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Functional Foods in Health and Disease

Epigenetic active phytoceuticals activate immune relevant miRNAs important in virus response systems

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ABSTRACT

Background: Viruses significantly affect global health. The few available viricidal and antiviral therapies are expensive and often associated with unwanted side effects. Functional foods as plant extracts (nutraceuticals) are epigenetically active, and multi-target antiviral compounds that affect several steps of the viral life cycle and host proteins. MicroRNAs (miRNAs) post-transcriptionally regulate host and viral gene expression and are responsible for the fine-tuning of gene expression by controlling the expression of their target messenger RNAs (mRNA) in host cells and viruses. Several plant ingredients have been proven to be active against RNA virus infections. Obviously, miRNAs play a central role in the regulation of gene transcription in viral replication and host immune defense. A healthy diet and nutraceuticals can support the fine-tuning of miRNAs.

Objectives: The aim of this study was to analyze the effects of epigenetic active phytoceuticals and/or functional foods on immune relevant miRNAs and mRNAs in a healthy human study population.

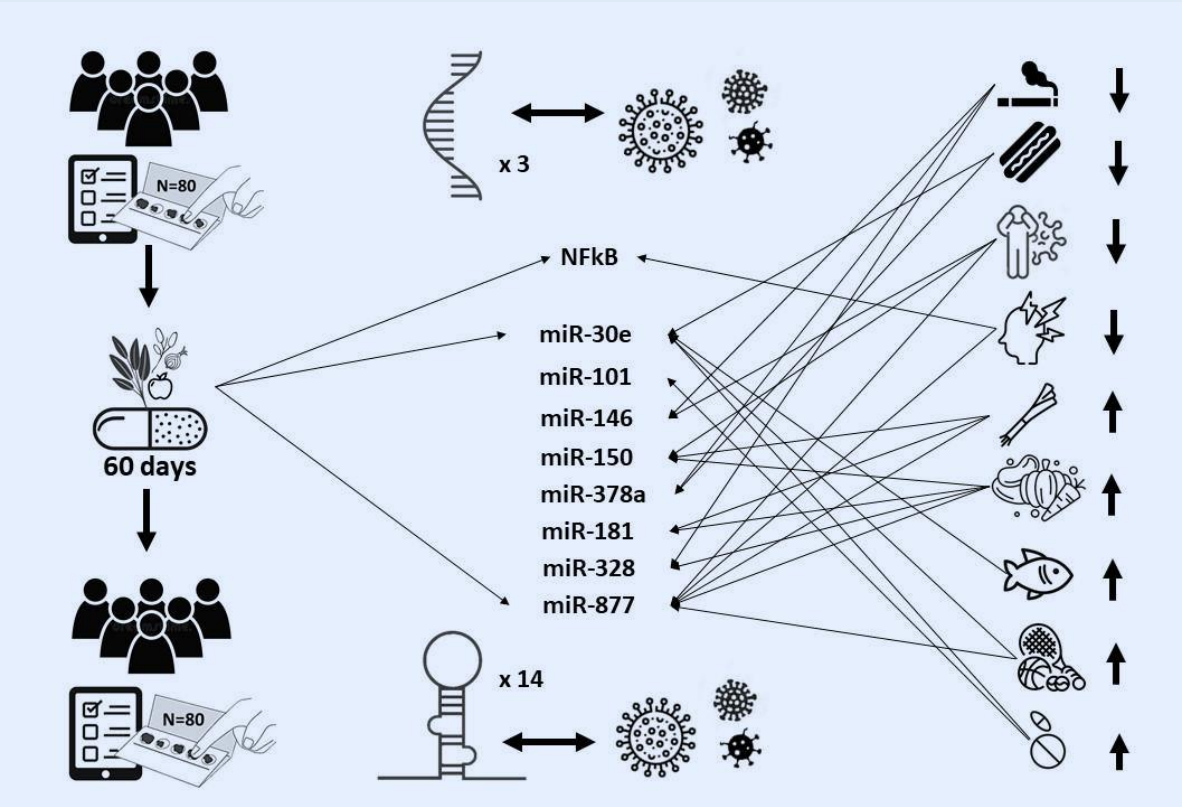
Methods: In a 2-month human intervention study (n=80), a mix of plant extracts (sage extract, green tea extract, berberine, apple extract, onion extract, elderberry extract, and grape skin extract) with proven activity against RNA viruses were examined for the change of miRNA and mRNA patterns involved in virus response systems. Lifestyle and nutritional behavior, including self-reported stress levels, as well as infection frequency as potential cofounders have been raised.

Results: The intervention with secondary plant extracts could modulate the expression of miRNAs involved in the virus defense (miR-30e, miR-877, miR-150) and the expression of NF-κB. The intervention also increased miRNA

expression patterns associated with a positive lifestyle. The intervention adapted miRNAs that correlate with an increased risk of infection, smoking, stress, and processed meat products.

Conclusion: Plant extracts can enhance the beneficial effects of a healthy nutrition and lifestyle by modulating miRNAs. MiRNAs are promising drug targets for maintaining immune homeostasis.

Keywords: antiviral, epigenetic active nutraceutical, miRNA, secondary plant extracts, virus response, plant ingredients, functional foods



INTRODUCTION

Instead of relying only on medication to treat viruses, functional food products are another way to combat symptoms and disease [1]. Since functional food science is using both a function-driven approach and a lifestyle approach to prevent and manage diseases, preventive or combination therapies with secondary plant substances (nutraceuticals) against viral diseases are becoming increasingly important [1].

