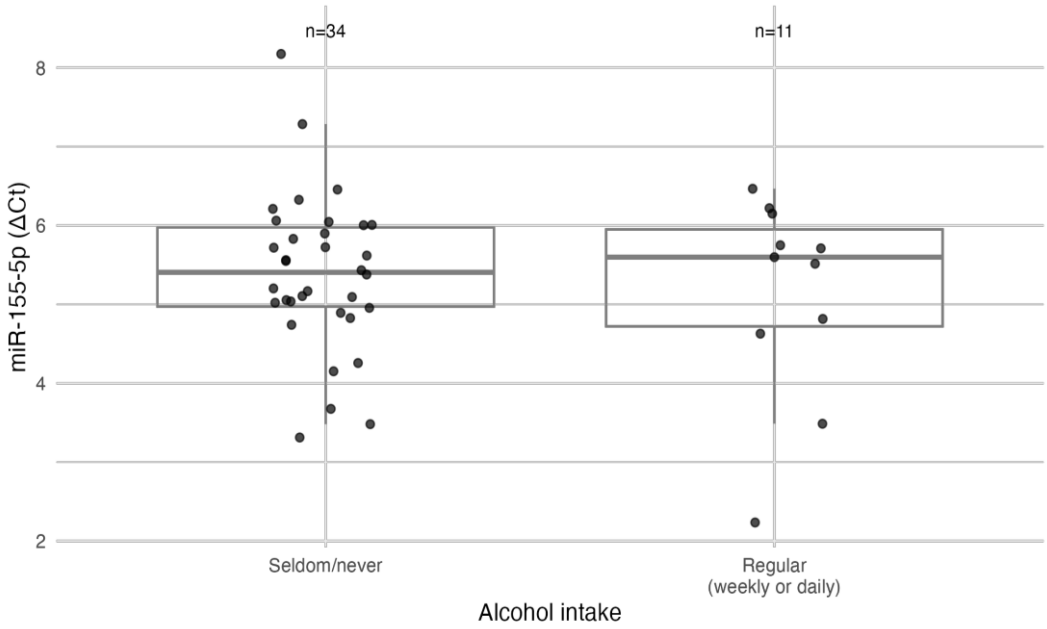

Figure 7: miR-155-5p ΔCt by binary alcohol intake (n=45 in plot; n=26 in adjusted model).

Note: Alcohol recoded as seldom/never (n = 34) vs. regular (weekly or daily; n = 11). No significant association was observed (β = 0.72, p = 0.377, q = 0.747). Model adjusted for age, BMI, sex. Individual data points shown; box indicates median and IQR.

4.6. Hypothesis-driven Dietary Models

Hypothesis-driven models were fitted to test the three pre-specified dietary hypotheses: (1) omega-3 fatty acid intake (fish_bin) in relation to miR-126a-5p and miR-130a-3p; (2) plant-based diet quality (vegetable and fruit intake, whole grain, fiber supplementation, and vegetable variety) in relation to the same protective miRNAs; and (3) UPF consumption (fast food and sweets) in relation to inflammatory miRNAs. Models were adjusted for age, BMI, and sex, and p-values were corrected using the Benjamini-Hochberg FDR procedure within each hypothesis block.

The only FDR-significant association in the entire analysis was identified within the plant-based hypothesis block: high vegetable variety was associated with lower miR-130a-3p ΔCt (WINSORIZED: β = -0.95, p = 0.002, q = 0.039; n=11, df = 6, R^2 = 0.87; 95% CI = -1.41 to -0.49) (Figure 8; see Table 5).

Although the model for miR-130a-3p yielded a high R^2 of 0.87 (β = −0.95, 95% CI = −1.41 to −0.49), this value should be interpreted cautiously, as only six residual df make overfitting a likely explanation rather than true explanatory power, and the confidence interval is wide relative to the point estimate, indicating considerable uncertainty around the true effect size.

Table 5: Hypothesis-driven regression results with p < 0.10.

miRNA	Exposure	n	β	p	q
miR-130a-3p	Vegetable variety (high vs. low)	11	−0.949	0.002	0.039*
miR-34a	Whole grain (weekly vs. rarely/never)	10	−1.814	0.025	0.203
miR-146a†	Fast food (weekly vs. rarely/never)	22	−0.853	0.034	0.135

*Note: Main analysis: WINSORIZED (unless marked). Models adjusted for age, BMI, sex. * FDR-significant (q < 0.05). † EXCLUDED mode. β = unstandardized regression coefficient. n=complete cases. q = BH-FDR corrected within hypothesis block. † miR-146a result from EXCLUDED sensitivity model (outlier removed).*

An identical effect was observed in the RAW dataset, indicating that this association is not driven by extreme values. Given that lower ΔCt reflects higher expression in the ΔCt framework, participants with high vegetable variety showed a tendency toward higher miR-130a-3p expression. However, the effective sample size of n=11 calls for caution; this finding should be considered preliminary and requires replication in a larger sample. No other plant-

based predictor, fish intake variable, or UPF variable reached statistical significance after FDR correction (all $q \geq 0.135$).

Full regression results for all three hypothesis blocks (omega-3, plant/fiber/antioxidant proxy, and UPF) are provided in Tables A1–A3 in the Appendix.

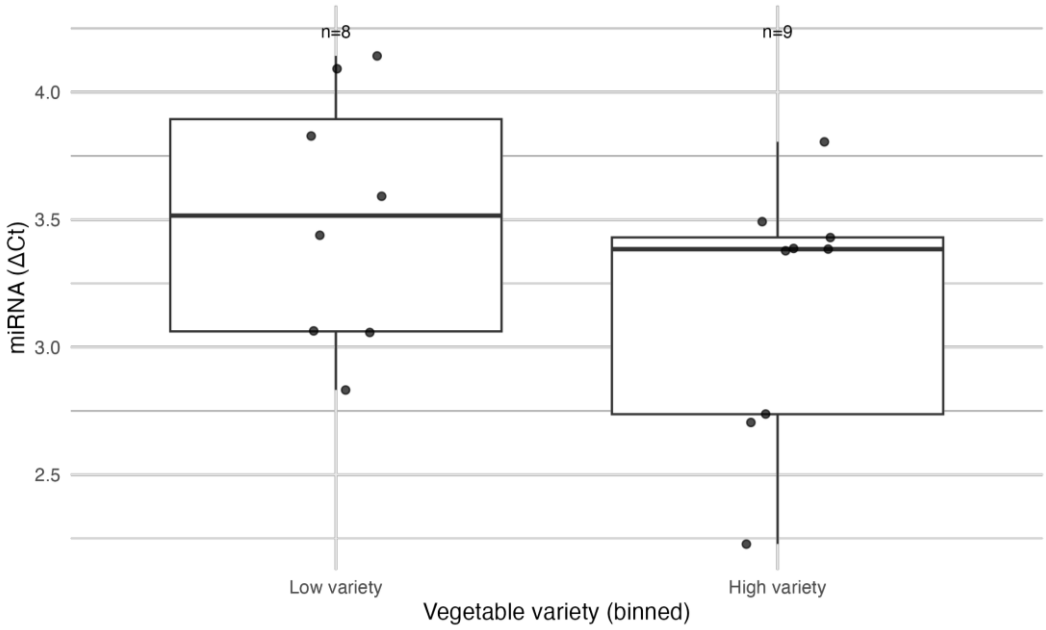

Figure 8: MiR-130a-3p ΔCt by vegetable variety (n=17 in plot; n=11 in adjusted model).

Note: High vegetable variety was associated with lower ΔCt values (higher expression; β = -0.95, p = 0.002, q = 0.039; the only FDR-significant finding). Note: the plot shows all observations with non-missing values for both variables; the model includes only complete cases across all covariates (age, BMI, sex). Individual data points shown; box indicates median and interquartile range.

4.7. Sex Differences in Diet and Lifestyle Variables

To assess whether dietary and lifestyle behaviors differed systematically between male and female participants, chi-square tests (or Fisher exact tests for 2×2 tables with low expected counts) were performed for thirteen variables, with p-values adjusted using the Benjamini-Hochberg FDR procedure. Variables examined included alcohol consumption, vegetable and fruit intake, fruit variety, whole grain intake, fish intake, fast food consumption, sweets consumption, fiber supplementation, green tea consumption, green tea capsule use, perceived stress, and smoking status.

No variable reached statistical significance after FDR correction (all $q \geq 0.686$). The most notable nominal difference was observed for alcohol intake ($p = 0.071$, $q = 0.686$): male participants showed higher rates of weekly and daily alcohol consumption (25.0% and 12.5%, respectively) compared to female participants (9.5% and 0.0%), while a greater proportion of

females reported seldom to never drinking (90.5% vs. 62.5%). A descriptive tendency was also observed for vegetable variety ($p = 0.146$, $q = 0.686$), with females showing a higher proportion in the upper variety categories (variety score 3: 45.0% vs. 13.6% in males), and for whole grain intake ($p = 0.304$, $q = 0.686$), with males more frequently reporting daily whole grain consumption (39.1% vs. 19.0%). All remaining dietary and lifestyle variables showed no meaningful distribution differences between sexes (all $p \geq 0.277$).

