universität wien

MASTERARBEIT/ MASTER'S THESIS

Titel der Masterarbeit/ Title of the Master's Thesis

„Correlations of 6 metabolic and aging related miRNA expressions with various lifestyle factors“

verfasst von/ submitted by

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angestrebter akademischer Grad/ in partial fulfillment of the requirements for the degree of
Master of Science (MSc)

Wien, 2017

Studienkennzahl lt. Studienblatt /
Degree programme code as it
appears on the student record sheet: A 066 838

Studienrichtung lt. Studienblatt /
Degree programme as it appears
on the student record sheet: Master Ernährungswissenschaften

Betreut von / Supervisor : Univ.- Doz. Dr. Alexander Haslberger

Ich erkläre hiermit, dass ich die vorliegende Masterarbeit selbstständig verfasst und keine anderen außer den angegebenen Quellen und Hilfsmittel benutzt habe.

Evgenia Guenova

I. Danksagung

Zu allererst möchte ich mich bei diejenigen bedanken, die mich während meines Studiums begleitet und in dieser Zeit unterstützt haben.

Mein Dank gilt zunächst an Univ.-Doz. Dr. Alexander Haslberger, der mir die Möglichkeit gab an einem interessanten neuen Projekt in seinem Labor der Epigenetik und Mikrobiota teilzuhaben.

Des Weiteren gilt mein Dank auch allen Mitgliedern der Arbeitsgruppe, besonders Ulrich Magnet, Elena Tomeva und Elisabeth Dum, die mir mit ihrer Erfahrungen bei Problemen im Labor aber auch Sonstiges weiterhelfen konnten und mir Mut zusprachen. Natürlich an allen bedanke ich mich dafür, dass im Labor für perfekte familiäre Stimmung sorgten. Ohne meinen Teamkollegen/innen wäre mein Arbeit nicht so schnell und sicher zu Ende gebracht gewesen.

Und vom Herzen möchte ich mich bei meinen Eltern bedanken, die mir große finanzielle und emotionale Unterstützung gaben und somit durfte ich mich ganz aufs Masterstudium konzentrieren. Ebenfalls möchte ich mich an meinem Freund Stoycho bedanken, der mich im Leben durch Hochs und Tiefs beistand.

Ebenso bedanke ich mich meiner Studienkolleginnen Birgit und Ursula, die auch meine Tutorinnen über den ganzen Mastestudium waren und glücklicherweise enstand aus uns ein geniales Motivationsteam. Umso wichtiger war es auch, dass GESTU mir immer begleitet haben und mir Möglichkeiten gaben, barrierfrei zu studieren.

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V. List of Abbreviations

AS	Atherosclerosis
IIS	insulin/IGF-1 signaling
AMP	adenosine monophosphate-activated protein
Nt	Nucleotide
MAPK	mitogen-activated protein kinase
TGF-b	transforming growth factor beta
mTOR	mechanistic Target of Rapamycin
EGFR (ErbB)	epidermal growth factor receptor
HRM	High Red Meat
NAD+	Nicotinamide adenine dinucleotide
SIRT1	sirtuin 1
NAMPT	Nicotinamide phosphoribosyltransferase
PGC1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
SREBP1c	sterol regulatory element-binding proteins 1c
LXR	liver X receptor
NF-kB	nuclear factor kappa B
HCC	Hepatocellular Carcinoma
NAFLD	Non Alcoholic Fatty Liver Disease
SOD2	Superoxid Dismutase 2
TRDX2	thioredoxin reductase 2
NASH	Non-alcoholic steatohepatitis
HCV	Hepatitis C Virus
CARHSP1	calcium-regulated heat stable protein 1
TNF-α	tumor necrosis factor alpha

MAP3K10	mitogen-activated protein kinase kinase kinase 10
BCL6 B-Cell	CLL/Lymphoma 6
CCL2	CC-chemokine ligand 2
ApoE	Apolipoprotein E
BAT	brown adipose tissue
WAT	white adipose tissue
CDKN2A	cyclin-dependent kinase Inhibitor 2A
MICA	MHC Class I Polypeptide-Related Sequence A
MICB	MHC Class I Polypeptide-Related Sequence B
NKG2D	transmembrane protein belonging to the CD94/NKG2 family of C-type lectin-like receptors
T2D	Type 2 Diabetes
IL1β	interleukin-1 beta
WD	high-fat high-cholesterol diet
FOH	WD supplemented with fish oil
MCP1	monocyte chemoattractant protein 1
PPAR	peroxisome proliferator-activated receptors
AKT	protein kinase B
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

VI. Summary

In recent years the population elderly people is growing but as well the epidemic of obesity. Both age and BMI have been important variables for the researchers to figure out their molecular pathways and its link to age-related and metabolic-related diseases. Therefore, changing lifestyle factors and gaining knowledge about the environment are good applications in preventing such diseases as those factors can alter epigenetic mechanisms including miRNAs. Nutrition, stress, sport, supplementations and drinking habits are such lifestyle factors and can affect positively as well as negatively the physiological status of humans. For instance, inflammations are one of the indicators which are able to influence miRNA pathways and modulate its expression levels. Hence miRNAs provide a lot of informations about current molecular processes which play a role at the posttranscriptional level and identifying its nutritional and evironmental modulators are aims of this master's thesis.

The expression levels of 6 chosen miRNAs with relevance to aging and metabolic syndrome such as obesity, hypertension, hypercholesterolemia and diabetes were analyzed with a quantitative real-time PCR method in blood spots of 120 subjects. Additionally participants were asked to fill out a questionnaire regarding their lifestyle habits without any interventions.

Statistical evaluations indicated higher expression of miR-21 in subjects with inflammations and allergic intolerance. Although intolerances refer to asthma and eosinophile oesophagitis but not atropy which expresses Ig E. Suprisingly, the analysis also showed a significant reduced expression of miR-21 in subjects taking supplementations of folat. We could not observe any significances in BMI and aging. Only miR-34a and miR-155 revealed a significant positive associations in BMI although miR-155 showed significant results between BMI < 21 and BMI 21,5-25 and as well as between BMI <21 and BMI>25 but not between BMI 21,5-25 and BMI > 25. Nevertheless, we observed similar molecular pathways of BMI-spesific miRNAs involved in activation of NF-κB signaling trough cytokines such as insulin-like growth factor-1, tumor necrosis factor and interleukin-6 and these have the ability to directly regulate PPARs and SIRT1. Regarding to aging higher expressions were found in miR-106b, miR-34a and miR-155 and lower expression in miR-151a. Furthermore daily

physical activity and multivitmins downregulated the expression of miR-34a and was positively associated with coffee consumption. Modulations in miR-106b by lifestyle factors were found in magnesium intake and meat consumptions. Analysis examined that miR-151a had positive association with physical activity and an u-shaped correlation with work stress. Lastly fish consumption of 4 times a week and dietary supplementations decreased the expression of miR-155. Still more accurate and reliable methods are needed, for example incorporating bioinformatics to improve the understanding of miRNAs networks and the influences by internal and external factors in a bigger study population.

VII. Zusammenfassung

In den letzten Jahren können Bevölkerungswachstum und hier insbesondere ein Wachstum der Bevölkerung hohen Alters aber auch steigende Zahlen an Übergewichtigen beobachtet werden. Alter und BMI werden von den naturwissenschaftlichen Forschern oft als Variablen herangezogen, um die molekularen Mechanismen metabolischer und altersbedingter Erkrankungen besser verstehen zu können. Die Umwelt und der Lebensstil können über epigenetische Regulationen, einschließlich der miRNA, bestimmte Erkrankungen präventiv beeinflussen, wodurch Veränderungen des Lebensstils und Umwelteinflüsse wichtige Faktoren für die Gesundheit darstellen. So können Ernährung, Trinkgewohnheiten, Stress, Sport und Supplementierungen, als Beispiele solcher Lebensstilfaktoren, positive sowie negative Auswirkungen auf den physiologischen Zustand des Menschen haben. Teilweise sind Entzündungen in der Lage die miRNAs hoch- bzw. runterzuregulieren, somit sind entzündliche Geschehnisse in der Lage Expressionsniveaus zu modulieren. Deswegen bietet die Expression von miRNAs viele Informationen über aktuelle molekulare Prozesse, die auf posttranskriptionaler Ebene wirken. Ziel dieser Masterarbeit ist es, die verschiedenen exprimierten miRNAs durch Ernährungs- und Umweltmodifikationen zu identifizieren. Die Expressionsniveaus sechs ausgewählter miRNAs, welche eine besondere Rolle im Alter und bei Erkrankungen des metabolischen Syndroms wie Fettleibigkeit, Hypertonie, Hypercholesterinämie und Diabetes spielen, wurden mittels quantitativer real time-PCR aus Blutproben von 120 Probanden analysiert. Zudem wurden die Teilnehmer aufgefordert einen Fragebogen über ihre alltäglichen Lebensstil auszufüllen.