The mechanism of action can be explained by their epigenetic regulatory ability. The regulation of the binding capacity of viral entry receptors and the regulation of immune-relevant genes are particularly important. The viral RNA in the cytoplasm can interact with host cell microRNAs (miRNAs) which can degrade the viral RNA and/or prevent viral replication. As such, host cellular miRNAs represent key cellular mediators of antiviral defense. Polyphenols, bioactive compounds found in plant-based foods, exert antiviral properties, partially due to their capacity to modulate the expression of miRNAs [2]. The initial defense against viruses is through the innate immunity. Pattern recognition receptors (PRRs) identify the type of intruder and activate intracellular signaling cascades, leading to the transcriptional expression of inflammatory mediators to coordinate the elimination

of pathogens and infected cells. To maintain a delicate balance between protective immunity and inflammatory pathology upon infection requires tight regulation of innate signaling pathways in the host [3]. Epigenetic reprogramming is discussed as a major possibility to control viral gene expression or latency [4]. MiRNAs in particular have emerged as potent modulators acting at the post-transcriptional levels.

Quality aspects for Functional Foods (FF) as preventive strategies in virus infections: The use of antiviral plants and their extracts has been part of human nutrition for thousands of years. Since the boundary between functional foods and medicines is not very clear, a precise definition of functional foods is required to differentiate them from medicines in order to make them accessible to patients, while still complying with all safety standards and effectiveness [5]. For that, the Functional Food Center has created a new system for categorizing functional foods. The new categorization system uses improved research on epidemiological and after market studies and evaluates the quality of evidence for the functional food product (FFP) as A, B, or C. A classification of A denotes the completion of aftermarket research, epidemiological studies, and certification of functional food status. Classification B denotes completion of epidemiological studies and certification of functional food status. Lastly, C indicates that the product has only been certified as functional [6]. Functional foods are defined as “Natural or processed foods that contain biologically-active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms." [5].

MiRNAs reflect the host – virus interaction: MicroRNAs (miRNAs) are short (22 nucleotides on average) non-coding RNAs that regulate up to 60% of the protein-coding genes in the human genome [7]·[8]. Together with specific proteins, they form an RNA-induced silencing complex (RISC) and bind to complementary target mRNAs to regulate gene expression [9]. Multiple works point to the critical role of miRNAs in the pathogenesis of various human diseases. Alterations of miRNA expression are observed in various types of cancer [10], in neurological diseases, and in immune regulation in infectious diseases [11]. Host miRNAs are known to be important regulators of virus replication and pathogenesis. They can interact with viruses through several mechanisms, including direct binding of viral RNA [12]. The primary effect produced by host miRNAs is to silence the viral RNA. MiRNAs also modulate host factors that confer an advantage on the viral pathogenesis [13]. Previous studies have shown that the expression of more than a hundred miRNAs was significantly altered in virus-infected cells [14]. An overview of the analyzed miRNA – Virus – nutrients are demonstrated in table 1. Examples of regulation are the modulation of mammalian target of rapamycin (mTOR) activity in T-cells through post-transcriptional regulation of mTOR mRNA by miRNAs or regulating the expression of cytochrome c oxidase. MiR-21-3p suppresses the replication of the coronavirus already in the first phases after infection. SARS-CoV-2 has been reported to interact with host miR-21–3p in the early stages of infection to inhibit its replication, resulting in a delayed immune response, supporting virus survival, and enhancing reproduction [12]·[15]. E.g. miR-21-3p is significantly increased in COVID-19 patients compared to healthy controls [16], and miR-181 family members are involved in inflammation regulation [17] and are considered as therapeutic targets due to their protective effects when down regulated [18], e.g. by quercetin [19]. The miR-328 plays a role in respiratory diseases [20] and can be up regulated by resveratrol [21]. MiR-146a is a dominant negative regulator of the innate immune response [22]. Quercetin increases

miR-146a expression [23]. Siniscalchi et al. 2021 has shown that the miR-378 can bind to the viral sequence and is able to repress plasmid-driven spike expression of SARS-CoV-2 [24]. MiR-30e-5p plays an integral role in the regulation of the innate immune response during viral infection [25]. Treatment with resveratrol on RPC (renal cells) can regulate miR-30a-5p, inhibit cell proliferation, promote apoptosis, and change cell cycle [26]. Thus, increased expression of miR-378 and miR-30e inhibits human NK cells cytotoxicity [27]. In addition, miR-181a, miR-30e, and miR-378 are involved in the direct regulation of the angiotensin-converting enzyme 2 (ACE2) [28], [29]. ACE2 is involved in modulating blood pressure and establishing blood pressure homeostasis. The mechanism for SARS-CoV-2 infection requires the binding of the virus to ACE2 receptor, well-known for its role in counteracting ACE [30]. MiR-101 acts as an antiviral host factor and as a key regulator of autophagy [31]. Berberine increases the expression of miR-101 and is able to suppress prostaglandin E2 (PGE2) signaling pathways [32]. MiR-150 has a binding site in the SARS-CoV-2 genome [33]. Critically ill patients infected with influenza A/H1N1 virus exhibit significant overexpression of circulating miR-150 compared to patients with milder disease [34]. Curcuminoids and Flavanones can modulate the expression of miR-150 [35]. MiR-877 is down regulated during respiratory viral infections [36].

Phytochemicals and the maintenance of immune homeostasis: Polyphenols could prevent SARS-CoV-2 infection by modulating the expression of miRNAs in the host cells [2]. In the last few decades, dietary polyphenols have become a focus of investigation because of their bioactive function in virus infection, prevention, and treatment. Due to their epigenetic active ingredients, nutraceuticals and functional foods have a broad potential to prevent the mechanisms of viral infections and modulating immune responses [4]. Phytochemicals exert their antiviral effects by directly controlling miRNA expression. Quercetin is a plant-based flavonoid derived from onions, citrus fruits, or apples. This phenolic compound negatively regulates LPS-induced TLR4 signaling. It reduces TLR4 expression, prevents NF-κB translocation into the nucleus, and significantly reduces the production of proinflammatory cytokines via MAP kinases and NF-κB pathway. Therefore, it has anti-inflammatory, antioxidant, chemopreventive, and neuroprotective properties [37],[38].