These findings suggest that dietary and lifestyle exposures were broadly comparable between male and female participants within this sample, supporting the use of sex as a covariate rather than a stratification variable in the regression analyses presented above.

Table 6: Sex differences in dietary and lifestyle variables.

Variable	n	Test	p	q (BH-FDR)
Alcohol consumption	45	Chi-square	0.071	0.686
Vegetable variety	42	Chi-square	0.146	0.686
Vegetable & fruit intake	43	Chi-square	0.277	0.686
Whole grain intake	44	Chi-square	0.304	0.686
Green tea intake	44	Chi-square	0.317	0.686
Fast food consumption	42	Chi-square	0.362	0.686
Fruit variety	44	Chi-square	0.400	0.686
Fish intake	45	Chi-square	0.574	0.811
Green tea capsules	42	Fisher exact	0.608	0.811
Perceived stress	45	Chi-square	0.814	0.977
Sweet consumption	45	Chi-square	0.967	1.000
Dietary fiber supplement	42	Fisher exact	1.000	1.000
Smoking status	44	Chi-square	n.a.	n.a.

Note: p-values from Chi-square or Fisher exact tests; BH-FDR correction applied across all 13 comparisons. Smoking excluded from inference due to near-zero expected cell counts

5. DISCUSSION

The present study investigated associations between dietary and lifestyle factors and the expression of six senescence- and inflammation-related circulating miRNAs with hepatic relevance (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p) in a preventive cohort subsample with available questionnaire data (n=48). The six miRNAs were selected based on their established roles in hepatic senescence, fibrosis, inflammation, and metabolic regulation, representing a panel with direct relevance to liver aging. The analytical approach combined exploratory multivariable regression with three pre-specified hypothesis-driven models (an Omega-3 hypothesis, a Plant/Fiber/Antioxidant hypothesis, and a UPF hypothesis), each targeting a distinct dietary or lifestyle exposure domain.

The study results indicate that nutritional modulation of the miRNA panel is selective and context-dependent, rather than broad and generalized. This is consistent with the emerging understanding from human intervention studies that diet-induced changes in circulating miRNAs reflect a complex response whose magnitude and direction depend on the metabolic health status of the individual, and are linked to changes in multiple diet-responsive miRNAs and their metabolic targets (Chodur & Steinberg, 2024).

The most compelling evidence from the study is the significant and biologically plausible association between vegetable variety and miR-130a-3p expression, underscoring the importance of plant-based dietary diversity.

The only association that survived Benjamini-Hochberg FDR correction across the entire analysis was observed between high vegetable variety and lower miR-130a-3p ΔCt values, indicating relatively higher miR-130a-3p expression in participants with greater dietary vegetable diversity. The consistency of this effect across RAW and WINSORIZED datasets, with identical point estimates, further supports the robustness of this association.

Consistent with its established role as an anti-fibrotic and insulin-sensitizing miRNA (see Introduction), miR-130a-3p suppresses the TGF-β/SMAD fibrogenic pathway and improves insulin sensitivity via GRB10 (growth factor receptor-bound protein 10) targeting, with higher expression associated with a more favorable metabolic liver phenotype. Its selective dietary responsiveness is therefore of particular interest within the present panel (Y. Wang et al., 2017; Xiao et al., 2014).

Given the very small effective sample size in the adjusted models (n=11) and the relatively large effect estimate, this association should be interpreted as a hypothesis-generating signal rather than definitive evidence, as model instability and overfitting cannot be excluded.

The biological plausibility of an association with vegetable variety is supported by multiple mechanistic pathways. Dietary vegetable diversity serves as a proxy for the intake of a broad range of bioactive plant compounds, including polyphenols (leafy greens, cruciferous vegetables, legumes, starchy vegetables, and other vegetables). Among polyphenols, resveratrol has received particular attention for its hepatoprotective properties. It activates SIRT1- and AMP-activated protein kinase (AMPK)-dependent pathways that regulate hepatic lipid metabolism and mitochondrial function (Price et al., 2012) and has been shown to improve liver steatosis in experimental models of fatty liver (Du et al., 2021). Notably, Kadhim et al. (2018) reported resveratrol-mediated downregulation of miR-130a (strand not specified) in hepatic mononuclear cells following Staphylococcal aureus enterotoxin B (SEB)-induced acute liver injury, which contrasts with the upregulation observed in the present study. This discrepancy may reflect differences in the cellular context (acute toxic injury vs. habitual dietary exposure) and underscores the complexity of polyphenol-miRNA interactions. While a link between higher vegetable variety, greater exposure to polyphenols and dietary fiber, and increased miR-130a-3p expression is mechanistically plausible, this interpretation remains speculative and should be viewed accordingly.

Besides polyphenols, vegetable variety also captures dietary fiber intake. Fiber is fermented by the gut microbiota to short-chain fatty acids (SCFA) such as butyrate, which act as endogenous histone deacetylase (HDAC) inhibitors and modulate host gene expression and inflammatory signaling. Although direct evidence for SCFA-mediated regulation of hepatic miR-130a-3p is lacking, this pathway illustrates how fiber-rich, plant-based diets could influence epigenetic control of liver-related miRNAs more broadly (Bishop et al., 2017; J. Wang et al., 2025).

Using vegetable variety as the relevant exposure is methodologically significant because it captures a broader range of bioactive compounds, reflecting a more comprehensive dietary pattern than single-nutrient measures. Binary or frequency-based classification of single foods (e.g., fish intake, fruit consumption) repeatedly failed to show significant associations in this dataset, while the variety index captured a qualitative dietary diversity dimension that appears more sensitive. This aligns with a broader shift in nutritional epidemiology away from single-nutrient models toward dietary pattern and diversity metrics, which better reflect the cumulative

and potentially synergistic effects of multiple bioactive compounds (Seifishahpar et al., 2025; Thompson et al., 2021).

However, the findings require cautious interpretation. The effective sample size of n=11 is extremely small (residual df = 6). With four covariates (age, BMI, sex, and the binary variety exposure) relative to 11 observations, the model is at high risk of overfitting, and the resulting β coefficient may overestimate the true population effect. The absence of outlier-driven inflation (confirmed by identical results in WINSORIZED models) is reassuring, but does not resolve concerns about model stability, generalizability, or potential confounding. Confidence intervals would be wide, and the study is underpowered to exclude the possibility of a type I error despite FDR correction. Overall, this finding should be viewed as a hypothesis-generating signal warranting replication in adequately powered studies (estimated $n \geq 100–150$ for adjusted models of this complexity) rather than as confirmatory evidence of a diet–miRNA relationship. The sample size limitation applies to all subsequent null findings discussed below.

Beyond the key finding, the three pre-specified dietary hypotheses yielded no significant results after FDR correction. The Omega-3 hypothesis is discussed first.

The Omega-3 hypothesis proposed that higher fish intake, as a proxy for omega-3 PUFA intake, would be associated with higher expression of miR-126a-5p and miR-130a-3p, both of which are downregulated in fibrotic and aging liver tissue. This hypothesis was not supported by the data (all q = 0.661). MiR-126a-5p is particularly relevant in this context: it regulates hepatic senescence and inflammation through targeting VCAN and modulating the NF-κB pathway, and is downregulated in aged liver with consequences for DNA damage response and hepatocyte function (Yan et al., 2019). A protective role of omega-3 PUFAs in maintaining miR-126a-5p expression is mechanistically plausible, as EPA- and DHA-derived lipid mediators have been shown to reduce NF-κB activity in liver macrophages and to promote an anti-inflammatory macrophage phenotype in chronic liver disease models (Videla et al., 2023).

However, several study design characteristics limit the ability to detect such an effect. First, fish intake was coded as a binary variable (weekly or more vs. rarely/never), which lacks resolution to differentiate omega-3 exposure intensity, the species consumed (fatty fish such as salmon vs. lean fish), or preparation method. Second, as noted above, the effective sample sizes of n=11 severely constrained statistical power. Third, miRNA levels measured in capillary blood do not exclusively reflect hepatic expression. Circulating miRNAs originate from multiple tissues and cell types, including immune cells, and changes in blood levels may

therefore not accurately capture diet-induced modulation of specifically hepatic miR-126a-5p (Mori et al., 2019). Future studies should employ validated biomarkers of omega-3 status, such as the erythrocyte-based omega-3 index, which more accurately reflects long-term EPA and DHA intake than questionnaire-based estimates (Dempsey et al., 2023; Wan et al., 2023), and should consider tissue-specific miRNA assessment, as circulating miRNA profiles represent a composite signal that cannot be attributed to a single organ (Mori et al., 2019).