Die statistischen Auswertungen zeigten eine höhere Expression an miR-21 bei Patienten mit Entzündungen und allergischer Intoleranz. Letzteres bezieht sich auf Asthma und eosinophile Ösophagitis, nicht aber Atropie, welche die Fähigkeiten besitzen IgE zu exprimieren. Ebenso zeigte die Analyse eine signifikant reduzierte Expression von miR-21 bei Patienten die Folsäure zu sich nahmen. Es konnte kein Zusammenhang zwischen miR-21, dem BMI und dem Alter beobachtet werden.

Nur miR-34a und miR-155 zeigten eine signifikant positive Assoziation mit dem BMI, wobei miR-155 indizierte signifikante Ergebnisse zwischen BMI <21 und BMI 21,5-25

sowie zwischen BMI <21 und BMI> 25, aber nicht zwischen BMI 21,1-25 und BMI> 25. Ebenso wurden ähnliche molekulare Mechanismen der miRNAs beobachtet, die bei der Gewichtkontrolle eine Rolle spielen. Die BMI-spezifische miRNAs modulieren Insulin-like Growth Factor-1, Tumor Necrosis Factor und Interleukin-6, was zu einer Aktivierung des Signalwegs von NF-κB führt und gleichtzeitig regulieren diese miRNAs direkt die PPARs, RXR und SIRT1.

In Bezug auf das Alter wurden höhere Expressionen in miR-106b, miR-34a und miR-155 und aber auch niedrigere Expressionen in miR-151a gefunden.

Darüber hinaus führte eine tägliche körperliche Aktivität und die Aufnahme von Multivitaminpräparaten zu einer Unterregulation von miR-34a, welche auch positiv mit dem Kaffeekonsum der Probanden assoziiert werden konnte. Signifikante Zusammenhänge wurden weiters zwischen miR-106b und Lebensstilfaktoren, wie der Magnesiumaufnahme und dem Fleischkonsum ersichtlich. Die Analyse zeigte eine positive Assoziation von miR-151a mit der körperlichen Aktivität sowie eine u-förmige Korrelation mit der Arbeitsbelastung. Außerdem verringerte sich die Expression von miR-155 bei Personen die 4-mal pro Woche Fisch aßen und ebenfalls bei Teilnehmern die Nahrungsergänzungsmittel zu sich nahmen.

Es wäre sinnvoll weitere Untersuchungen mit genaueren und zuverlässigeren Methoden, wie zum Beispiel der Bioinformatik vorzunehmen, um ein besseres Verständnis der komplizierten Netzwerke von miRNAs sowie deren Beeinflussung durch interne und externe Faktoren zu erlangen.

1. Introduction

1.1. Metabolic Syndrome

The metabolic syndrome is comprised of several metabolic abnormalities such as central obesity, dyslipidaemia, hyperglycaemia and hypertension. It is further defined by the presence of other components, including elevated levels of circulating triglycerides, reduced levels of HDL-cholesterol, impaired fasting glycaemia, elevated circulating inflammatory substances such as C-reactive protein, tumor necrosis factor-α interleukin-6 and thrombotic markers such as plasminogen activator inhibitor type 1. This syndrome can also be characerized by reduced levels of anti-inflammatory molecules such as adiponectin. [Maury E. et al., 2010]

Besides of circulating cholesterol and lipid profiles it can result in non-alcoholic fatty liver disease and is associated with increased risk of type 2 diabetes and cardiovascular disease. [Rottiers V and Näär A M, 2012] Atherosclerosis is seen as a severe outcome of metabolic syndrome due to inappropriate ratios of lipid profiles and its accumulation resulting to chronic inflammation and finally to cardiovascular diseases. The main mediator is the cholesterol loading in macrophage which were differentiated from monocytes in respond to inflammatory factors and oxidative stress. In the pathological stage of this process macrophages are recruited to take up oxLDL to form lipid-overloaded foam cells which play a crucial role in the formation of atherosclerotic plaque and the progression of AS. [Li X et. al., 2016]

1.2. Aging

Aging is a highly complex process where over time the accumulation of cellular and molecular damage leads to the functional decline of tissues and organs that may lead to increased susceptibility of disease and mortality. [Noren Hooten N et al., 2013]

This progressive deterioration of cellular processes are involved in almost every cellular and biological functional pathway. [Harries L. W., 2014] It has been discussed that gene expression patterns that modulate senescence might be the result of secondary effects of mechanisms that are important during the cellular growth and the development of

organisms. There is a complete investigation of C. elegans model on factors that modulate its lifespan. Common mechanisms for aging are the signalings through the IIS pathway, heat-shock factors, AMP-activated protein kinases, mitogen-activated protein kinases, sirtuins, target of rapamycin and mitochondria. [Smith-Vikos T and Slack F J, 2012]

1.3. Lifestyle

Some lifestyle factors such as nutrition, behavior, stress, physical activity, working habits, smoking and alcohol consumption may influence all three major epigenetic mechanisms which are described in the next section. The term lifestyle is used to define the "typical way of life or manner of living characteristic of an individual or group". [Alegría-Torres J A et al., 2011]

Epigenetics is expected to help explaining how gene expression is modulated by lifestyle and environmental factors and give insights of individual responses to environmental situations and acquired risk factors. As lifestyle and epigenetic mechanisms are highly modifiable and it is a great challenge to determine how tightly epigenetic markers are dependent on lifestyle factors as well as how much epigenetic mechanisms can be modified after positive or negative lifestyle changes. [Rüegger S and Großhans G, 2012]

The situation of metabolic syndrome with its symptoms is rapidly worsening due to our progressive lifestyle changes, especially due to increased stress, decreased physical activity, increased incidence of obesity, endocrine disruptors and other negative factors. [Kunes J et al., 2015] The extent to which cells are able to respond to these lifestyle changes lies in large part with the plasticity of the cells. Epigenetic modifications and interventions during pregnancy may be an important strategy to prevent metabolic disorders in later life as the developing fetus is a prime target of these modifications. Despite the recognized role for epigenetics in fetal programming for metabolic syndrome still there are some limitations in this field. [Wang J et al., 2012]

1.4. Epigenetics and Metabolic Syndrome

Epigenetics is defined by the regulation of gene expression without alteration of the DNA sequence. External or enviromental factors including stress, toxins, enviromental pollutants, nutrients and medical history can modulate gene expression and its phenotypic traits. In several studies it is even mentioned that epigenetic phenotypes are stably heritable as well. Three major epigenetic mechanisms are involved in these regulations: DNA methylation, histone posttranslational modifications and noncoding RNAs. [Xu W et al., 2016] About 30% of all human genes are estimated to be potential miRNA targets. [Sevignani C et. al., 2006] Other more recent study assumed that 30%–80% of human genes are predicted to be influenced by miRNAs. [Lu J and Clark AG, 2012]

Phenotypic changes cannot be explained solely by the changes in DNA sequence. It was proved in several studies that gene environmental interactions such as nutrient exposure and genetic background can have a dramatic impact on the development of the metabolic syndrome. [KuneŠ J et al., 2015] Disruption of epigenetic mechanisms can result in oxidative stress, obesity, insulin resistance, diabetes and vascular dysfunction in humans. [Wang J et al., 2012]

Nutrigenomic studies aim to understand how lifestyle factors can influence metabolic pathways and homoeostatic control. These regulations are changed in the early phases of diet-related disease as severel studies shared these observations. As a consequence epigenetic biomarkers must support evidence-based dietary intervention strategies for restoring health and fitness as well as preventing diet-related diseases. If the same circulating miRNAs which are found to be responsive to dietary modulations but also are altered in different pathological contexts then they are concluded as non-specific to a given disease. This appearance can be explained by the fact that not all miRNAs are stable in the blood and thus measurable and that only a handful of them is exported from cells. [Ross S A and Davis C D, 2014] Circulating miRNAs are thought to be released into circulation in response to stress, injury or tissue damage. Still more investigations need to be done because the exact release mechanisms and the uptake of microRNAs into multivesicular bodies are not fully understood. [Meurer S et al., 2016]

One study identified only 114 miRNAs consistently expressed in 1500 serum and plasma samples [Blondal et al., 2013], although more than 2000 miRNAs are reported in the database for all microRNAs.[Hammond S M, 2015] For this reason an altered miRNA level in the blood may be more related to a general alteration of the metabolism and to systemic inflammation that affect common organs in various pathological states like cardiovascular disease, diabetes, ageing, obesity and others than to a specific disease. Therefore the identification of lifestyle interventions that are able to reverse the level of altered circulating miRNAs may indicate a positive impact on the metabolism. These identified miRNAs which plays a role in dietry and physical lifestyle might be able to treat or reduce the progression of a disease.