Table 1: Overview of the miRNAs reflecting host – virus interaction and their interaction with nutrients and/or phytoceuticals.

miRNA	characteristics	disease	up/ down regulation	Ref.	Phytoceuticals / nutrient interaction	up/ down regulation	Ref.
miR-21	inhibits replication of coronavirus in the first phase	SARS-CoV-2	up	[12], [15] [16]	PUFAs Vitamin E Vitamin D	down	[39]
miR-181	cell proliferation, apoptosis, autophagy, mitochondrial function, Tumor necrosis factor-α (TNFα) mRNA stability, and immune response	SARS-CoV-2	down	[17] [18] [33]	Quercetin	down	[19]
miR-328	role in respiratory diseases; liver inflammation; predictive role in the short-term prognosis of stroke	liver failure, stroke	up	[20] [40],[41]	Resveratrol	up	[21]
miR-146a	dominant negative regulator of the innate immune response		down	[22]	Quercetin	up	[23]
	can control inflammatory and proinflammatory reactions, regulating immune cell proliferation	pathogenic infections		[42]			

miRNA	characteristics	disease	up/ down regulation	Ref.	Phytoceuticals / nutrient interaction	up/ down regulation	Ref.
	TLR4-responsive, a NF-κB-associated gene			[43]			
	preventing sepsis-induced NF-κB signaling			[44]			
	immunopathogenesis of chronic hepatitis B infection	chronic hepatitis	up	[45]			
	targets cytokines such as type I IFNs, TNF-α, IL-6, IL-12, and IL-10			[38]			
miR-378	can bind to the viral sequence and is able to repress plasmid-driven spike expression	SARS-CoV-2 also variant B.1.1.7 and 501Y.V2		[24]			
miR-30e-5p	integral role in the regulation of the innate immune response, induced by viral molecular patterns	viral infections		[25]	Resveratrol	on RPC (renal cells) can regulate miR-30a-5p, inhibit cell proliferation, promote apoptosis, and change cell cycle	[26]
miR-378, miR-30e	IFN-α activation suppresses expression to release cytolytic molecular mRNAs for their protein translation and then augments cytotoxicity of NK cells. Thus, increased expression inhibits human NK cells cytotoxicity			[27]			
miR-101	antiviral host factor and as a key regulator of autophagy			[31]	Berberine	upregulation, is able to suppress prostaglandin E2	.[32]
	binding site	SARS-CoV-2		[33]			
	reduces the replication	Herpes simplex virus		[46]			
	abrogates viral life cycle by targeting the mTOR pathway,	influenza A virus	up	[47]			
	downregulates mTOR transcripts in later stages of infection	mTOR	down	[47]			
miR-150	binding site	SARS-CoV-2		[33]	Curcuminoids	regulation	[35]
	overexpression of circulating miR-150 compared to patients with milder disease	influenza A/H1N1 virus	over-expression	[34]	Flavanones	regulation	[35]
	correlate with IL-1ra, IL-2, IL-6, CXCL8, IFN-γ, CXCL10 and G-CSF						
	HBV-related liver diseases	liver diseases	up	[48]			
miR-877		respiratory viral infections	down	[36]	Polyphenols	suppression of FOXM1	[49]
	inversely related to age	aging	up in younger people	[36]	Rutin	up regulation in Cancer Cell Apoptosis	[50]
	regulate overproduction of IL-8 and IL-1β in mesangial cells activated by secretory IgA	nephropathy	regulation	[51]			

Anthocyanins are flavonoids with three phenol rings found in berries (elderberries), grapes and potatoes. They have anti-inflammatory effects, decrease TLR4 expression, inactivate NF-κB, and reduce pro-inflammatory mediators, such as iNOS and TNF-α. Anthocyanins exhibit epigenetic modulation capacity by inducing histone H3 acetylation and modulating HDAC and HAT activity [52]. Kolehmainen et al. 2012

showed in a human intervention study with anthocyanins a reduction of the inflammation score in the blood of patients, which was reflected by decreasing serum levels of IL-6, IL-12, and high sensitivity C reactive protein [53]. Resveratrol is a phenolic stilbene derivative extracted from grape skins, berries, and peanuts [37]. It inhibits the inflammatory factors TNF-α, IL-6, MAPKs [54] and modulates AP-1 activity in THP-1 human monocytes. Reduction in IkB phosphorylation and NF-κB activity reduces NF-κB induced pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, and the free radicals, NO and ROS, and LTs and PGs levels [43]. Chojnacka, K. et al 2020 showed that high concentrations of sage extract could inhibit SARS-CoV-2 replication [55] and block the enzymatic activity of 3CLpro [56]. In addition to antiviral activity, *S. officinalis* has been reported to induce antifungal, antibacterial, and antimalarial effects. The antiviral activity of *S. officinalis* is most likely mediated by two antiviral diterpenoids, safficinolide, and sageone [4].

Epigallocatechin-3-Gallate (EGCG) is one of the best-known polyphenolic catechins, found primarily in green tea, onions, apple skin, and plums [57]. It has remarkable anti-inflammatory, antioxidant, anti-cancer, and anti-angiogenetic effects.

Berberine is an isoquinoline alkaloid isolated from many medicinal herbs such as Rhizoma coptidis and Cortex phellodendri. The antiviral activity has been demonstrated for strains of the chikungunya virus (CHIKV) and Influenza (H1N1). Berberine reduces virus-induced mitogen activated protein kinase (MAPK) activation, including extracellular signal-related kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK), resulting in a stronger reduction of viral titers [4]. Berberine derivatives have also been evaluated for their ability to suppress TNF-α-induced nuclear factor (NF)-κB activation [32]. Phloretin, a natural phenolic compound, is a dihydrochalcone found in apples. It exhibits a wide variety of activities such as anti-oxidative, anti-inflammatory, anti-microbial, anti-allergic, anti-carcinogenic, anti-thrombotic, and hepatoprotective effects, and is also involved in the activation of apoptotic associated gene expression and signal transduction in molecular pathways [58]. Phloretin has antioxidant properties and affects the synthesis of pro-inflammatory molecules like PGE2, IL-8, IL-6, MCP1, and ICAM-1. Phloretin has also been shown to prevent TNF-α-stimulated upregulation of adhesion molecules (VCAM-1, ICAM-1, and E-selectin) [4].