Similarly, the UPF hypothesis was not supported.

The hypothesis that higher UPF or fast-food consumption would be associated with upregulation of inflammation-related miRNAs (miR-155-5p, miR-146a, miR-21) was not supported. In the EXCLUDED dataset, a nominal association between fast food consumption and miR-146a ($\beta = -0.85$, $p = 0.034$) was observed, but this did not survive FDR correction ($q = 0.135$) and was absent in the RAW and WINSORIZED models, making a meaningful interpretation not possible.

Consistent with its established role as a counter-regulatory brake on NF-κB-driven inflammation (see Introduction), miR-146a acts in opposition to miR-155-5p by targeting IRAK1 and TRAF6 (Roos et al., 2016). A lower miR-146a expression in frequent fast-food consumers would have been consistent with a pro-inflammatory dietary phenotype reducing this counter-regulatory miRNA. The nominal effect, if real, would align with prior reports linking processed food consumption to increased systemic inflammation (Lane et al., 2022). However, the lack of replication across sensitivity analyses and the failure to survive multiple testing correction prevent any such conclusion. The UPF exposure measurement is also limited: fast food frequency captures only one dimension of UPF intake, excluding industrially processed snacks, ready meals, and sweetened beverages.

It is worth noting that the sample studied here is a preventive health cohort (Liveration study) with generally health-conscious participants. The prevalence of daily fast-food consumption was low, and the variance in UPF intake may have been insufficient to generate detectable miRNA effects. Population-based studies with greater dietary heterogeneity would be better positioned to detect such associations.

Turning to lifestyle exposures, alcohol, smoking, and stress were examined as potential modulators of miRNA expression.

Among lifestyle variables, the most notable nominal signal was observed between daily alcohol consumption and miR-155-5p ($\beta = 4.07$, $p = 0.017$, $q = 0.551$). Consistent with its established role as a pro-inflammatory miRNA via NF-κB-driven activation in Kupffer cells (see Introduction), miR-155-5p amplifies TNF-α production and contributes to steatohepatitis in the context of alcohol-induced liver injury (Bala et al., 2016). An association between heavier alcohol consumption and elevated miR-155-5p expression is therefore mechanistically plausible.

It should be noted that the direction of this nominal association is counterintuitive: based on established evidence for alcohol-induced upregulation of miR-155-5p via NF-κB activation (Bala et al., 2011), higher alcohol consumption would be expected to correspond to lower ΔCt. The observed opposite direction is most plausibly a small-sample artefact rather than a true biological effect: with only three participants in the daily-drinking group, a single individual with an extreme ΔCt value (driven by age, medication or biological variability unrelated to alcohol) is sufficient to shift the entire group comparison. Additional unmeasured confounding, including social and behavioral correlates of drinking patterns, may further blur the signal.

However, the sensitivity analysis in which alcohol was recoded as a binary variable (seldom/never vs. regular use combining weekly and daily categories) yielded no significant association. This finding is important: it indicates that the nominal effect in the main analysis was driven specifically by the extreme category of daily drinkers (n=3), whose small group size makes the estimate highly unstable. Combining weekly and daily drinkers into a single regular use category was therefore statistically justified, and the resulting null finding confirms that the original nominal association should not be over-interpreted. In a larger study, the alcohol–miR-155-5p relationship could be re-examined with greater resolution of drinking patterns and volume.

Regarding smoking, a nominal trend between current smoking and miR-155-5p was observed in exploratory models but was similarly not robust to multiple testing correction. Smoking is a well-established inducer of systemic and pulmonary inflammation and has been linked to miRNA dysregulation in lung, cardiovascular, and hepatic tissues (Karabegović et al., 2023). The limited number of current smokers in this sample (n=6) substantially restricted statistical power for this subgroup. Stress showed no associations with any miRNA outcome, consistent with the small and categorical nature of the stress variable used.

In addition to the pre-specified hypotheses, simplified dietary models revealed two nominal associations of interest.

In the simplified dietary models, daily vegetable and fruit consumption showed a nominal positive association with miR-21 ΔCt ($\beta = 1.11$, $p = 0.047$, $q = 0.666$), which was not significant after FDR correction. Under the convention used in this study, a higher ΔCt corresponds to lower relative miRNA expression; the direction of this association therefore suggests a trend toward lower miR-21 expression with higher plant food intake. Consistent with its established role as a pro-fibrotic and pro-inflammatory miRNA targeting PTEN and PDCD4 (see Introduction), a reduction in miR-21 in individuals with high daily plant food intake would be consistent with an anti-fibrotic dietary phenotype, although this observation remains preliminary given the lack of FDR significance.

Similarly, whole grain intake showed a nominal negative association with miR-34a ($\beta = -1.81$, $p = 0.025$, $q = 0.203$), suggesting potentially lower miR-34a expression in participants with frequent whole grain consumption. miR-34a is a p53-regulated miRNA that promotes hepatocyte apoptosis and lipid accumulation, and is elevated in MASLD (Hochreuter et al., 2022). However, at $q = 0.203$, this association also fails to meet FDR significance and must be treated as a hypothesis-generating observation only.

The observed patterns can be placed in the context of existing nutrimiromics research.

The findings regarding the association between plant-based dietary patterns and miRNA expression are consistent with previous research by Pointner et al. (2024) and the wider nutrimiromics literature, particularly in relation to miR-21 and miR-155-5p. That study demonstrated that dietary patterns characterized by high vegetable, fruit, and legume intake were associated with decreased expression of miR-21 and increased expression of let-7a and miR-328, a miRNA profile consistent with reduced inflammatory and pro-fibrotic signaling. The directional alignment with the nominal miR-21 trend observed in the present study, despite the smaller sample and different dietary exposure operationalization, suggests directional consistency across studies, although replication in adequately powered samples is needed.

Pointner et al. (2024) also identified miR-155-5p as a miRNA sensitive to lifestyle exposures, particularly those involving inflammatory activation. The nominal alcohol–miR-155-5p association in the present study is consistent with this characterization. In contrast to single-miRNA approaches, the nutrimiromics framework emphasizes the co-regulation of miRNA

clusters in response to dietary exposures, which may explain why some associations emerge more robustly in studies assessing broader dietary patterns than in models focused on individual food items or binary frequency indicators as used here.

The relevance of the investigated miRNA panel to biological aging is further supported by the MiRNA-3Age model developed by Schneider et al. (2025), in which miR-21 and miR-155-5p were identified as key components of a miRNA-based estimator of biological age. The inclusion of these miRNAs in an aging model underscores their sensitivity to cumulative lifestyle and physiological influences and provides a rationale for investigating their dietary modifiability. If diet can measurably shift the expression of age-associated miRNAs, even modestly, this would support the concept of nutritional epigenomics as a lever for modulating biological aging trajectories. The present data provide preliminary, directionally consistent evidence for this possibility, while clearly demonstrating the methodological requirements (larger cohorts, longitudinal designs) necessary to establish it with confidence.

In the wider literature, associations between plant-based dietary patterns and hepatoprotective miRNA profiles have been reported in both observational and intervention contexts. Polyphenol-rich diets and Mediterranean dietary patterns have been linked to modulation of miRNAs involved in NF-κB signaling, oxidative stress response, and autophagy (DeLucas et al., 2024). The direction of the observed association between vegetable variety and miR-130a-3p is consistent with this broader evidence base, strengthening its biological plausibility despite differences in study design and exposure measurement. It should be noted that FDR correction was applied within each hypothesis block; a study-wide correction across all tested combinations would likely yield no significant results, underscoring the exploratory nature of the findings.

An additional methodological consideration concerns the role of sex in the present analysis.

No statistically significant sex differences were found in dietary and lifestyle exposures (Section 6.7) or miRNA ΔCt values (Section 6.3), supporting the decision to include sex as a covariate rather than stratifying by sex. Stratification would have reduced group sizes to approximately 23–25 participants per stratum, further limiting the already low statistical power. Although males reported nominally higher alcohol consumption (25.0% weekly + 12.5% daily vs. 9.5% + 0.0% in females), the difference is consistent with known population-level patterns and was not large enough to meaningfully affect the results.

It is worth noting that the relationship between sex and miRNA expression is itself an active area of investigation. Sex hormones, particularly estrogens, regulate hepatic lipid metabolism and inflammation in a sex-specific manner (Della Torre, 2020), and sex-specific miRNA profiles have been reported in liver disease contexts (Chan et al., 2022). In the present sample, the lack of FDR-significant sex differences in miRNA ΔCt values may reflect the relatively balanced sex distribution (25 males, 23 females) and the age range of participants (55.2 ± 13.7 years), a period during which sex hormonal differences may be reduced following menopause in many female participants. This potential confound should be considered in future studies that include a broader age range or explicitly stratify by menopausal status.