Still there is a number of technical issues associated with miRNA profiling in biofluids. Their use as indicators in nutritional studies needs to be urgently validated as a number of relevant studies demonstrate that miRNAs might be promising biomarkers to monitor the impact of lifestyle or dietary interventions. [Ross SA and Davis CD, 2014] Most of the BMI-associated miRNAs are likely originated from the liver. Blood cell parameters are useful medium to distinguish sex-associated miRNAs. [Ameling S et al., 2015]

1.5. Epigenetics and Aging

Identification of stable miRNA biomarkers in biofluids have great potential as a noninvasive diagnostic tool and also give better understanding of physiological changes that occur with age. Altered expression of circulating miRNAs have been associated with age- related diseases. [Noren Hooten N et al., 2013] Because of the close relationship between diseases and longevity miRNAs may serve as biomarkers of human aging. [Smith-Vikos T et al, 2016]

In most individuals biomarkers of aging can change in a predictable direction with aging and when it is assessed early in life it may predict subsequent longevity better than chronological age alone. Not only it can be used as progonostic tool but also may give informations about the intrinsic mechanism of aging as a biological process figuring out what may accelerate or decelerate aging. It could be useful for identifying persons at risk of developing clinically adverse health outcomes.

[Smith-Vikos T et al, 2016] During aging it has been categorized following common features: changes in genomic instability by accumulating DNA damage and simultanously decreased DNA repair response, the progressive shortening of telomeres as chromosomes age, changes in fine control of gene expression through epigenetic modifications, changes to proteostatic processes such as ubiquitination, protein folding and trafficking, deregulation of nutrient sensing pathways such as mTOR and IGF1 signaling, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and changes in intercellular communication such as increased inflammatory responses and disruptions in cytokine expressions. It is known that miRNAs take part in this interlinked complex. [Harries L W, 2014] During cellular metabolism, the mitochondrial genome is at particular risk from the reactive products of respiration. Normally the mitochondrion is protected from the adverse effects of free radicals and other reactive species by some antioxidant enzymes such as superoxide dismutase 2 (SOD2) and thioredoxin reductase 2 (TRDX2). Some miRNAs can interact with these enzymes and change its expressions. [Li X et al., 2013]

1.6. MiRNAs

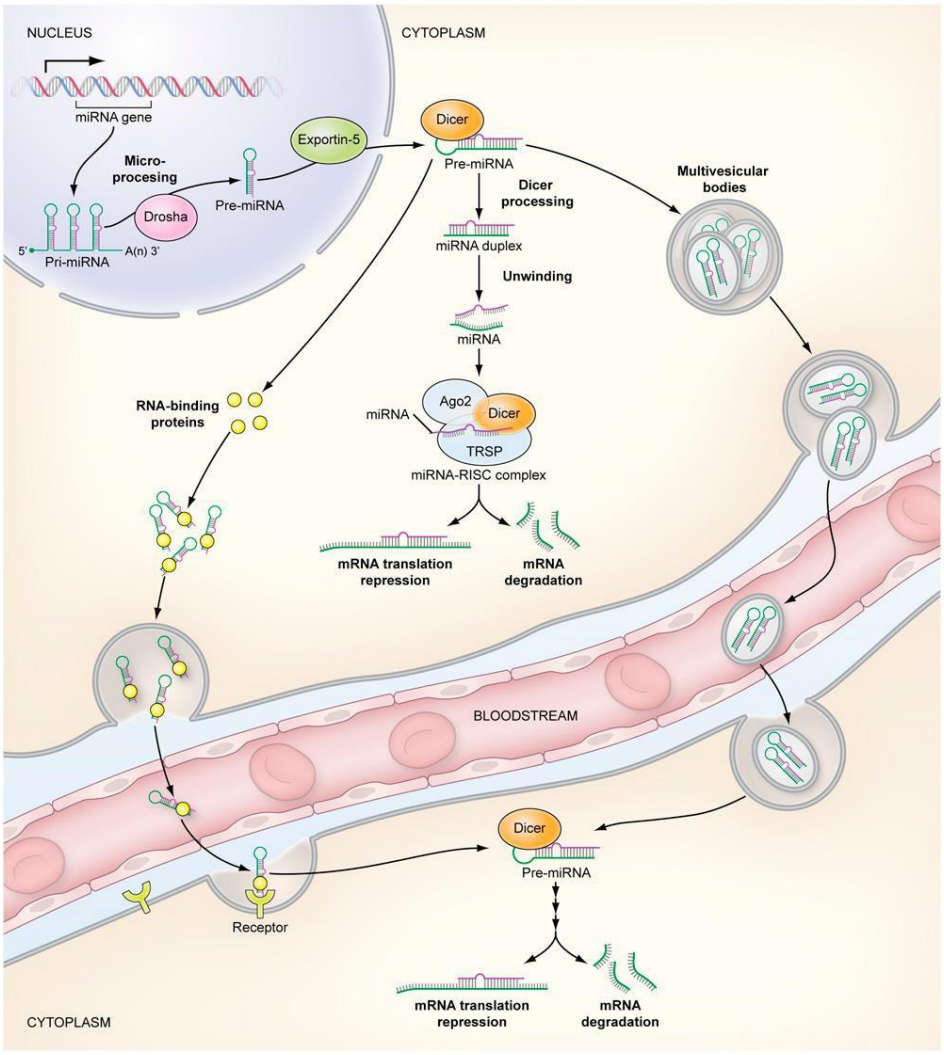

Figure 1. Biosynthesis of miRNAs [Ross S A and Davis C D, 2014]

MiRNAs are widespread in cells and tissue and regulate gene expression at the post-transcriptional level. The mechanisms which are mediated by small non-coding RNAs act through their respective pathways to induce DNA methylation or histone modifications to silence or enhance gene expression. The biogenesis of mature miRNA from its miRNA gene involves both the nucleus and cytoplasm in mammalian cells. [Wang J et al., 2012] The genomic location of miRNAs is diverse, around 40% in introns, 30 % in exons, 30% in uncertain transcriptional regions and 20% in genomics repeats. MiRNA genes are often found at fragile sites that can be subjected to a variety of mechanisms including deletions, amplifications or mutations. These alterations

modify the expression profile of miRNAs related to specific miRNA loci which enable to change the expression of their mRNA target genes following a different mechanism. Even if the expression of miRNA is normal but mutations or a single-nucleotide polymorphism is present in its sequence, the mRNA regulatory role can be altered. [Gavrilas L I et al., 2016] At the canonical biogenesis pathway miRNAs are transcribed from intergenic, intronic or polycistronic genomic loci by RNA polymerase II in the nucleus. The produced primary miRNA transcript forms a stem-loop structure which is long capped and polyadenylated with 80 nucleotids in length. [Rottiers V and Näär A M, 2012] [Wang J et al., 2012] Afterwards they are recognized and processed by Drosha (type-III RNase) and its associated protein DGCR8 (Pasha) to generate precursor miRNA with a length of 70 nucleotids. [Wang J et al., 2012] MiRNAs from non-canonical miRNA pathway are transcribed directly as short endogenous hairpin RNAs (shRNAs) or derive directly through splicing from introns that can refold into hairpins. The trimmed precursor (pre-miRNA) hairpins from both canonical and non-canonical miRNA pathways are then transported by an Exportin-5 and Ran-GTP-dependent process to the cytosol. In the cytoplasm they are cleaved by the enzyme complex of Dicer (type-III ribonuclease) and TRBP (transacting RNA-binding protein) to form the mature double-stranded miRNA with approximately 22 nucleotides. [Rottiers V and Näär A M, 2012] This duplex is unwound to form a passenger strand miRNA for degradation and the other strand is a mature single-stranded miRNA which carries its functions for the next step. This guided miRNA strand is incoporated into an RNA-induced silencing complex (RISC) containing several components, including Dicer, TRBP, Argonaute proteins, GW182 protein and fragile X mental retardation protein (FMRP1). [Wang J et al., 2012] Finally the RISC-miRNA assembly is then guided to specific target sequences in messenger RNAs. The initial recognition of mRNAs by the RISC-miRNA complex is driven primarily by Watson-Crick base-pairing of 2-8 nucleotides in the mature miRNA with the 3'-untranslated region of the specific mRNA target sequences. Further additional base-pairing results in greater affinity and targeting efficiency. [Rottiers V and Näär A M, 2012] Depending on the type of the complementery binding, partially or fully, of these evolutionarily conserved molecules results in degradation or inhibition of translation, respectively. [Gavrilas L I et al., 2016] This biological process shows that miRNAs have versatile functions in

physiological processes and play crucial roles in growth, development and health.[Wang J et al., 2012] MiRNAs are intigrated in many biological functions such as stem cell self-renewal, cell proliferation, apoptosis and metabolism. [Smith-Vikos T et al., 2016]