In this study, we tested in a 2-month intervention the effects of a combination of seven epigenetic active, secondary plant extracts for the change of miRNA and mRNA patterns involved in virus response systems. We also raised a nutrition and lifestyle questionnaire, including self-reported stress levels and infection frequency since this is known that nutrition and stress levels play an important role in the regulation of the immune system. MiRNAs reflect the complex regulation of gene transcription in viral replication and host immune defense. A healthy diet and a mix of nutraceuticals can support the fine-tuning of miRNAs.

METHODS

Study Design: For this study, 60 participants, men (23%) and women (77 %) (age 45.8 ± 13.07 years; BMI 24.04 kg/m^2), were recruited for the intervention group and 20 participants, men (35 %) and women (65 %), for the control group (age 38.25 ± 11.13 years, BMI 24.97 kg/m^2). The intervention lasted 2 months with a daily intake of one capsule of a nutraceutical mixture (Virmune®/ Vi-OFFENCIO®) of Vitamin D3 (20 µg/400 % NRV), Vitamin B9 (600 µg/ 4000 % NRV), Zinc (14 mg/ 400 % NRV), sage extract (140 mg), green tea extract (125 mg), berberine (4 mg), apple extract (40 mg), onion extract (140 mg), elderberry extract (110 mg), and grape skin extract (140 mg). The supplement is approved for the European market, the dosage of the ingredients corresponds to the requirements.

Before (T0) and after (T1) the intervention, capillary blood samples (DBS) were collected, and a questionnaire regarding food frequency in portions and lifestyle in times per day/ week was queried. Individuals with a history of chronic illness, being infected with Covid-19 within the past two months, or taking medication were excluded from participation. All study participants have given their written consent to the use of their data.

Sample collection, DNA and RNA extraction: Capillary blood was sampled from the fingertip using the *safety Lancet Extra 18G* (Sarstedt, Germany), collected on *Whatman® protein saver cards* (Sigma-Aldrich, Austria) and dried overnight at room temperature. Samples were stored at -20 °C until analysis was conducted.

Total RNA Extraction: The RNA was extracted using the the MagMAX™ FFPE DNA/RNA Ultra Kit (Applied Biosystems TM by Thermo Fisher Scientific) and KingFisher™ Duo Prime Magnetic Particle Processor (96-well deep well setting). Samples were stored at -20 °C.

MiRNA and mRNA expression: Changes in miRNA expression, involved in antiviral capacity and the immune system (miR-21, miR-155, miR-30e, miR101, miR146a, miR-378a, miR-150, miR-181, miR-127, miR-328, miR-877, miR-132, miR-122, miR-139), and mRNA expression for genes associated with inflammation (INFγ, NFkB, TNFα) were determined using commercially available primers (Thermofisher, USA). As endogenous controls miR-24 and miR-93 were used (using ΔΔCt method). CDNA of mRNA was done using *LunaScript RT SuperMix Kit* (Biolabs, Germany). The *TaqMan™ Advanced microRNA cDNA Synthesis Kit* and *TaqMan™ Advanced microRNA assays* under the default settings on *QuantStudio™ 3* (Thermofisher, Netherlands) were used. Samples were run in 10 µL reactions in doubles. The cDNA samples were stored at -20 °C. All target mRNAs were normalized to β-Actin as an endogenous control. Fold changes for mRNA and miRNA were calculated using the ΔΔ cycle threshold (ΔΔCT) method, with fold changes expressed relative to the mean values for the control group, the mean ΔCt of the samples was normalized to the mean of the housekeeping genes.

Statistical analysis: All data are presented as mean ± standard deviation (SD). Data were analyzed using IBM SPSS Statistics for Windows Version 26 for MacOs (IBM Corp., Armonk, NY, USA) and graph pad prism (Version 6). Kolmogorov-Smirnov-Test was used to revise the data for normal distribution. Paired t-Test was used to compare the 2 different time points. Nonparametric tests were performed when normal distribution was not obtained. Pearson or Spearman test were used to calculate correlations. For group comparison either Man-Whitney U test or ANOVA were used. The significance level was $p < 0.5$.

RESULTS

Characterization of study groups: 44 participants of the intervention group and 20 of the control group successfully completed the study. 32% of the participants were male and 68% of participants were female. 11 participants reported being a smoker.

Effect of the Intervention - expression of miRNAs: In the phytoceutical intervention group significant up regulation for miR-30e (p=0.014, Figure 1A) and down regulation of miR-877 expression (p=0.017, Figure 1B) were seen. MiR-150 expression was down regulated but not in a significant extend (p=0.096).