Overall, the results of this study are consistent with a model in which dietary quality, particularly the diversity of plant-based food intake, is selectively associated with the expression of circulating miRNAs relevant to liver aging and fibrosis, rather than exerting a uniform influence across all investigated miRNA targets. The single FDR-significant finding (vegetable variety and miR-130a-3p) is biologically plausible, directionally consistent with prior evidence, and supported by mechanistic literature on polyphenol–miRNA interactions. The multiple nominal trends (miR-21 with vegetable/fruit intake, miR-34a with whole grains, miR-155-5p with alcohol) collectively suggest a dietary quality dimension that spans the investigated miRNA panel.

From a clinical and public health perspective, these findings, if confirmed in larger studies, would support the value of dietary diversity as a component of liver health promotion strategies targeting molecular aging markers. Vegetable variety, as an exposure, is actionable through dietary counseling, is associated with multiple established health benefits, and is measurable via standardized dietary assessment instruments. The identification of miR-130a-3p as a potentially diet-responsive miRNA in the context of hepatic metabolism adds to the growing evidence base for nutrition-targeted epigenomic approaches in preventive medicine.

Despite the lack of significant associations for the omega-3, UPF, and most lifestyle hypotheses, these areas remain underexplored in the present dataset. The present findings highlight the methodological challenges inherent to nutrimiromics in small preventive cohorts and emphasizes the need for adequately powered studies with refined exposure assessment to characterize the full landscape of diet–miRNA interactions in human liver aging.

6. LIMITATIONS AND FUTURE RESEARCH

Several methodological limitations must be considered when interpreting the findings of this study.

Sample size and statistical power. First, the questionnaire subsample comprised n=48 participants, and effective sample sizes for adjusted regression models were substantially smaller due to missing miRNA values, particularly for miR-126a-5p and miR-130a-3p, where n_used dropped to approximately 11. Models with four predictor variables and n=11 have very low statistical power and are prone to unstable coefficient estimates. The sole FDR-significant finding should therefore be interpreted as preliminary. A formal missingness analysis indicated that missing values in miR-34a were associated with lower BMI ($p = 0.013$) and lower vegetable variety ($p = 0.043$), suggesting that missingness in this miRNA may not be entirely random (i.e., potentially MAR (missing at random) or MNAR (missing not at random) rather than MCAR (missing completely at random)). Analyses involving miR-34a should therefore be interpreted with caution, as the available sample may disproportionately represent participants with higher BMI and greater dietary variety, potentially introducing bias in the estimated associations.

Cross-sectional design. As an observational cross-sectional study, no causal inference is possible. Associations between dietary exposures and miRNA expression may reflect reverse causation, unmeasured confounding, or coincidental co-variation. Longitudinal or intervention studies are required to establish directional relationships.

Circulating versus tissue-specific miRNA expression. MiRNA levels in this study were measured from capillary whole blood collected on Whatman filter cards, not from liver tissue. Circulating miRNAs originate from multiple tissues and cell types, and blood-based profiles do not exclusively reflect hepatic expression. While the six miRNAs investigated have well-documented roles in liver biology, changes in their circulating levels may also be driven by non-hepatic sources such as immune cells, adipose tissue, or vascular endothelium (Mori et al., 2019). The extent to which capillary blood miRNA levels correspond to hepatic miRNA expression in healthy individuals remains unclear, and this limits the organ-specificity of any observed associations. While dried blood spots are increasingly used for miRNA profiling, the comparability of DBS-based miRNA quantification with standard plasma-based methods has not been systematically validated for the specific panel used in this study.

Dietary assessment. Dietary data were collected via a simplified questionnaire with categorical frequency items, which cannot capture portion sizes, preparation methods, or detailed nutritional composition. This limitation may have led to exposure misclassification, potentially obscuring true associations; future studies should consider using more detailed dietary tools such as food diaries or 24-hour recalls.

Residual confounding. Regression models were adjusted for age, BMI, and sex. Although additional variables such as physical activity and supplement intake were assessed in the questionnaire, they were not included as covariates to preserve model stability given the small effective sample sizes. The binary or categorical coding of dietary exposures may further have introduced residual confounding by grouping participants with meaningfully different intake patterns into the same category.

ΔCt as miRNA expression measure. ΔCt values represent relative expression normalized to a reference gene, and a lower ΔCt value corresponds to higher relative expression. This directionality must be considered when comparing findings across studies using different normalization approaches.

Multiple testing and type II error. Despite Benjamini-Hochberg FDR correction within each analysis block, the large number of tested exposure-miRNA combinations, combined with the small sample size, may have reduced statistical power and increased the risk of false negatives (type II error), such that true associations may have gone undetected.

Future research directions. Studies with larger sample sizes, prospective designs, and more comprehensive dietary assessment are needed to confirm the preliminary associations observed here. Targeted follow-up of the vegetable variety and miR-130a-3p finding in adequately powered cohorts would be particularly valuable. Integration of plasma metabolomics or untargeted food biomarker approaches could provide more objective exposure assessment and help elucidate the specific dietary components driving any observed miRNA modulation.

7. CONCLUSION

The aim of this study was to investigate whether dietary and lifestyle exposures are associated with the expression of six liver-associated miRNAs linked to cellular senescence (miR-21, miR-34a, miR-155-5p, miR-146a, miR-126a-5p, miR-130a-3p) in a preventive health cohort. MiRNA expression was measured from capillary blood collected on Whatman filter cards in a subsample of n=48 participants with available questionnaire data. The analytical approach combined exploratory multivariable regression with three pre-specified hypothesis-driven models targeting omega-3 fatty acid intake, plant-based diet quality, and UPF consumption.

The main hypothesis, that diets rich in secondary plant compounds are associated with higher expression of protective miRNAs, was partially supported. High vegetable variety was significantly associated with higher miR-130a-3p expression after FDR correction ($\beta = -0.95$, $q = 0.039$), a miRNA with established roles in hepatic lipid metabolism and anti-fibrotic signaling. This finding was robust across sensitivity analyses and biologically plausible. The secondary hypotheses (omega-3 and UPF) were not supported, most likely due to the small effective sample sizes (n=11 for key models), the limited resolution of categorical dietary assessment, and the low variance in dietary exposures within this health-conscious cohort. Nominal associations between vegetable and fruit intake and miR-21, whole grains and miR-34a, and alcohol use and miR-155-5p suggest additional diet-responsive targets that require confirmation.

However, several limitations should be considered. The cross-sectional design does not allow causal conclusions, miRNA levels measured from capillary blood may not accurately reflect intrahepatic miRNA expression, and the influence of unadjusted confounders cannot be ruled out. Replication in larger cohorts with prospective designs and more precise dietary assessment tools would be needed to confirm these findings. If supported by future research, dietary vegetable diversity could become a practical target for strategies aimed at promoting liver health at the molecular level.

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Use of AI-based tools

This thesis represents the independent and original work of the author. AI-based tools (Perplexity, Claude, ChatGPT) were used only to support the writing and analytical process, including improving clarity and style, suggesting alternative wordings, assisting with the translation of selected passages (e.g. the abstract), supporting the revision of individual sections, and providing guidance on statistical coding and output interpretation. The study was conceived, all data were processed and analyzed, and all findings were interpreted by the author alone. All content is traceable and fully supported by appropriately cited sources in line with good scientific practice.

9. APPENDIX

9.1. Questionnaire

Lifestyle Questionnaire

Please complete this questionnaire and send it with the Bloodspot Protein Saver Card to the Vienna Lab.
Please use the enclosed return envelope.

Patient Information:

Test Requested: ☐ Prevention Panel ☐ Follow-Up Panel

First Name: _____

Last Name: _____

Date of Birth: _____

Gender: _____

Height (in m): _____

Weight (in kg) or BMI: _____

Waist Circumference (measurement above belly button): _____

Address: _____

Phone: _____

Email: _____

Requesting Practitioner:

Full Name: _____

Email: _____

Phone: _____

Please send test results to following email:

_____

Warranty, liability and research

The test results do not present a diagnosis for disease or other malfunction, but shall exclusively serve to support a nutritional counselling. LAULARMED carries out the tests under Austrian scientific nutritional standards and does not assume any liability for the information provided by the patient. The results from the test can be used by HealthBioCare to improve clinical research. All data is stored and used in accordance with EU data protection guidelines. It is only assumed that human samples are analyzed. HBC analyzes do not include a test to verify this. The test results provided by LAULARMED do not present a diagnosis of the health state of the person.