Limitation

It should be taken into account that demographic characteristics as confounder can have an influnce in amounts of miRNAs. It has also become obvious from studies analysing circulating miRNAs in the context of metabolic diseases that relevant miRNAs might be associated with the demographic characteristics of the subjects. It is demonstrated that Caucasian and Asian populations have unique metabolic profiles especially Asian populations are at elevated risk of diabetes and have specific nutritional habits. [Prabu et al., 2015] [Zhu H and Leung SW, 2015]

1.7. Selected miRNAs in Nutrition, Metabolic Syndrom and Aging

1.7.1. MiR-21

1.7.1.1 Inflammation

The expression of several key proteins in more general pathways associated with growth and development (MAPK, TGF-b, mTOR, ErbB and the Wnt pathways), the immune system (T and B cell signaling) and stress (p53 pathway) can be inhibited by miR-21. [Bye A et al., 2013] An overexpression of this miRNA is related to oxidative stress and inflammation. In animal models with obesity and atherosclerosis miR-21 promotes oxidative DNA damage and induce inflammations. [Rüegger S and Großhans H, 2012]

IL-6/Stat3 binds to 5’ promoter region of miR-21 and increases the expression of miR-21 in both human hepatocytes and HSC. [Roy S et al., 2014] This miRNA has a dominant role in controlling the inflammatory response by targeting PDCD4 that acts as a molecular switch between the proinflammatory (NF-kB) and anti-inflammatory (IL-

10) responses. [Lai L et al., 2013] Several studies support the observation of a positive correlation between serum levels of miR-21 and C-reactive proteins. [Bye A et al., 2013] Inflamm-aging is described as an increase of the general level of inflammation and simultaneously decreased immune capacity with age. It results in activation of several signaling pathways, including the TOLL-like receptor (TLR) and the NF-kB pathways. MiR-21 belongs to the inflamma-miR group and can also modulate levels of pro-inflammatory cytokines in pancreatic beta cells and also showed altered expression in plasma from patients with cardiovascular disease in cerebrospinal fluid and in extracellular fluid from patients with Alzheimer's disease. Generally it is associated with chronic age-related diseases. [Harries L W, 2014]

1.7.1.2 Age

There are contradictory findings regarding to aging with miR-21. Some studies found positive correlations with age especially when the young group had a broad age range from 20-65 and the older cohort was aged 66-95. But no significant differences could be highlighted when the young cohort is aged around 30 years and the older cohort is approximately 64 years. A positive aspect of miRNA examinations are that race and sex did not significantly correlated with miRNA expression the same way as age did. This was proven by three-way analysis of variance using sex, race and age. [Noren Hooten N et al., 2013] Other studies investigated the association of plasma miRNA levels with the phenotypes age, BMI, and sex. Seven miRNAs were significantly associated with age of which miR-21 exhibited one of the strongest correlation. [Ameling S, 2015]

1.7.1.3. Nutrition

Investigations on nutrition and miRNAs started to gain relevant informations about the function of the nutritional compounds and patterns on miRNA expressions. For instance, miR-21 was positively associated with weekly consumption of raw and cooked vegetables in stool and with the same but non-significant trend in plasma. On the other hand miR-21 was less expressed in wine and liquor consumers. [Tarallo S et al., 2014] In some studies mRNA targets of miRNAs are included and was discovered

that miR-21 (and miR-34) are found to be modulated by at least five dietary factors that have also been shown to influence downstream targets involved in cancer pathways. [Ross S A and Davis C D, 2014] Walnut intake could also alter the miRNA expression profile. Recently it has been investigated the association between the high intake of red meat and alterations of some miRNA levels in rectal mucosa tissue. In a randomized cross-over trial resulted upregulation of oncomir miR-21 following an HRM diet. [Gavrilas L I et al., 2016]

1.7.1.4. Supplementations

Few studies have evaluated whether miRNAs could be used to monitor the beneficial effect of specific supplementations. It was found that in type 2 diabetic patients receiving one-year supplementation with resveratrol-containing grape extract (GE-RES), the observed down-regulation of pro-inflammatory cytokines in PBMC was concomitant with higher levels of miRNAs involved in the regulation of the inflammatory response (miR-21, miR-155 and miR-34a). [Tome-Carneiro et al. 2013] Several studies reported that Diindolylmethane (DIM) act as anticancer therapeutic agents. It is an active compound that is generated in the stomach through the metabolic conversion of Indole-3-carbinol (I3C) present in cruciferous vegetables such as cabbage, broccoli, cauliflower, kale, radish, turnip and brussels sprouts.

I3C seem to reverse upregulated miR-21 that was observed in vinyl carbamate-induced lung cancer in mice. It was also identified that PTEN, PDCD4, and reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) are potential targets of miR-21.Other study showed that I3C could downregulate miR-21 in pancreatic carcinoma cells. Overexpression of miR-21 mediated I3C-induced sensitivity towards gemcitabine and reduced the expression of its target, PDCD4, which was upregulated by I3C.

Other nutritional compounds such as Ginsenoside Rh2 and grape seed extract rich in flavonoids could decrease the expression of miR-21 whereas it is known to be upregulated in CRC. [Gavrilas L I et al., 2016]

1.7.1.5. Physical Activity

Some miRNAs, including miR-21, are involved at fitness levels of some individuals. MiR-21 was enhanced in male participants with low VO2max. There were no correlations between traditional risk factors for cardiovascular disease like blood pressure, cholesterol, smoking habit or obesity and miR-21. Non-fasting individuals performed exercise within 24 h before sampling and found that miR-21 were increased in healthy subjects with low VO2max. Similarly to other fitness related variables and traditional CVD risk factors suggested that miR-21 and others may act as new potential independent biomarkers of fitness level and predicting cardiovascular risk. It has to be mentioned that only a weak correlation was observed between miR-21 and VO2max. No correlations were found between miR-21 and exercise habits like frequency, intensity and duration of self-reported regular physical activity, nor with selfreported time since last exercise in the validation cohort. A release of miR-21 from the endothelium in healthy subjects with low VO2max could be related to subclinical artherosclerosis hypoxia or inflammation. However, it is reported that miR-21 is correlated with the FINDRISC score, which predicts the risk of being diagnosed with type 2-diabetes in the next ten years. [Bye et al., 2013] Exercise was able to modulate the levels of miR-21 through modulating apoptosis, immune function, protein membrane trafficking and transcription. Still more clarification is needed to be done for better understanding of its mechanisms. In this context circulating miRNAs may serve as potential biomarkers or mediators of physiological adaptations. [Meurer S. et al., 2016]

1.7.2. MiR- 34a

1.7.2.1 Obesity

In obese and aging mice miR-34a was overexpressed while the level of NAD+ was decreased. MiR-34a can directly hinder the activities of SIRT1 and reduces its protein levels that regulates cholesterol homeostasis. This findings suggest that a hepatic overexpression of miR-34a reduces the levels of NAMPT(a rate-limiting enzyme for NAD+ biosynthesis), SIRT1 and NAD+ and increases the acetylation levels of SIRT1-

targeted transcriptional regulators, including important metabolic regulators like peroxisome proliferator-activated receptor γ co-activator 1a (PGC1a), sterol regulatory element-binding proteins 1c (SREBP1c), liver X receptor (LXR), farnesoid X receptor, and nuclear factor kappa B (NF-kB) and p53 which are the main molecular modulators of cholesterol, lipid and energy homeostasis and thereby linked to relevant molecular mechanisms of obesity. [Xu W et al., 2016] SIRT1 represses p53-dependent transcripional activation of miR-34a promoter through histone deacetylation resulting in deacetylation of p53 and activates FXR. FXR transcriptionally activates small heterodimer partner which isolates p53 and thus inhibits miR-34a transcription. [Rottiers V and Näär A M, 2012] The upstream transcriptional target of miR-34 is tumor suppressor p53 that may induce cell cycle arrest. [Roy S et al., 2014]