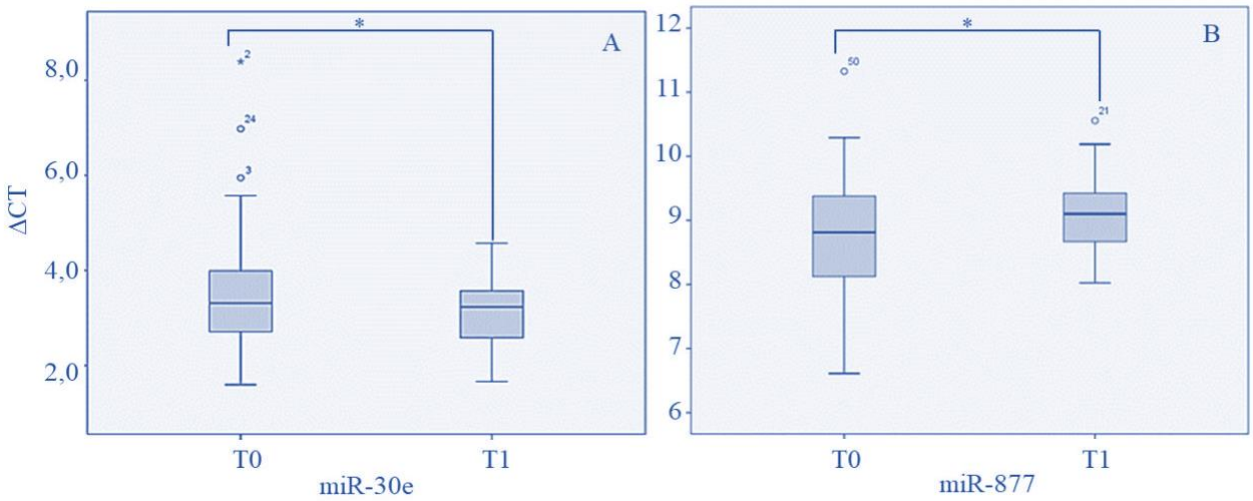


Figure 1: Significant differences after the 2-month intervention for miR-30e5 (A) and miR-877 (B). Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile. The asterisk indicates a significant difference (p= 0.05).

Expression of mRNAs: The phytoceutical intervention could significantly increase the mRNA gene expression of NF-κB relevant for mechanisms involved in inflammatory regulation (p=0.022).

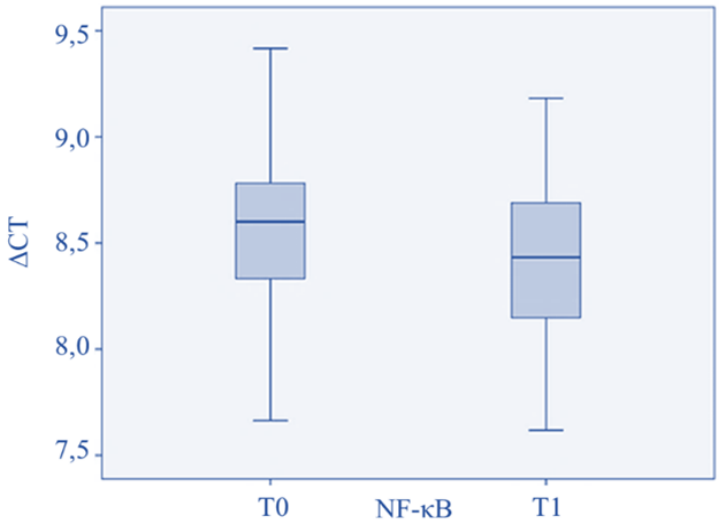


Figure 2: Significant differences after the 2-month intervention for NF-κB. Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile. The asterisk indicates a significant difference (p= 0.05).

Differences of miRNA in participants with previous supplementation of vitamin D and C and zinc: 45.45% of the total study participants took vitamin D, C, and zinc supplements before the start of the study. One participant reported vitamin C intake, one reported multivitamin supplement intake, seven participants reported vitamin D intake, and 22 participants reported the intake of two different supplements. Expression of miR-30e (p=0.01, Figure 2A and B) and miR-101 (p=0.014, Figure 2C and D) was significantly down regulated in the study population group with a prior supplementation.