Acknowledged by **(please sign below)**:

Name: _____

Date: _____

1. Ethnicity

(Mark the appropriate categories)

Ethnicity	Myself	Mother	Father
African (Sub-Sahara) or African American			
North Asian			
South Asian (incl. Southeast Asian Islands)			
New Guinea and Australian Aboriginal			
Caucasian ("white")			
Middle East			
Northeast Asian and Arctic			
Pacific Islander			
Native American			
No answer			

2. Have you suffered from any of the following ailments more than twice in a year?

- ☐ Influenza
- ☐ Inflammation of the skin or mucus membrane
- ☐ Inflammatory disorder of the stomach or intestines
- ☐ Muscle or joint disorders
- ☐ Bladder infection
- ☐ Genital warts
- ☐ Warts on the skin or feet
- ☐ Depression
- ☐ High blood pressure
- ☐ Other: _____
- ☐ None

3. Have you been diagnosed with any of the following metabolic disorders?

- ☐ Diabetes Mellitus Type I
- ☐ Diabetes Mellitus Type II
- ☐ Thyroid Disfunction
- ☐ Pancreatic Disorder
- ☐ Gout
- ☐ Lipid Metabolic Disorder
- ☐ Impaired Glucose Tolerance
- ☐ None of the above

4. Have you been diagnosed with an intolerance to the following foods or ingredients?

- ☐ Grains containing gluten
- ☐ Wheat
- ☐ Shellfish and products with shellfish
- ☐ Eggs and products with eggs
- ☐ Fish and fish products (other than fish gelatin)
- ☐ Fructose
- ☐ Peanuts and peanut products
- ☐ Soy (beans) and soy products
- ☐ Milk and dairy products (including sheep and goat milk)
- ☐ Lactose
- ☐ Nuts and products produced with nuts
- ☐ Celery and products containing celery
- ☐ Mustard and mustard products
- ☐ Sesame seeds and products containing sesame seeds
- ☐ Sulphur dioxide and related products
- ☐ Lupines and products made from lupines
- ☐ Mollusks such as snails, muscles, squid, octopus, and related foods
- ☐ Other: _____

5. Do you currently take medication for treatment of the following?

- ☐ Diabetes
- ☐ High blood pressure
- ☐ Thyroid disorders
- ☐ Depression / Burnout
- ☐ Lipid Metabolic Disorder
- ☐ Hormone replacement therapy
- ☐ Contraception
- ☐ Other: _____

6. Do you adhere to a specific nutrition plan or diet?

- ☐ Vegetarian
- ☐ Vegan
- ☐ Low Carb (max.____g carbohydrates/day)
- ☐ Ketogenic (max.____g carbohydrates/day)
- ☐ High Carb (min.____g carbohydrates/day)
- ☐ Paleo
- ☐ Hypoallergenic
- ☐ Gluten free
- ☐ Lactose free
- ☐ Intermittent fasting / Dinner canceling
- ☐ Calorie reduction
- ☐ Other: _____
- ☐ None

7. How many days a week do you eat fast food, ready-made meals, or traditional foods (such as roast pork)?

- ☐ Every day of the week
- ☐ Most days in the week
- ☐ Seldom / Never

8. How often do you eat vegetables? (Please provide the appropriate frequency for day, week, and month.)

- ☐ ____ / Day
- ☐ ____ / Week
- ☐ ____ / Month
- ☐ Never

9. Which types of vegetables do you prefer?

- ☐ Leafy Green (Salad, spinach, chard, bok choy, ...)
- ☐ Cruciferous (Broccoli, cabbage, brussels sprouts, ...)
- ☐ Legumes (Peas, lentils, ...)
- ☐ Starchy (Potato, sweet potato, ...)
- ☐ Other (Tomato, pepper, celery, eggplant, zucchini, ...)

10. How often do you eat fruits? (Please provide the appropriate frequency for day, week, and month.)

- ☐ ____/Day
- ☐ ____/Week
- ☐ ____/Month
- ☐ Never

11. Which types of fruit do you prefer?

- ☐ Berries (Strawberry, blackberry, raspberry, ...)
- ☐ Pitted (Cherry, plum, nectarine, peach, ...)
- ☐ Seeded (Apple, pear, quince, ...)

12. How many days per week do you consume dairy products (including sheep and goat milk)?

- ☐ Never
- ☐ Occasionally to every other day
- ☐ Every day
- ☐ More than once a day

13. How many days per week do you consume whole grain products?

- ☐ Seldom to never
- ☐ Once a week
- ☐ 2–3 times a week
- ☐ 4–6 times a week
- ☐ Daily
- ☐ More than once a day

14. Which side dishes do you eat often (every other day or daily)?

- ☐ Rice
- ☐ Potatoes
- ☐ Noodles
- ☐ Whole grain bread
- ☐ Bread
- ☐ Quinoa/buckwheat/amaranth
- ☐ None

15. How many eggs do you eat in a week?

- ☐ None
- ☐ 1–2 per week
- ☐ 3–4 per week
- ☐ 5–6 per week
- ☐ Seven or more per week

16. How many times a week do you eat fish?

- ☐ Seldom to never
- ☐ Once per week
- ☐ 2–3 times per week
- ☐ 4 or more times per week

17. How often do you eat white meat (turkey or chicken)?

- ☐ Once per week
- ☐ 2–3 times per week
- ☐ 4 or more times per week
- ☐ Daily
- ☐ More than once a day

18. How often do you eat red meat (pork or beef)?

- ☐ Seldom to never
- ☐ Once per week
- ☐ 2–3 times per week
- ☐ 4 or more times per week
- ☐ Daily
- ☐ More than once a day

19. How often do you consume processed meats/sausages?

- ☐ Seldom to never
- ☐ Once per week
- ☐ 2–3 times per week
- ☐ 4 or more times per week
- ☐ Daily
- ☐ More than once a day

20. Do you take the following supplements?

Vitamins:

- ☐ Biotin
- ☐ Folic acid
- ☐ Multivitamin
- ☐ Vitamin B12
- ☐ Vitamin B3
- ☐ Vitamin C
- ☐ Vitamin D

Minerals:

- ☐ Iron
- ☐ Calcium
- ☐ Magnesium
- ☐ Selenium
- ☐ Zinc

Lifestyle:

- ☐ Amino acids/Protein
- ☐ Green tea capsules
- ☐ Prebiotic extracts
- ☐ Probiotic extracts
- ☐ Fiber
- ☐ Garlic extract
- ☐ L-Carnitine
- ☐ Timeblock®
- ☐ Other: _____
- ☐ None

21. How much water do you drink per day?

- ☐ Less than 1 liter
- ☐ 1 – 2 liters
- ☐ 2 – 3 liters
- ☐ More than 3 liters

22. Which beverages do you prefer (and consume daily)?

- ☐ Tea (black, green)
- ☐ Tea (black, green) sweetened with sugar
- ☐ Tea (herbal)
- ☐ Tea (herbal) sweetened with sugar
- ☐ Tea (fruit)
- ☐ Tea (fruit) sweetened with sugar
- ☐ Coffee
- ☐ Coffee sweetened with sugar
- ☐ (Mineral) Water
- ☐ Juice
- ☐ Diluted juice
- ☐ Nectar
- ☐ Concentrated juice
- ☐ Lemonade without caffeine
- ☐ Cola
- ☐ Energy drinks
- ☐ Other: _____

23. What kinds of alcohol do you drink and how often?

	Beer	Wine	Liquor
Never			
Only on occasion			
1 – 2 times per month			
3 – 5 times per month			
1 – 2 times per week			
Daily			

24. Do you smoke or have you smoked in the past?

- ☐ No, I do not smoke and have never smoked
- ☐ I used to smoke (over 10 years ago)
- ☐ I used to smoke (less than 10 years ago)
- ☐ Yes, currently less than a pack a day
- ☐ Yes, currently more than a pack a day

25. How many of the listed sweets or snacks do you consume more often than 3 times per week?

- ☐ Chocolate / Pralines
- ☐ Cake/Baked goods
- ☐ Cookies
- ☐ Gummy bears / Gummy candies
- ☐ Candies / Sweets
- ☐ Ice cream
- ☐ Pudding / Creams
- ☐ Chips
- ☐ Popcorn
- ☐ Salted nuts
- ☐ Other: _____

26. Do you use the following sweeteners daily?

- ☐ Stevia
- ☐ Erythritol
- ☐ Retail sugar
- ☐ Birch sugar (Xylitol)
- ☐ Aspartame
- ☐ Agave nectar
- ☐ Rice syrup
- ☐ Coconut flower syrup / sugar
- ☐ Honey

27. What mealtimes are in your dietary schedule?

	Weekdays	Weekends
Breakfast		
Morning snack		
Lunch		
Afternoon snack		
Dinner		
Late night		

28. How often in the week are you physically active (shopping on foot, walking, working in the garden)?

- ☐ Seldom to never
- ☐ Once per week
- ☐ 2 – 5 times per week
- ☐ 6 – 7 times per week

29. How often in the week do you engage in a sport (over 30 minutes)? Please mark the following

	Endurance	Strength
Seldom to never (max. 2 times per month)		
1 – 2 times per week		
More than 2 times a week		

30. How high would you assess your positive stress (eustress) at work, in your free time or in your family in reflection of the past month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