Taken altogehter SIRT1 is a key sensor and regulator of metabolic states as it responds to NAD+ levels in the cell and directly deacetylates and modulates both histone and non-histone targets to alter the expression of transcriptional programmes governing cholesterol, lipid and energy homeostasis. [Rottiers V and Näär AM, 2012]

Therefore it is highly expressed in patients with non alcoholic fatty liver disease, non alcoholic steatohepatitis and type 2 diabetes. Patients with HCC and NAFLD had significantly higher serum level of miR-34a which could be a potentional noninvasive biomarker of diagnosis. [Roy S et al., 2014] When more evidence will be provided in vivo by activation of SIRT1 through antisense inhibition of miR-34a could potentially represent an alternative therapeutic target for the treatment of NAFLD and other obesity-related diseases. [Rottiers V and Näär AM, 2012]

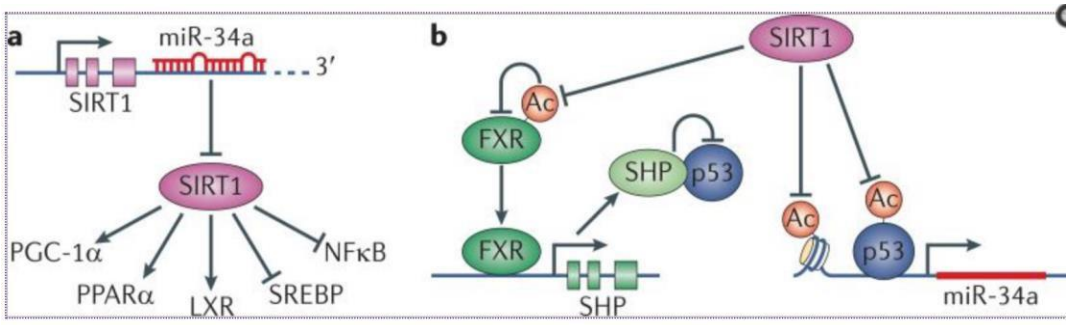

Figure 2. The molecular interaction of miR-34a, SIRT1, FXR and p53 [Rottiers V and Näär A M, 2012]

1.7.2.2. Age

A study provided evidence that there is a significant positive correlation of miR-34a with age suppressing important downstream targets and leading to telomere shortening and cardiomyocyte dysfunction and apoptosis. [Meder B et al., 2014] The miR-34 family target several key mRNAs in DNA damage response itself, for instance transcripts involved in cell cycle arrest in the G1-phase or cellular apoptosis such as CDK4 and CCNE2. Neither long nor short telomere length are good indications of aging as it must be carefully controlled in a spesific range by the cell. With lower repeat numbers the cell will undergo premature senescence and with higher repeat numbers the cell may not be able to adequately control cellular "lifespan" resulting in diseases. This miRNA regulates antioxidant enzymes that play an essential role in aging, SOD2 and TRDX2. It is expected that as older the individual as more miR-34a is upregulated. [Li et al., 2011]

1.7.2.3. Alcohol

A microarray analysis compared the expession of miR-34a in ethanol-exposed liver tissues and normal tissues in rat models. The hepatic cells with ethanol treatment resulted in overexpression of miR-34a following increased proliferation, migration and transformation. [Roy S et al., 2014]

1.7.2.4. Nutrition and supplementations

Many miRNAs including miR-34a were associated with dietary and lifestyle factors, for example, meat/fish consumption, weekly intake of vegetables and fruit, but not consistently in different sampling materials e.g. stool and plasma. In the peripheral blood, few studies have evaluated whether miRNAs could be used to monitor the beneficial effect of specific supplementations. As it is already mention above it was found that in type 2 diabetic patients receiving one-year supplementation with resveratrol-containing grape extract the observed down-regulation of pro-inflammatory cytokines in PBMC was linked with higher levels of miRNAs such as miR-34a. [Tome-Carneiro et al., 2013]

1.7.3. miR- 92a

1.7.3.1. Nutrition

An overexpression of miR-92a resulted in repressed production of proinflammatory cytokines such as TNF- alpha and IL-6. In the case of meat comsumer exhibited lower expression of miR-92a and therefore higher levels of proinflammatory cytokines were found in human plasma. [Lai L et al., 2013] A study demonstrated that three different groups of dietary habits vegans, vegetarians and omnivorous had various expression of miR-92a in plasma and stool samples. Vegans and vegetarians showed a sigificant higher expression compared to omnivores. It has also been indicated that miR-92a expression was inversely related to dairy products including cheese consumption. [Tarallo et al., 2014]

1.7.3.2. Supplementation

Dietary supplementations are able to influence miRNA expressions in human serum due to altered inflammatory pathways. Several miRNAs including miR-92a could be mediated by zinc intake which adds new perspectives for treatment of inflammatory-related diseases. [Ryu et al., 2011] Other active nutritional compound Diindolylmethan (DIM) downregulated miR-92a which is associated with receptor activator of NF-kB ligand signalling, EMT and cancer progression to inhibit differentiation of osteoclasts and osteoblasts in prostate cancer metastasis. This substance is present in cruciferous vegetables such as cabbage, broccoli, cauliflower, kale, radish, turnip and brussels sprouts. [Tarallo et al., 2014]

1.7.3.3. Inflammation

The Toll-like receptors (TLRs) count as one of the important immunsystem modulators which take part in fighting against invading pathogens. MicroRNAs are involved in this TLR signaling. A simulation of multiple TLRs leaded to reduced levels of miRNA-92a and some other members of the miRNA-92a family in macrophages. Moreover miR-92a

mimics and knockdowns work in opposite way. In addition mitogenactivated protein kinase kinase 4 (MKK4) was found to be directly targeted by miR-92a. A knockdown of MKK4 inhibited the activation of JNK/c-Jun signaling and the production of TNF-alpha and IL-6. [Lai L et al., 2013]

1.7.4. MiR-106b

1.7.4.1. Age

Like with miR-34a, the members of the miR-106b cluster have also been associated with cellular senescence which results from the activation of the CDKN2A-ARF and p53 components of the DNA damage checkpoint. [Harries L W, 2014] It is estimated that in eldery this miRNA is decreased by 40,4% in T cells. [mirbase.org]

1.7.4.2. Obesity

A study revealed that the prominent BMI-associated miRNAs belong to different families organized in clusters such as miR-106b. The relative high amount of liver-specific miRNAs whose blood levels were found to be positively correlated with BMI. After releasing miRNAs from lysing hepatocytes into the circulation a subclinical or/and manifest NAFLD could be diagnosed which in turn is strongly positively associated with an increased BMI. [Ameling S, 2015] MiRs-106b are categorized to governors of cholesterol efflux, uptake, synthesis and HDL metabolism. [Witwer K W, 2012]

1.7.4.3. Stress

When it comes to the term stress exact mechanisms correlated with miRNAs are known. Stress induced targets can be described as heat shock, oxidative stress, viral infection and DNA damage. The expression of this stress-induced targets can overcome miRNA-defined threshold. Generally the relative levels of miRNAs and mRNA targets determine how much target protein is produced under normal conditions where the

threshold is stated meaning that the level of target gene repression is not depending only on the cellular concentration of miRNAs alone but also on the concentration of mRNA target relative to the miRNA. This is illustrated in the case of two extracellular ligands MICA and MICB, which are induced by stresses. An immune activating receptor, NKG2D, which is present on Natural Killer cells and T cells recognizes these ligands and allow the elimination of tumor and virus-infected cells. [Stern-Ginossar N et al., 2008]

1.7.4.4. Supplementations

MiR-106b acts as a typical oncomir and some studies could prove that consuming EGCG reduced the amount of this miRNA. [Masika J et al., 2015]
A systematic review identified that 10 g fiber intake per day decreases the risk of colorectal cancer by 10% and has further protective effects on overall health. It is mainly caused by increased production of butyrate, a short-chain fatty acid, processed during fermentation of dietary fiber in the intestine. Butyrate induces expression of p21 a key regulatory molecule of cell cycle arrest by miR-106b downregulation. This informations provide mechanistic insights that antiproliferative and proapoptotic activity of butyrate may be in part explained by changes in miRNA activity. [Gavrilas L I et al., 2016]