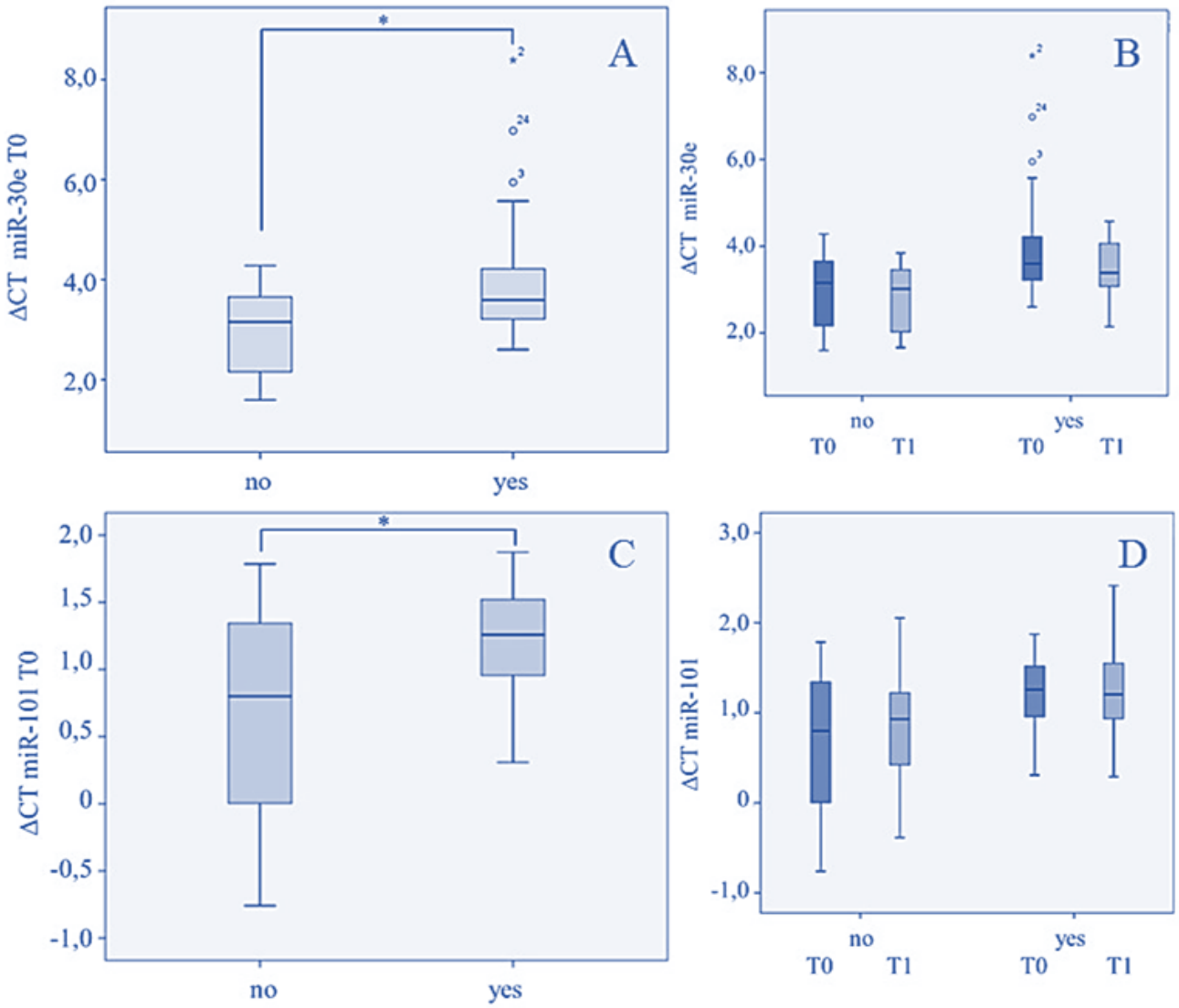


Figure 2: Significant differences of miR-101 and miR-30e5 in a study population with prior supplementation with vitamin D and C, and Zinc at T0 (left side A and C) and T0 compared to T1 (right side B and D). Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile. The asterisk indicates a significant difference (p= 0.05).

Correlation of miRNAs with lifestyle factors and nutrition: Smoking and increased unhealthy eating habits correlated, among other things, with degreased miR-146a expression**.** 11 of the study participants reported being a smoker. MiR-146a was significantly down regulated (p=0.014, Figure 3) and miR-30e (p=0.057), but not statistically significantly, in the smoking study population group compared to T0 and T1. After the intervention, the values of the smoking group adjusted to those of the non-smokers. At time point T0 miR-30e (R=-8.56, p=0.007), miR-146a (R=-0.39, p=0.028), miR-328 (R=0.39, p=0.026), and miR-378a (R=0.43, p=0.013) correlated negatively to an increased consumption of unhealthy snacks and miR-122 correlated positive (R=0.71, p=0.01). Also, the consumption of sweets correlated significantly with the expression of miR-30e (R=0.33, p=0.045). After the intervention the correlations haven't been significant anymore. A high intake of processed meat correlated negatively with the expression of miR-378a (R=-0.38; p=0.035) at T0. MiR-378a (R=-0.38, p=0.035) and miR-30e (R=0.39, p=0.035) correlated negatively to the consumption of processed meat.

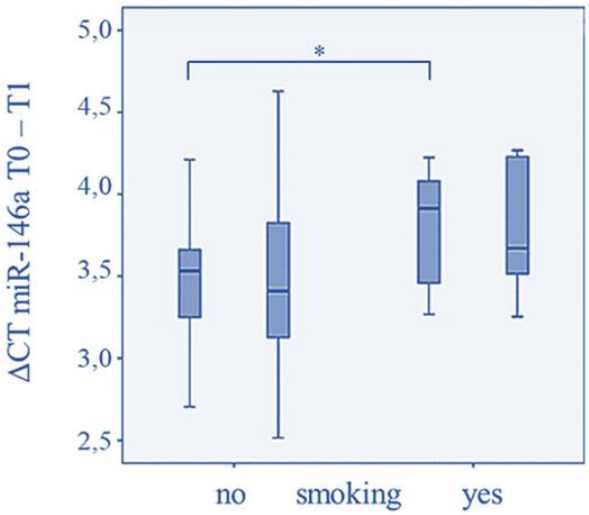


Figure 3: The miR-146a expression adapts to the non-smoker values as a result of the intervention. Rectangular marks represent mean levels, medians are indicated with a line, whisker ranges between 5th and 95th percentiles and box limits between the 25th and 75th percentile. The asterisk indicates a significant difference (p= 0.05).

The intervention enhanced correlations between miRNA and a healthy lifestyle, such as increased vegetable consumption or physical activity: Participants stated their weekly consumption of "other vegetables" like tomatoes, carrots, or eggplant. Leeks and leafy green vegetables were queried separately. Consumption of "other vegetables" correlated at T0 positively with miR-150 (R=0.33, p=0.035), miR-181a (R=0.322, p=0.04; Figure 4), and miR-328 (R=0.354, p=0.023). These correlations were more significant after the intervention at T1: miR-155 (R=0.863, p=0.001), miR-150 (R=0.97, p=0.001), miR-181a (R=0.733, p=0.016), miR-127 (R=0.68, p=0.029), miR-328 (R=0.76, p=0.011), and miR-877 (R=0.65, p=0.042). Leafy green vegetables were positively correlated with miR-101 at T1 (R=0.75, p=0.018). Leeks had correlations with the expression of the same miRNAs as "other vegetables", but only significantly at T1: miR-155 (R=0.731, p=0.016), miR-150 (R=0.765, p=0.01), miR-181a (R=0.782, p=0.008), miR-877(R=0.641, p=0.046). MiR-30e5 correlated negatively with the total amount of exercise per week (R=-0.31, p=0.045). Stress levels correlated negatively with miR-877 (R=-0.663, p=0.037) and TNF alpha expression (R=-0.384, p=0.012). The weekly consumption of fresh fish corelated positively with the expression of miR-30e5 (R=0.709, p=0.001).

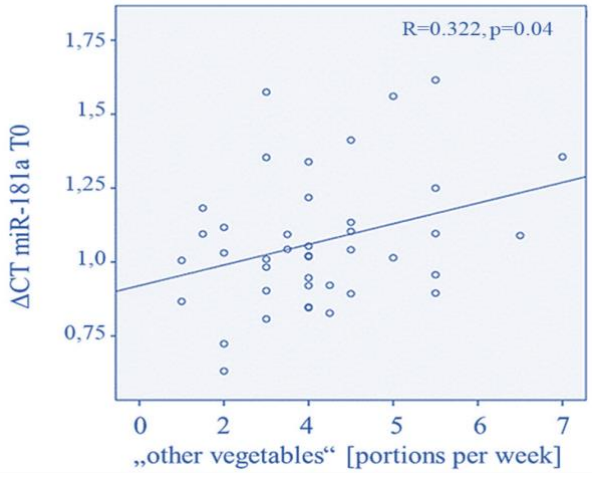


Figure 4: MiR-181a correlates to the stated vegetables consumption.