31. How high would you assess your negative stress (distress) at work, in your free time or in your family in reflection of the past month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

32. How high would you rate your current sense of well-being with that of last month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

33. How is your contentment with your current lifestyle in general?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

34. How is your contentment with your current lifestyle in reflection of the last month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

35. How high do you assess your quality of life in general?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

36. How high would you assess your quality of life in reflection of the last month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

37. How would you rate your work performance in general?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

38. How would you rate your work performance in reflection of the last month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

39. How would you rate your performance in your private life in general?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

40. How would you rate your performance in your private life in reflection of the last month?

- ☐ Low
- ☐ Moderate
- ☐ High
- ☐ Very high

41. Do you suffer from anxiety more often than twice a year?

- ☐ Yes
- ☐ No

42. Would you like to:

- ☐ Lose weight
- ☐ Maintain your weight
- ☐ Increase your weight
- ☐ Follow lifestyle recommendations
- ☐ Follow personalized supplement recommendations

43. What are your expectations for this analysis?

- ☐ Status quo of your cell health
- ☐ Root cause analysis of your ailments
- ☐ Intervention / Intervention check

Thank you for completing the questionnaire

9.2. RNA extraction protocol

7 PROCEDURE

7.1 PHASE I – RNA EXTRACTION FROM DRIED BLOOD SPOTS

7.1.1 OBJECTIVE

This phase describes the procedure for isolating high-quality total RNA, including microRNA (miRNA), from dried blood spot (DBS) samples using the MagMAX™ FFPE DNA/RNA Ultra Kit in combination with the KingFisher™ DUO magnetic particle processor. The protocol involves overnight enzymatic digestion followed by magnetic bead-based purification to ensure high yield and integrity of RNA for downstream applications.

7.1.2 REAGENT PREPARATION (BEFORE DAY 1)

Prepare wash buffers **prior to first use** of the kit:

- **RNA Wash Solution Buffer**: Add 46 mL **isopropanol** to the RNA Wash Solution Buffer Concentrate, mix thoroughly, and store at room temperature.
- **Wash Solution 2**: Add 168 mL **ethanol** to the Wash Solution 2 Concentrate, mix, and store at room temperature.

7.1.3 DAY 1 – BLOOD SPOT PUNCHING AND PROTEASE DIGESTION

Protease Solution Preparation:

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

Procedure:

1. Excise **4 × 4 mm** punches from each patient DBS card using a sterile puncher and place into a labeled 1.5 mL RNase-free microcentrifuge tube using tweezers and a metal rod.
2. Sterilize tools (puncher, rod, tweezers) between each sample by immersing in ethanol and briefly passing through a flame.
3. Add **235 µL** of freshly prepared protease solution to each tube, ensuring the sample is fully submerged.
4. Incubate the tubes **overnight** at **55 °C**, **850 rpm** in a ThermoMixer.

7.1.4 DAY 2 – NUCLEIC ACID PURIFICATION (KINGFISHER DUO)

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.

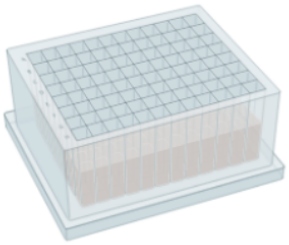


PLATE SETUP

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

Note[1]: After DNase digestion, the instrument prompts the addition of **450 µL RNA Rebinding Buffer** to Row A of the RNA plate.

Execution on KingFisher DUO:

1. Centrifuge digestion tubes, compress the DBS punches with a pipette tip, and transfer up to **200 µL** of supernatant to Row A of the **DNA plate**.
2. Insert the **12-tip magnetic comb** into Row F.
3. Ensure KingFisher™ DUO is configured with the correct deep-well magnetic head and select the **"DBS RNA and DNA extraction"** protocol.
4. Start the run and follow the prompts:
   - **After ~15–20 minutes**:
     - Remove DNA plate, add **20 µL beads** + **650 µL RNA Binding Buffer** to each well in Row A
     - Return the plate and resume run
   - **After ~50–55 minutes**:
     - Remove RNA plate, add **450 µL RNA Rebinding Buffer** to each Row A well, **vortex gently**
     - Return the plate and resume run
5. After run completion, transfer eluates from Row H of each plate to labeled 1.5 mL RNase-free tubes.
6. Add **100 µL nuclease-free water** to DNA samples if further dilution is needed.
7. Store all eluates at **–20 °C**.

Waste Disposal:
Discard deep-well plates, tip combs, and liquid waste in accordance with SOP Section 5.7.

9.3. cDNA-Synthesis

7.2 PHASE II – MIRNA CDNA SYNTHESIS

7.2.1 OBJECTIVE

This phase outlines the reverse transcription of miRNA extracted from dried blood spots into complementary DNA (cDNA) using the TaqMan® Advanced miRNA cDNA Synthesis Kit. The protocol follows a four-step enzymatic conversion: polyadenylation, adaptor ligation, reverse transcription, and pre-amplification (miR-Amp), optimized for low-input samples. The final product is diluted for downstream qPCR.

7.2.2 PREPARATION BEFORE STARTING

- Verify that samples belong to miRNA-based panels (e.g., Metabo, Kombi, Stress, Sport, EBV, ViRepair, Virmune) — **Aging Panel is excluded**.
- Label **three PCR tubes** per sample:
  - **RT** (poly(A), adaptor ligation, RT)
  - **miR-Amp** (pre-amplification)
  - **1:10 dilution** (diluted product)
- Prepare **0.1× TE Buffer** for miR-Amp dilution:
  - Mix 1 part 1× TE with 9 parts nuclease-free water
- Thaw reagents on **ice**; mix gently and briefly centrifuge before use

7.2.3 POLY(A) TAILING REACTION

This step adds a polyadenylated (poly[A]) tail to mature miRNAs, enabling universal primer binding during reverse transcription. The reaction is temperature-sensitive and must be conducted using RNase-free techniques.

PREPARATION NOTES:

- Thaw RNA samples and **TaqMan® cDNA Synthesis reagents** on ice.
- Mix all reagents by **gentle vortexing** and briefly centrifuge to collect liquid and eliminate air bubbles.
- **Do not thaw assay primers/probes** until needed.
- ⚠ **IMPORTANT:** The 50% PEG 8000 reagent (used in the next step) must be at **room temperature**—warm to 32 °C if crystallized.

POLY(A) REACTION MIX PREPARATION

Prepare the following master mix in a 1.5 mL RNase-free microcentrifuge tube:

Component	Per Reaction	12 Reactions (with 10% overage)
10× 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 Volume	3.0 µL	39.6 µL

- **Mix gently by vortexing, then centrifuge briefly.**

REACTION SETUP:

1. Dispense **3.0 µL** of Poly(A) Reaction Mix into each RNase-free PCR tube.
2. Add **2.0 µL** of purified RNA sample to each tube.
    - Total reaction volume: **5.0 µL**
3. Mix by pipetting up and down; avoid introducing bubbles.
4. Seal the tubes and centrifuge briefly to collect contents and remove air.

THERMAL CYCLING CONDITIONS (SIMPLIAMP PCR SYSTEM):

Use the black tube adaptor plate and run the following program:

Step	Temperature	Time
Polyadenylation	37 °C	45 min
Stop Reaction	65 °C	10 min
Hold	4 °C	Hold

7.2.4 ADAPTOR LIGATION REACTION

This step ligates a universal adaptor to the polyadenylated 3' end of the miRNA, enabling targeted reverse transcription in the next phase.

IMPORTANT – PEG Handling Notes:

- Use **50% PEG 8000** at **room temperature**.
- If crystallized, incubate at **32 °C for 5–10 minutes** to dissolve.
- **Pipetting technique for PEG:**
    - Aspirate slowly; hold the pipette tip in solution for ~10 seconds.
    - Dispense slowly; hold plunger down for ~10 seconds to fully empty the tip.

LIGATION REACTION MIX PREPARATION

Component	Per Reaction	12 Rxns (with 10% overage)
5× DNA Ligase Buffer	3.0 µL	39.6 µL
50% PEG 8000	4.5 µL	59.4 µL
25× 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 Volume	**10.0 µL**	**132.0 µL**

REACTION SETUP

1. Vortex and briefly centrifuge the master mix to eliminate bubbles.
2. Add **10 µL** of Ligation Reaction Mix to each tube containing **5 µL** of poly(A) tailing product (from previous step).
    - **Final volume: 15 µL**
3. Mix gently by pipetting, seal, and centrifuge tubes briefly.

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

→ **Proceed immediately to Reverse Transcription (RT).**

7.2.5 REVERSE TRANSCRIPTION REACTION (RT)

This step synthesizes first-strand cDNA from adaptor-ligated miRNA using a universal primer.