1.7.5. MiR-151a

1.7.5.1 Age

It has demonstrated in aging studies a decrease of miR-151a in age-related phenotypes and age-related diseases. In addition it has an important role in inflammatory pathways specifically in low-grade systemic chronic inflammation including NF-κB, TNFα, IL-1 and INF-α. MiR-151a inhibits inflammation-associated aging in young individuals. [Noren Hooten N et al., 2013] A more recent study could reproduce the results and confirmed that the miR-151a expression is decreased by age in serum and can modulate inflammatory pathways and DNA damage responses as refered above including IL-6 and IL-10. [Harries L W, 2014] There may be differences between the old and the

oldest old who manifest exceptional longevity. Interestingly its expression has been positively correlated with patient survival. Still it needs further investigations because on certain diseases resulted into inconsistent expression patterns. [Noren Hooten N et al., 2013]

1.7.6. MiR-155

1.7.6.1. Metabolic disease

Many studies provided evidences that miR-155 is implicated in numerous biological processes including haematopoiesis, inflammation, immunity, renal function and vascular smooth muscle as it interacts in the regulation of type 1 Angiotensin II receptor (AT1R). Transfection of miR-155 into human primary lung fibroblasts decreased the endogenous expression of the AT1R compared with nontransfected cells. A single nucleotide polymorphism (+1166 A/C) in the human AT1R was known to be associated with hypertension, cardiac hypertrophy and myocardial infarction but it is located within an untranslated region therefore its impact could not be substantly proved. Still it is believed that this polymorphism overlaps with the miR-155 target site in the 3′UTR of AT1R mRNAs and translationally represses the protein. This subject with overexpressed miR-155 inhibits impaired AT1R accompanied by low blood pressure. Otherwise miR-155 deficiecy shows a higher risk of hypertension. Therefore it is important to take it into consideration that this miRNA has an important function on blood pressure. [Faraoni I et al., 2009]

1.7.6.2. Inflammation

It recieved high prestige as it has been done many publictions on miRNA-155. One of its main task is maintaining the innate and adaptive immunity. Commonly it is often investigated in association with chronic inflammatory diseases derived from abnormal immune response. After alcohol feeding miR-155 expression in Kupffer cells was identified through TNF which promote liver inflammation. A typical trait in mouse models of NASH and patients with HCV is a high miR-155 expression which results in

development of HCC by regulating C/EBP β levels and Wnt signaling respectively. Remarkably, higher expression of miR-155 in peripheral monocytes is shown in patients with chronic therapy resistant HCV-infection whereas no change was found in patients who responded to treatment. [Roy S et al., 2014] The TRF1 gene is a target of so called inflamma-miR especially miR-155. Like miR-34 family it may regulate telomere length therefore it plays a role in DNA damage response. Upregulation of miR-155 has been noted in extracellular fluid from patients with Alzheimer's disease and in the synovial fluid of patients with rheumatoid arthritis. [Harries L W, 2014]
This prominent miRNA encoded in chromosome 21 and transcribed from the B-cell integration cluster. Noteably in foam cells and other clinical speciments from patients with Atherosclerosis showed an increased level of miR-155 in a dose- and time-dependent manner. The molecular mechanism behind this expression happens through calcium-regulated heat stable protein 1 (CARHSP1), which controlls the stability of tumor necrosis factor alpha (TNF-α) mRNA. [Li X, 2016] It must be taken into account that for inflammation timing becomes particularly important in case of acute stress responses. NF-κB upregulates the transcription of miR-155 along with other inflammatory responsive genes through a signaling cascade in macrophages. This mature miRNA peaks at much later time approximatly after 24 hours while other immediate early-response genes, the expression of primary miRNA transcripts take less time to accumulate and its signal appear within 2 hours. If the concentration of mature miRNAs reach a certain level in cells than a delay between the emerging presence of mature miRNAs and the beginning of target repression is expected. Remarkably the mRNA targets of miR-155 belongs themselves to pro-inflammatory signaling molecules like transcription factor NF-κB that upregulate this particular miRNA. For this reason miRNAs activated by NF- κB reset the proinflammatory signaling pathway after activation. This controlled expression in macrophages is believed to promote a strong inflammatory attack to pathogens and at the same time keeping the host uneffected as possible. [Leung AKL and Sharp PA, 2010] Some other studied confirmed this results that elevated miR-155 relieves chronic inflammation by a negative feedback loop and plays a protective role during atherosclerosis-associated foam cell formation via the miR- 155–CARHSP1–TNF-α pathway. On the other hand various investigations have indicated that miR-155 has opposite effects in different cell types, pathological stages,

and animal models of AS. As it can have an anti-inflammatory properties by targeting MAP3K10 in macrophages but also may promote AS as it has been observed to derepress BCL6-mediated inhibition of CCL2 transcription in the bone marrow cells of ApoE–/– mice. [Li X et al., 2016] Inflammatory mediators such as IFN-β polyriboinosinic–polyribocytidylic acid (poly IC) or Tumor Necrosis Factor-α (TNF-α) can induce miR-155 in macrophages and monocytes. It also can be induced by baterial lipopolysaccharide (LPS) in a human cell line through c-Jun Nterminal kinase (JNK) pathway and partially through the mitogen-activated protein kinase (MAPK) pathway. [Faraoni I et al., 2009] Not only overexpression may result in pathologic appearance but also a low expression seems to exhibit aberrant phenotype such in defective B and T cell immunity. Investigations with miR-155 deficient mice have shown an impaired antigen-presenting function of dendritic cells, reduced germinal center function, T cell dependent antibody response and cytokine production. [Faraoni I et al., 2009]

1.7.6.3. Age

MiR-155 indicated similar expression patterns in both senescent cells and in aging PBMCs. It has been investigated that miR-155 decreased during cellular senescence but as an oncogenic miRNA it acts differently and mostly upregulated in various cancers. The explanation might be its location in a fragile locus named B cell integration cluster that is an integration site of viral-induced lymphomas. [Noren Hooten N et al., 2010]

2. Objectives

The aim of the study was to examine whether the 6 relevant metabolic miRNAs miR-21, miR-34a, miR- 92a, miR-106b, miR-151a and miR-155 have influence by external lifestyle factors, for instance, by different eating habits, drinking consumptions, physical activity, stress levels, supplementations and inflammation. Also our great interest is to analyze important variables with greater variances such as Body Mass Index and Age with these miRNAs that may lead to some changes in their expression levels. The final evaluation could be used as early predictiors that could prevent some pathological changes derived from unhealthy lifestyle factors.

3. Materials and Methods

3.1. Study design

Participants were asked to fill out a questionnaire regarding their lifestyle, health status and nutrition and simultaniously blood samples were collected with a Dried Blood Spots. 132 participants were recruited of which one is a reference person.

qPCR examination of 6 relevant miRNAs which plays a role in metabolic disorders and aging including miR-21, miR-34a, miR- 155, miR-106b, miR-151a and miR-92a from dried blood spots was performed. The participants were divided according to their age into a group under the age of 50 years and the other group were above 50 years old. Similarly the individuals were divided according to their BMI into 3 groups- the first group had a BMI under 21 kg/m^2, the second group's BMI ranged between 21-25 kg/m^2 and the third group had a BMI of over 25 kg/m^2. All three BMI groups did not obtain the exact classifications especially between underweight and normal weight as it was more important to achieve a consistent distribution of all 132 individuals. The criteria smoking was excluded. All the informations about their lifestyle

patterns were collected through a questionnaire from each individual. miRNA-24 were recommended by Thermofisher to be used as endogenouse control also known as „housekeeping gene“. Finally statistical evaluations on correlations between each miRNAs to following variables were done:

- Food and drinking patterns: meat, fish, fruit/vegetables, alcohol, coffee and tea consumptions
- Supplementations of multivitamins or single supplementation of vitamins or food compounds
- Level of stress
- Level of physical activity

3.2. Sample Collection

Participants warmed up one of their fingers with mechanistic movements of their other hand to stimulate blood circulation and make it easier to collect blood drops. Before puncturing the finger with a lancet (Safety-Lancet Extra 18G, Sarstedt) desinfection was done with an alcohol sponge. Afterwards Blood droplets were collected in Whatman® protein saver cards(Sigma-Aldrich®) and left to dry at room temperature until further use.