Stated infection risk: The expression levels of miR-146a (R=-0.305, p=0.047) miR-150 (R=0.334, p=0.029), miR-877(R=-0.366, p=0.016) and INFγ (R=-0.393, p=0009) correlated with the stated infection incidence. Study participants with a stated increased infection risk also stated that they took supplements more frequently.

ACE SNP frequency: 22.7% of the study population had the single nucleotide variance (SNV) homozygote (G/G), 15.9% were homozygote (C/C), and 61.4% had the heterozygote variance C/G.

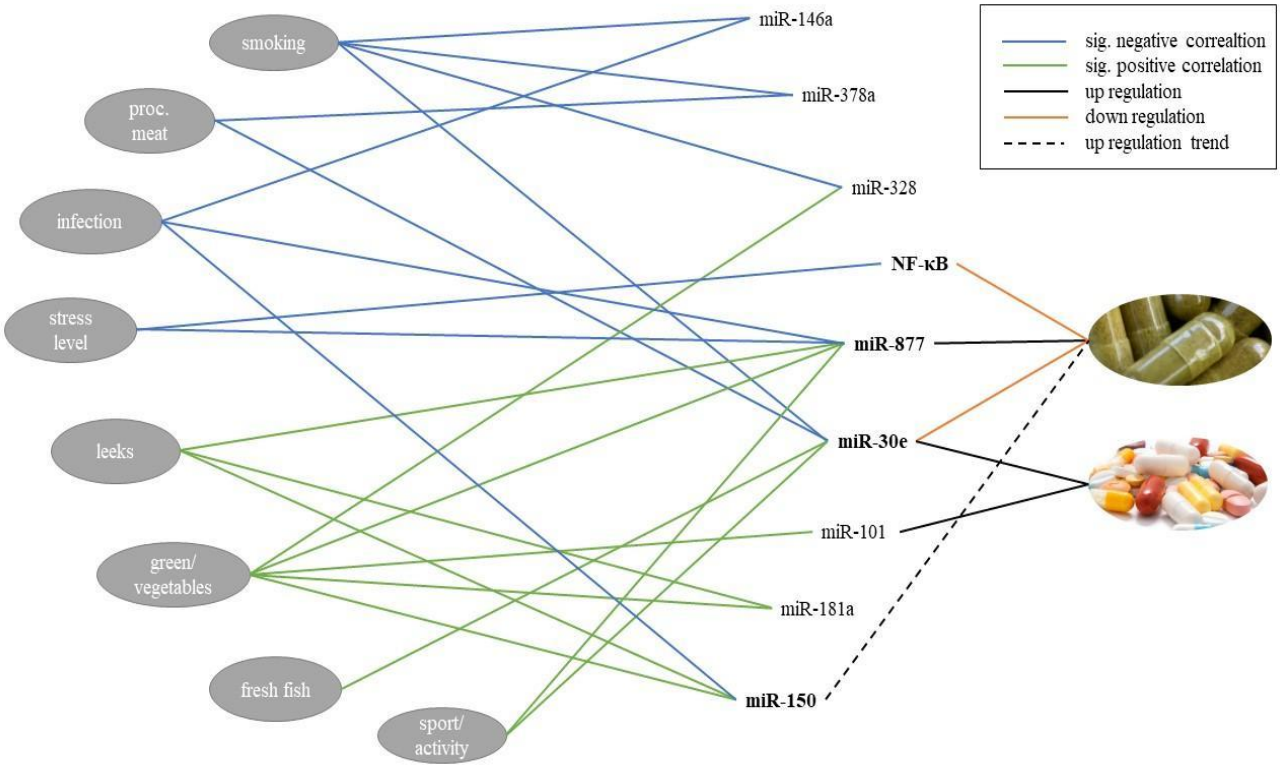


Figure 5: Overview of the miRNA expression levels, lifestyle factors and correlations.

DISCUSSION

Numerous reports have illustrated the large diversity in responses and clinical outcomes to SARS-CoV-2 infection. One possible variable that has not been sufficiently considered is the individual differences in patients' miRNA profiles [2]. We observed significant negative correlations for total infection incidence of the last 3 months and with miR-877, miR-150, and miR-146a. Roganović et al. 2021 showed that inflammation-modulating miR-146a is among the first miRNAs induced by immune response to a virus, and that miR-146a deficiency may contribute to severe COVID-19 states [59]. Smoking and snacking had a significant negative effect on miR-146a, miR-328, miR-378a, and miR-30e5 at T0. In the case of miR-146a, the initial poor miR-146a status among smokers improved with the intake of Vi-OFFENCIO®. Therefore, we assume that the supplementation could potentially influence the course of COVID-19 disease. The consumption of processed meat correlated negatively to miR-378a and miR-30e5p. An unhealthy lifestyle could additionally contribute to the risk of a severe virus course.

With the progress of nutriepigenomics, the relationship between diet and epigenetic regulatory factors such as miRNAs has become clearer. Beside nutrition and lifestyle factors, many natural compounds, like polyphenols, have been shown to influence miRNA expression. The use of natural compounds to regulate miRNA is a promising therapeutic agent. Both variants, phytoceutical-induced regulation of host miRNAs and food-derived

miRNAs, are promising possibilities in miRNA control and disease treatment [60]. An overview of miRNA expression levels in this study and their analyzed correlations are shown in figure 5.