RT REACTION MIX PREPARATION

Component	Per Reaction	12 Rxns (with 10% overage)
5× RT Buffer	6.0 µL	79.2 µL
25 mM dNTP Mix	1.2 µL	15.84 µL
20× Universal RT Primer	1.5 µL	19.8 µL
10× RT Enzyme Mix	3.0 µL	39.6 µL
RNase-free Water	3.3 µL	43.56 µL
Total Volume	15.0 µL	198.0 µL

REACTION SETUP

1. Vortex and briefly centrifuge the RT master mix.
2. Add **15 µL** of RT mix to each ligation tube containing **15 µL** of sample.
    - Final volume: **30 µL**
3. Mix by pipetting, seal, and centrifuge briefly.

THERMAL CYCLING CONDITIONS:

Step	Temperature	Time
Reverse Transcription	42 °C	15 min
Enzyme Inactivation	85 °C	5 min
Hold	4 °C	∞

→ **Store RT product at –20 °C for up to 2 months if not proceeding immediately.**

9.4. qPCR

7.3 PHASE III – REAL-TIME PCR (QPCR) ANALYSIS OF MIRNA

7.3.1 OBJECTIVE

This phase outlines the procedure for detecting and quantifying miRNA expression using the TaqMan® Fast Advanced Master Mix and miRNA-specific assays on the QuantStudio™ 3 (QS3) system. The method supports a wide range of diagnostic panels and ensures consistency for both baseline and follow-up analysis.

7.3.2 PRE-RUN SETUP

Before proceeding, ensure the following tasks are completed:

- **Identify target samples**: Check the Probenliste to confirm panel type (e.g., Metabo, Kombi, Stress, Sport (+), Prevention, EBV, Virmune, Virepair, etc.).
- **Verify miRNA assays** are required for each panel. Cross-check with the list:
  Übersicht miRNAs Panels_19.02.2024.xlsx (Location: HBCServer\SOP\Routine)
- **Determine the correct positive controls**:
  - Use **UM** for most panels
  - Use **UK** for EBV
- **Design your plate/run** in the **QuantStudio Design Software**:
  - Use an existing .edt template from HBCServer/QS3/miRNAs/
  - Adjust plate layout via **"Plate" → "Advanced Setup"**
  - Ensure the correct miRNA targets are selected

→ **Important**: If a sample belongs to **multiple panels**, verify which miRNAs are required and load **all targets for that sample on the same plate** to avoid duplication.
All follow-up panels must include the same miRNAs as in the baseline panel.

7.3.3 PANEL-TO-miRNA MAPPING

miRNA	Metabo	Kombi	Prevention	Stress	Sport/+	EBV	Virmune	Virepair
H miR-24-3p								
H miR-93-5p								
H miR-103a-3p						x		x
miR-21-5p								
miR-155-3p								
miR-19b-3p								
miR-20a-5p								
miR-22-5p								
miR-30e-3p								
miR-101-3p								
miR-146a-5p								x
miR-378a-3p								
miR-505-3p								
let-7a-5p								
let-7g-5p								
miR-16-5p								
miR-15a-5p								
miR-106b-5p								
miR-30e-5p								
miR-122-5p								

miR-132-3p								
miR-150-5p								
miR-139-5p								
miR-181a-5p								
miR-127-3p								
miR-151a-5p								
miR-328-3p								
miR-877-5p								
miR- 31-5p								
miR-200c-3p								
								-
miR-125b-5p								
								-
miR-199a-5p								
miR-29b-3p								
miR-155-5p								
miR-1307-3p								
miR-10a-5p								
UM control								
UK control								

7.3.4 EXPERIMENTAL PROCEDURE

STEP 1 – INSTRUMENT & TEMPLATE SETUP

- Turn on the **QS3** instrument
- Write down your **manual pipetting layout**
- Thaw **diluted 1:10 miR-Amp samples** and **TaqMan® miRNA assays** on ice

STEP 2 – MASTER MIX PREPARATION

Prepare one master mix per miRNA target in **labeled 1.5 mL tubes**:

Component	1 Rxn	6.5 Rxns
TaqMan® Fast Adv. MM	5 µL	32.5 µL
TaqMan® miRNA Assay	0.5 µL	3.2 µL
Nuclease-Free Water (NFW)	2.0 µL	13.0 µL
Total Volume	**7.5 µL**	**48.7 µL**

Note: If you're running 12 different miRNAs, you'll need 12 master mixes.

STEP 3 – PLATE LOADING

1. Place a clean **96-well PCR plate** on a plate rack
2. Dispense **7.5 µL of each Master Mix** into the designated wells
    - Use **reverse pipetting**
    - Use **one tip per Master Mix**
3. Add **2.5 µL of 1:10 diluted miR-Amp product** into corresponding wells
    - Use a **new pipette tip for each sample**
4. Add **positive control (UM/UK)** samples to the assigned wells
5. Seal the plate securely with **optical sealing film**
    - Ensure firm contact across and between wells
6. Centrifuge briefly to eliminate air bubbles

STEP 4 – RUNNING THE QPCR

1. Load the sealed plate directly into the **QS3** (no well rack required)
2. On the QS3 Software:
    - Navigate to:
      Network Drive > miRNAs > [Current Year] > [Month]
    - Load the appropriate .edt template
    - Verify and start the run

STEP 5 – EXPORTING RESULTS

- After run completion, **export the run** as a .eds file
- Save the file in the same folder used for setup:
  \\HBCServer\QS3\miRNAs\YYYY\MM\

7.4 PHASE IV – MIRNA DATA ANALYSIS

7.4.1 OBJECTIVE

This phase describes the analysis of miRNA expression data after qPCR amplification using QuantStudio™ 3 and Thermo Fisher Cloud. The procedure ensures consistency across panels by using validated endogenous controls and reference samples, enabling accurate interpretation of ΔCt or RQ values.

7.4.2 PRE-ANALYSIS CHECKLIST

Before starting data analysis, ensure the following:

- The .eds file has been **transferred to the server**: \\QS3\miRNA\[Current Year]\[Current Month]\filename.eds
- **Target names in the run** (e.g., miRNA assay IDs) match exactly with those used in **Thermo Fisher Cloud panel definitions**
- Samples are correctly labeled and traceable to the panel order
- Housekeeper miRNAs (e.g., **miR-24**, **miR-93**) are successfully amplified

7.4.3 STEP-BY-STEP DATA ANALYSIS

A. In QuantStudio Design & Analysis Software

1. Open the .eds run file with **QuantStudio Design & Analysis Software**
2. Inspect **amplification plots** for:
    - Overall curve quality
    - Consistent amplification of **housekeeper miRNAs**
    - Absence of non-specific signals or outliers
3. Perform any necessary **adjustments**, then **Save** the file

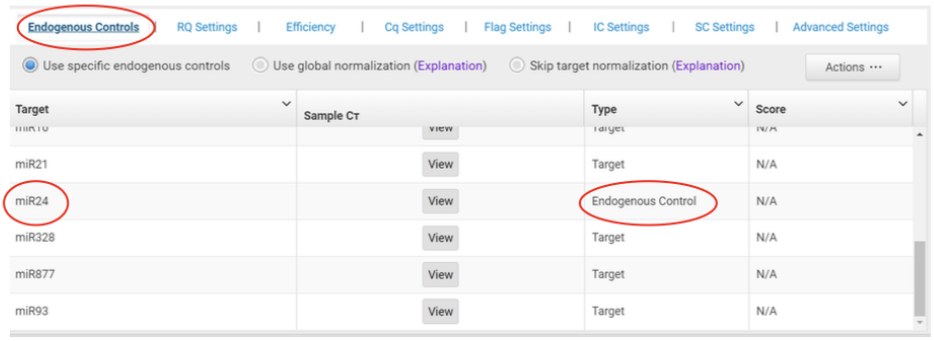
B. In Thermo Fisher Cloud

1. Go to Thermo Fisher Cloud and log in
2. Navigate to your corresponding **Study** (linked to the panel used)
3. Click **Import Run** → Select your .eds file from the server
4. In **Plate Setup**:
    - Check that all **targets and samples** are relevant to the selected panel
    - Delete any **non-panel-related** targets/samples
    - Click **Save**

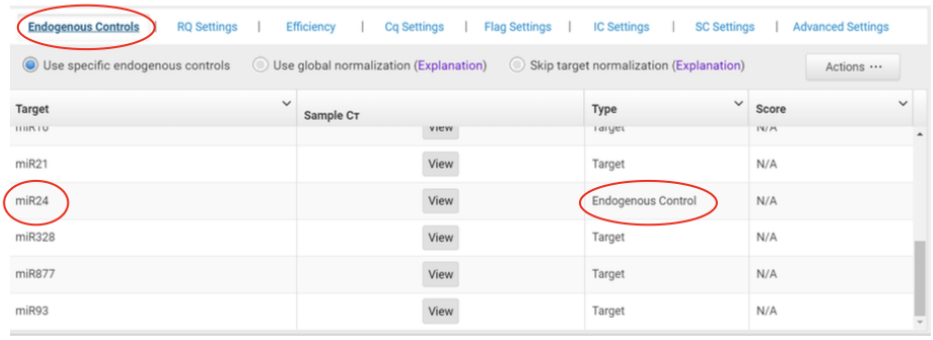
7.4.4 ANALYSIS SETTINGS IN THERMO FISHER CLOUD