3.3. Relative miRNA expression analysis

3.3.1. Total RNA extraction

The extraction was carried out using the miRNeasy Micro Kit by Qiagen and QIAzol Lysis Reagent with a small adjustion in the protocol thereby samples are able to be purified from Dried Blood Spots. Firstly a steril cut out of one whole blood spot was done which then was placed in 1000 µl Quiazol. The screw caps tube with its samples were bead homogenized at 5500 rpm for 45 seconds with a break of 10 seconds inbetween. Then the samples were transfered into a new 2 ml collection tube where 200

µl Chloroform were added to lysate. The following next steps were conducted exactly as it is described in the protocol.

3.3.2. Quantification

The purified RNA samples were measured with Pico100 (Picodrop Limited, Hinxton UK) to determine their quality and quantity. Further handling for analyzing miRNAs a value over 5ng/µl was taken into account. The samples were stored at -20 °C until further examinations.

3.3.3. cDNA synthesis

This method is conducted for quantifying miRNA by adding poly(A) tail(3') and an adaptor(5') as the first step to amplify all miRNAs in a single reverse transcription reaction. During the reverse transcription reaction an universal RT primer binds to the 3'poly(A) tail so that finally cDNA is created. The last step of cDNA synthesis is called miR-Amp reaction where the amount of cDNA increases by adding universal forward and reverse primers. All important reagents were used from TaqMan® Advanced miRNA cDNA Synthesis Kit. [Thermofisher, Applied Biosystem]

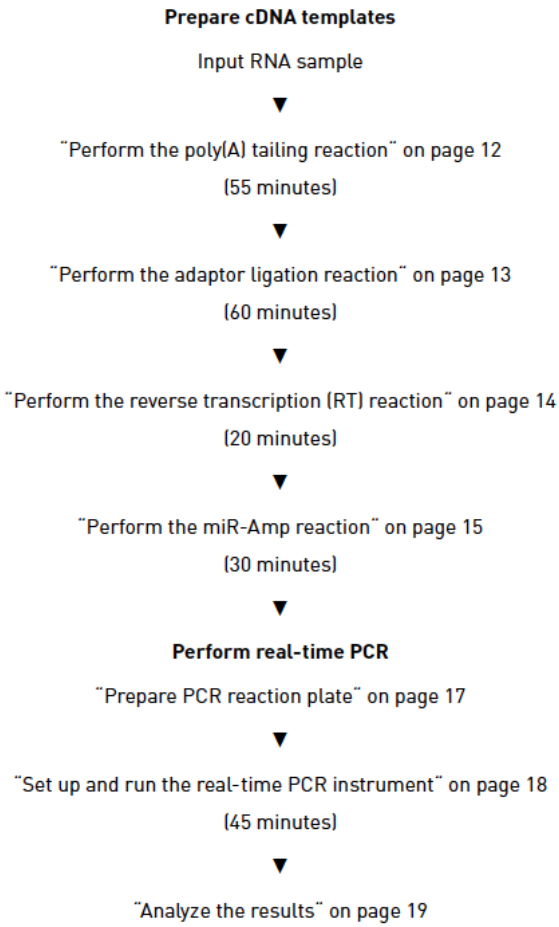

Figure 3. Overview of cDNA synthesis and qPCR of miRNAs.

3.3.3.1. Poly (A) tailing reaction

Total RNAs, including miRNAs, are extended by poly (A) polymerase and ATP to maintain a poly (A) tailing reaction on 3' end of the specific sequence. [Shi R et al., 2012]

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
10X Poly(A) Buffer	0.5 µL	2.2 µL	5.5 µL
ATP	0.5 µL	2.2 µL	5.5 µL
Poly(A) Enzyme	0.3 µL	1.3 µL	3.3 µL
RNase-free water	1.7 µL	7.5 µL	18.7 µL
Total Poly(A) Reaction Mix volume	**3.0 µL**	**13.2 µL**	**33 µL**

[1] Volumes include 10% overage.

Table 1. Pipetting scheme of Poly(A) tailing reaction

Step	Temperature	Time
Polyadenylation	37°C	45 minutes
Stop reaction	65°C	10 minutes
Hold	4°C	Hold

Table 2. The regulatory loop of miR 34a, SIRT1, FXR and p53

3.3.3.2. Adaptor ligation reaction

A poly (T) adaptor is added to the miRNA complementary to a poly (A) tail a condition called reverse transcription where it is converted into cDNA.
[Shi R et al., 2012]

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X DNA Ligase Buffer	3 µL	13.2 µL	33 µL
50% PEG 8000[2]	4.5 µL	19.8 µL	49.5 µL
25X Ligation Adaptor	0.6 µL	2.6 µL	6.6 µL
RNA Ligase	1.5 µL	6.6 µL	16.5 µL
RNase-free water	0.4 µL	1.8 µL	4.4 µL
Total Ligation Reaction Mix volume	**10 µL**	**44 µL**	**110 µL**

[1] Volumes include 10% overage.
[2] 50% PEG 8000 is very viscous, follow the Important statement below to ensure accurate pipetting.

Table 3. Pipetting scheme of adaptor ligation reaction.

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

Table 4. Optimized cycling protocol for adaptor ligation reaction

3.3.3.3. Reverse Transcription reaction

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X RT Buffer	6 µL	26.4 µL	66 µL
dNTP Mix (25 mM each)	1.2 µL	5.3 µL	13.2 µL
20X Universal RT Primer	1.5 µL	6.6 µL	16.5 µL
10X RT Enzyme Mix	3 µL	13.2 µL	33 µL
RNase-free water	3.3 µL	14.5 µL	36.3 µL
Total RT Reaction Mix volume	**15 µL**	**66 µL**	**165 µL**

[1] Volumes include 10% overage.

Table 5. Pipetting scheme of Reverse Transcription reaction

Step	Temperature	Time
Reverse transcription	42°C	15 minutes
Stop reaction	85°C	5 minutes
Hold	4°C	Hold

Table 6. Optimized cycling protocol for reverse transcription reaction

3.3.3.4. miR-Amp reaction

For the PCR amplification it is necessary to use a miRNA-specific forward primer and a universal poly(T) adaptor reverse primer.

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
2X miR-Amp Master Mix	25 µL	110 µL	275 µL
20X miR-Amp Primer Mix	2.5 µL	11 µL	27.5 µL
RNase-free water	17.5 µL	77 µL	192.5 µL
Total miR-Amp Reaction Mix volume	**45 µL**	**198 µL**	**495 µL**

[1] Volumes include 10% overage.

Table 7. Pipetting scheme of miR-Amp reaction

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

Table 8. Optimized cycling protocol for miR-Amp reaction

3.3.4. Real-time reverse transcription polymerase chain reaction execution of real-time PCR

So qPCR TaqMan® Fast Advanced Master Mix and specifically ordered TaqMan® Fast Advanced miRNA Assays by Thermofisher were carried out to determine the quantification of each selected miRNA in every samples.

Complementary DNA is used as template for the qPCR reaction after RNA is transcribed into cDNA by reverse transcriptase from total RNA.

Component	1 Rxn	4 Rxns[1]
TaqMan® Fast Advanced Master Mix (2X)	10 µL	44.0 µL
TaqMan® Advanced miRNA Assay (20X)	1 µL	4.4 µL
RNase-free water	4 µL	17.6 µL
Total PCR Reaction Mix volume	**15 µL**	**66 µL**

[1] Volumes include 10% overage.

Table 9. Pipetting scheme of real-time reverse transcription polymease chain reaction

After pipetting the samples in the PCR device were the forward and reverse primers annealed to complementary sequences along the denatured cDNA template strands. There are variouse types of primer binding sites which depend on the target miRNA sequence. So they can achieve the specificity.

TaqMan® MGB probes are present in TaqMan® Advanced miRNA Assay that contain three chemical substances, a reporter dye which binds at the 5′ end of the probe, a non-fluorescent quencher (NFQ) dye and a minor groove binder (MGB) at the 3′ end of the

probe. For the qPCR instrument in our laboratory a laballed oligoprobe FAM, 6-carboxyfluorescein, was used as an appropriate reporter dye to help detecting the quantity of our chosen miRNAs. The NFQ dye does not fluoresce which allows the real-time PCR system to measure the reporter dye contributions more accurately. MGB increases the melting temperature (Tm) without increasing the probe length. During the arrangement of the required three target sequence-specific oligonucleotides, the signal is emitted only after the probe hydrolysis. The DNA polymerase only cleaves probes that hybridize to the target sequence. It results a separation of the reporter dye from the quencher dye and the fluorescence increases by the reporter dye. This happens only if the probe is complementary to the target sequence and amplified during PCR. Polymerization of the strand continues until the blocked 3' end of the probe therefore the extension stops.