Nersisyan et al. 2020 found that miR-21-3p has the highest probability of binding the human coronavirus RNAs and is dramatically up regulated during SARS-CoV infection up to 8-fold [12]. However, tour study participants were not infected and the intervention with the phytoceuticals showed no significant effect on miR-21 after 2 months. In addition, it is described that miR-21 inhibits TNFα-induced CD40 expression via SIRT1-NFkB signaling pathway. But unfortunately, we couldn't confirm this since we could not observe any correlation between TNFα - and miR-21 expression [61]. This indicates that miR-21 and TNFα may not be prognostic markers, but only play a role after infection with SARS-CoV infection. After the intervention, study participants showed significant changes in the expression of miR-30e (p=0.014) and miR-877 (p=0.017). MiR-150 was down regulated, but not in a significant way (p=0.096). These miRNAs are important in the regulation of the innate immune system and in pathogen recognition via toll-like receptor 4 (TLR4) [62], [25]. A down regulation of miR-150 was shown by Jiang et al. 2020 to suppress inflammatory cytokines [63]. An activated innate immune response can limit viral entry, but excessive immune activation can lead to systemic inflammation and severe disease [64]. MiRNAs are described by Gantier et al. 2007 to fine-tune the homeostasis of the innate immune response [65].

Multiple polyphenolic compounds demonstrate their synergistic antioxidant effects. Polyphenolic compounds can interact with other substances to produce antioxidant effects that are often more potent than either substance alone [66]. The main substances found to produce synergistic effects with polyphenolic compounds are active antioxidants such as polysaccharides, vitamins C/E and carotenoids, and extracts from natural raw materials such as kelp extract and sweet potato extract [66]. 45.45% of the study participants supplemented before the intervention with zinc, Vitamin C, and D. Study participants with a stated increased infection risk also stated that they took supplements more frequently. Study participants with a prior supplementation of vitamin C, D, or zinc had a lower expression level of miR-30e and miR-101 compared to the group with no prior supplementation. In both groups, the results adjusted. MiR-30e* is a positive regulator of the antiviral innate immune responses [67]. Up regulation of miR-30e has been shown to inhibit replication of influenza B viruses [68]. Upregulation of miR-101 during an influenza A virus infection abrogates the viral life cycle by targeting the mTOR pathway [47]. In the group without prior supplementation, miR-101 was down regulated after the intervention, assuming that both supplementations, phytoceuticals, and/ or vitamins and zinc decrease expression of miR-101. Also, the stated consumption of greens and "other vegetables" was positively corelated with the expression of miR-101. MiR-30e5 correlated positively with the stated consumption of fresh fish and exercise, and negatively with snacking frequency, consumption of processed meat, and smoking. Mice that were exposed to cigarette smoke for 4 weeks demonstrated an up-regulation of miR-101 in their lungs [69]. In our study group, smoking was significantly negatively correlated with the expression of miR-328, miR-146a, and miR-378a. Suzuki et al. 2016 showed that serum levels of miR-146 where elevated in smokers compared to non- or ex-smokers [70]. MiR-146a is a key regulator in the interplay between DNA damage response (DDR), cell senescence, and inflammation, and is described when up regulated to be proinflammatory [71]. MiR-146a, miR-378, and miR-328 correlated also negatively with snacking. Both miRNAs are therefore particularly sensitive to negative lifestyles. After the intervention these correlations weren't detectable anymore. These

results underscore that the intervention with phytoceuticals may reverse the effects of the miRNA expression patterns of an unhealthy lifestyle and counteract the proinflammatory effects. When we analyzed miRNAs that were particularly correlated with a negative lifestyle, we also observed miRNAs (miR-181a, miR-101, miR-150, and miR-877), that correlated with positive lifestyle aspects. For these miRNAs, the correlation even increased after the intervention with phytoceuticals. MiR-181a negatively correlated with the proinflammatory cytokines IL-6 and TNFα, and positively with the anti-inflammatory cytokines TGFβ and IL-10 [72]. We could observe a positive correlation of miR-181a, miR-150, and miR-877 to the consumption of stated weekly number of leeks, greens, and "other vegetables" at both time points. The results emphasize the importance of a healthy lifestyle regulating the expression of miRNAs to activate immune-relevant miRNAs important for viral response systems. The phytoceutical intervention amplifies this effect, reversing the effects of the miRNA expression patterns of an unhealthy lifestyle.

CONCLUSION

Viruses have a long history in human evolution. The adaptation of the immune system to limit viruses in their replication and to contain the infection is regulated through many pathways. MiRNAs play an important role in this regulation. The same micro RNAs can also be regulated through a healthy diet, functional foods, and plant compounds. Using this mechanism, individual nutritional components can be used specifically to prevent viral infections and strengthen the immune system. Especially in times of increased risk, the use of combined functional foods should be recommended for everyone as a preventive approach but also to cotreat an existing infection.

List of Abbreviations: ACE2: Angiotensin-converting enzyme, CHIKV: chikungunya virus, COVID-19: Coronavirus disease, DBS: dried blood spot, DDR: DNA damage response, EGCG: Epigallocatechin-3-Gallate, ERK: extracellular signal-related kinase, FF: functional foods, FFP: functional food products, H1N1: Influenza, HAT: Histone acetyltransferases, HDAC: histone deacetylase, HSV: Herpes simplex viruses, ICAM-1: Intercellular Adhesion Molecule, IFNs: Interferon gamma, IFN-γ: Interferon gamma, IL-10: Interleukin, IL-12: Interleukin, IL-1ra: Interleukin 1 receptor antagonist, IL-6: Interleukin, iNOS: inducible Nitric oxide synthase, JNK: c-Jun NH2-terminal kinase, LPS: Lipopolysaccharides, MAP: Mitogen-activated protein, MAPKs: mitogen-activated protein kinase, MCP1: Monocyte Chemoattractant Protein-1, miRNA: microRNA, mRNA: messenger ribonucleic acid, mTOR: mammalian Target of Rapamycin, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, NK cell: Natural Killer Cells, PGE2: prostaglandin E2, PRR: Pattern recognition receptors, PUFAs: Polyunsaturated fatty acid, RISC: RNA-induced silencing complex, RNA: Ribonucleic acid, RPC: renal cells, SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2, SIRT1: Sirtuin-1, TGFβ: Transforming Growth Factor beta, TLR4: toll-like receptor, TNFa: tumor necrosis factor-α

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