Once the run is imported and plate setup is finalized, proceed with analysis configuration:

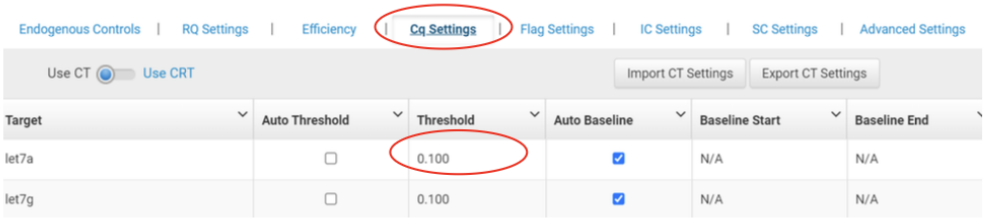
1. **Navigate to the Analysis Settings tab**
2. **Set Endogenous Control(s)**



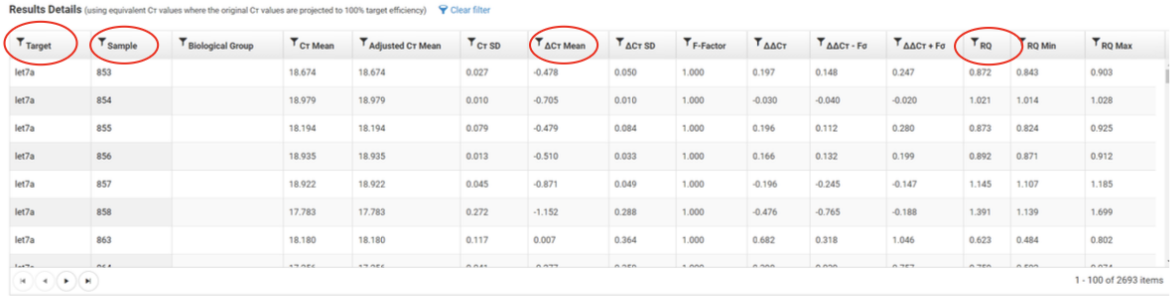
3. **Set the Reference Sample**



4. **Set the threshold value** for **all targets to 0.100**



5. Click **Finish** and then click **Analyze**
6. In the **Analysis** tab:
    - View results as **ΔCt Mean** or **RQ Values**, depending on the panel
    - Use filter tools to select individual **targets** or **samples**



7.4.5 PANEL-SPECIFIC ANALYSIS CONFIGURATION

Panel	Endogenous miRNA(s)	Reference Sample	Reported Value
Metabolic / Kombi / Bluezones / Intervention / Prevention	miR-24 & miR-93	UM	ΔCt Mean
Stress / miRNA & Stress	miR-24	UM	ΔCt Mean
Sport / Kraftsportstudie	miR-24	UM	ΔCt Mean
EBV	miR-24	UK	RQ
ViRepair	miR-24 & miR-93	UM	ΔCt Mean
Virmune	miR-24 & miR-93	UM	ΔCt Mean

7.4.6 ADDITIONAL EVALUATION – VIREPAIR & EBV PANELS

For **ViRepair** and **EBV**, data must also be recorded in a standardized Excel sheet.

8 Excel Instructions:

1. Enter **Sample Name**, **Sample Number**, and **Previous Sample Number** (if applicable) in **Columns A–D**
2. Enter **RQ results for EBV miRNAs** in **Columns E–J**
3. Enter **ΔCt results for ViRepair miRNAs** in **Columns K–V**
4. Enter **ΔCt results for EBV miRNAs (dual housekeeping)** in **Columns W–AB**
5. Notify **Berit** that new data entries have been added

9.5. Statistical results

9.5.1. Omega-3 hypothesis

Table A1. Full regression results: Omega-3 hypothesis. Exposure: fish_bin (weekly vs. rarely/never). Models adjusted for age, BMI, sex. n=11. q = BH-FDR within hypothesis block.

Exposure	miRNA	Model	n	β	p	q
Fish (weekly vs. rarely/never)	miR-126a-5p	RAW	11	−0.202	0.661	0.661
	miR-126a-5p	WINSORIZED	11	−0.202	0.661	0.661
	miR-130a-3p	RAW	11	−0.350	0.378	0.661
	miR-130a-3p	WINSORIZED	11	−0.350	0.378	0.661

Note: RAW = original values; WINSORIZED = outlier values trimmed to IQR bounds. No outliers detected in miR-126a-5p or miR-130a-3p; RAW and WINSORIZED results are identical.

9.5.2. Plant/Fiber/Antioxidant proxy hypothesis

*Table A2. Full regression results: Plant/Fiber/Antioxidant proxy hypothesis. Models adjusted for age, BMI, sex. q = BH-FDR within hypothesis block. * FDR-significant (q < 0.05).*

Exposure	miRNA	Model	n	β	p	q
Vegetable variety (high vs. low)	miR-130a-3p	RAW	11	−0.949	0.002	0.039*
	miR-130a-3p	WINSORIZED	11	−0.949	0.002	0.039*
	miR-126a-5p	RAW	11	−0.517	0.283	0.684
	miR-126a-5p	WINSORIZED	11	−0.517	0.283	0.684
	miR-21	RAW	25	0.239	0.514	0.686
	miR-21	WINSORIZED	25	0.239	0.514	0.686
	miR-34a	RAW	20	0.257	0.712	0.796
	miR-34a	WINSORIZED	20	0.246	0.723	0.796
Whole grain (weekly vs. rarely/never)	miR-34a	RAW	10	−1.814	0.025	0.203
	miR-34a	WINSORIZED	10	−1.814	0.025	0.203
	miR-21	RAW	11	0.743	0.352	0.684
	miR-21	WINSORIZED	11	0.743	0.352	0.684

Fiber supplement (yes vs. no)	miR-130a-3p	RAW	11	0.754	0.116	0.620
	miR-130a-3p	WINSORIZED	11	0.754	0.116	0.620
	miR-34a	RAW	20	−0.742	0.295	0.684
	miR-34a	WINSORIZED	20	−0.732	0.296	0.684
	miR-21	RAW	25	−0.446	0.293	0.684
	miR-21	WINSORIZED	25	−0.446	0.293	0.684
	miR-126a-5p	RAW	11	−0.175	0.771	0.796
	miR-126a-5p	WINSORIZED	11	−0.175	0.771	0.796
Vegetables/fruit (every day vs. rarely)	miR-21	RAW	26	0.664	0.381	0.684
	miR-21	WINSORIZED	26	0.664	0.381	0.684
	miR-34a	RAW	21	1.304	0.422	0.684
	miR-34a	WINSORIZED	21	1.286	0.425	0.684
	miR-126a-5p	RAW	11	0.438	0.470	0.684
	miR-130a-3p	RAW	11	0.396	0.461	0.684

Note: RAW = original values; WINSORIZED = outlier values trimmed. Vegetables/fruit: only every day vs. rarely comparison shown; occasionally/every other day results available on request. EXCLUDED model not available for miR-130a-3p × vegetable variety (n too small after outlier removal).

9.5.3. UPF hypothesis

Table A3. Full regression results: UPF hypothesis. Exposures: fast_food_bin, sweets_bin (weekly vs. rarely/never). Models adjusted for age, BMI, sex. n=22–26. q = BH-FDR within hypothesis block.

Exposure	miRNA	Model	n	β	p	q
Fast food (weekly vs. rarely/never)	miR-155-5p	RAW	26	0.605	0.224	0.448
	miR-155-5p	WINSORIZED	26	0.434	0.337	0.449
	miR-155-5p	EXCLUDED	22	−0.375	0.407	0.483
	miR-146a	RAW	26	−0.257	0.581	0.775
	miR-146a	WINSORIZED	26	−0.423	0.267	0.449
	miR-146a	EXCLUDED	22	−0.853	0.034	0.135
Sweets (high vs. low)	miR-146a	RAW	26	0.557	0.145	0.448

miR-146a	WINSORIZED	26	0.479	0.128	0.449
miR-146a	EXCLUDED	22	0.283	0.361	0.483
miR-155-5p	RAW	26	−0.105	0.805	0.805
miR-155-5p	WINSORIZED	26	−0.061	0.875	0.875
miR-155-5p	EXCLUDED	22	0.233	0.483	0.483

Note: RAW = original values; WINSORIZED = outlier values trimmed; EXCLUDED = participant with outlier removed. No result reached FDR significance (all q > 0.05).