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

Table 10. Optimized cycling protocol for real-time reverse transcription polymease chain reaction

The data was then analyzed with StepOnePlus Software which present the startpoint of the threshold of each samples

3.3.5. Statistical Analysis correlated with Questionnaire

A questionnaire filled out by each participants with different life stages and body shapes provide important informations through their various lifestyle factors. These final data were added in statistical program to find correlations of each single variables of lifestyle factores with all miRNAs of our interest.

In every statisical calculation miRNA 24 as endogenous control and a blood sample of a reference person were considered in each PCR-plate to obtain the end results needed.

After normalizing the Ct-values with Microsoft® Excel® 2010 these data were analyzed with IBM® SPSS® Statistics Version 20. The different types of statistical test were chosen depending on their normal distribution which were checked with Kolmogorov-Smirnov Test and Q-Q plots. Afterwards correlations between metric variables were done with Kendall's Tau, Pearson or Spearman Correlations. For linear variables to evaluate their mean value between groups were examined with Student's t-test.

3.3.6. Molecular Pathways of MiRNAs

In this study we looked for common molecular pathways from 3 miRNAs after examining correlations of each miRNAs with other parameters such as BMI and age. An internet tool called Loom Miroculus was used with the possibility to figure out more about the molecular network of miRNAs with its related disease and target genes. Words were typed in the searching field such as metabolic disease, age and all miRNAs of our interest.

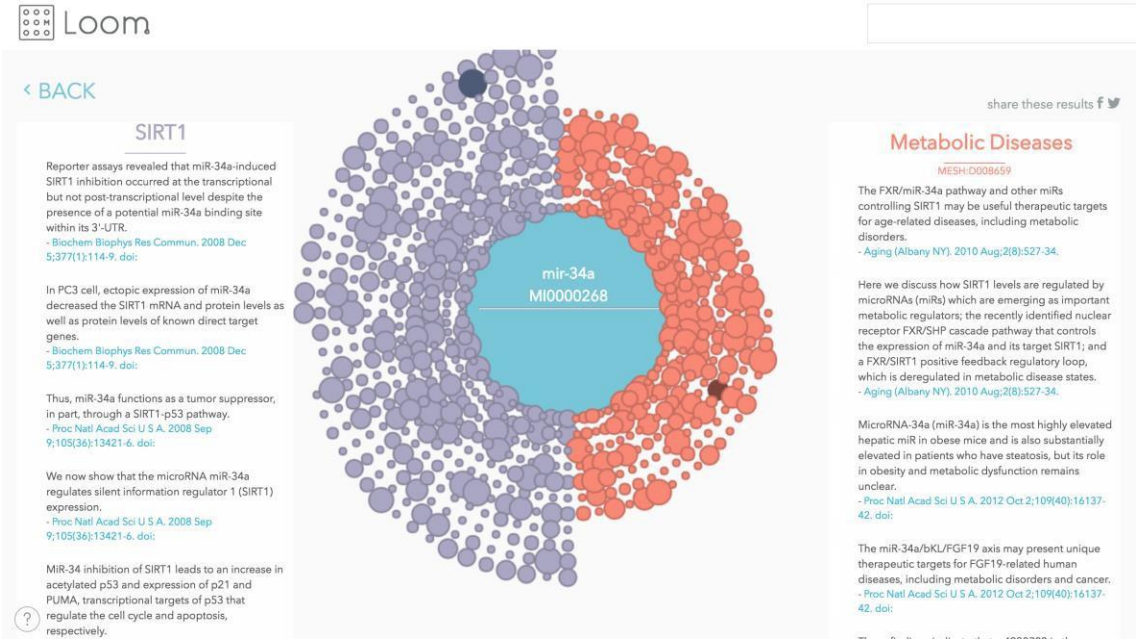

Figure 4. Loom map a molecular network between miRNAs, target genes and disease [https://loom.miroculus.com/]

4. Results

4.1. Subjects characteristics

	Female	Male	Total	Total St.dev.
Age	**114** **Min.:22** **Max.:72**	**76** **Min.:27** **Max.:79**	**190**	
BMI	**114** **Min.:15,82** **Max.:47,75**	**76** **Min.:21,22** **Max.: 53,19**	**190**	
miR-21	**75**	**43**	**118** **Min.:0.228227** **Max.:4.762467**	**0.924196**
miR-34a	**72**	**45**	**117** **Min.:0.254092** **Max.:54.865317**	**10.68571**
miR-92a	**71**	**47**	**118** **Min.:0.127970** **Max.:2.449840**	**0.494331**
miR-106b	**74**	**46**	**120** **Min.:0.044026** **Max.:2.117625**	**0.3935098**
miR-151a	**68**	**42**	**110** **Min.:0.131730** **Max.:18.808223**	**3.0687298**
miR-155	**51**	**36**	**87** **Min.:0.3025396** **Max.:3.2002286**	**0.4496054**

Table 11. Amount of participants in total and in both genders in our study.

190 participants were split into 3 groups according to their BMI„BMI <21“, „BMI 21,1-25“ and „BMI <25,1“ and two groups were divided on the basis of their age into „< 50 years old“ and „ > 51 years old“.

BMI groups:

	N for BMI <21	N for BMI 21,1-25	N for BMI >25,1
miR-21	31	48	39
miR-34a	31	47	39
miR-92a	27	50	41
miR-106b	31	49	40
miR-151a	25	45	40
miR-155	20	29	38

Table 12. Amount of participants split in three BMI groups.

Age groups:

	young	old
Inflammation exists	34	20
No inflammation	60	63
miR-21	56	62
miR-34a	58	59
miR-92a	56	62
miR-106b	56	64
miR-151a	47	63
miR-155	41	46

Table 13. Amount of participants split into young and old ages.

Inflammations:

	N for inflammation	N for no inflammation
miR-21	34	80
miR-34a	33	79
miR-92a	32	81
miR-106b	34	81

miR-151a	35	72
miR-155	35	52

Table 14. Amounts of participan's samples used for each miRNAs analysis.

4.2. Relative miRNA expressions

4.2.1. MiR-21

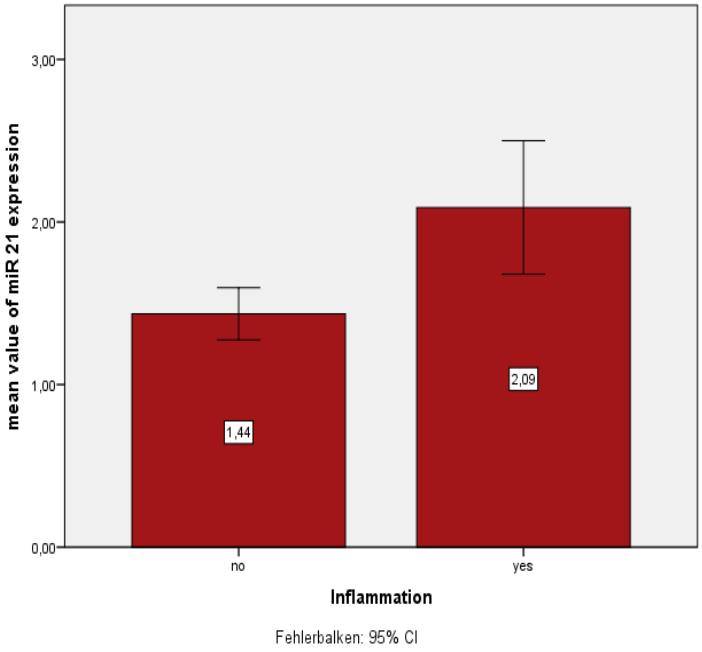

Figure 5. MiRNA expression between groups with and without inflammations.

It is evaluated that individuals with inflammations have a strong significantly higher expression of miRNA 21 than subjects indicating no inflammations. The group with inflammations consist of 34 persons and without inflammations are 80 participants. It is shown that datas from both groups have a normal distribution and therefore a student's T-test of mean values was determined. (p=0.000, M difference= 0.180369891, F=11.799, N= 114)

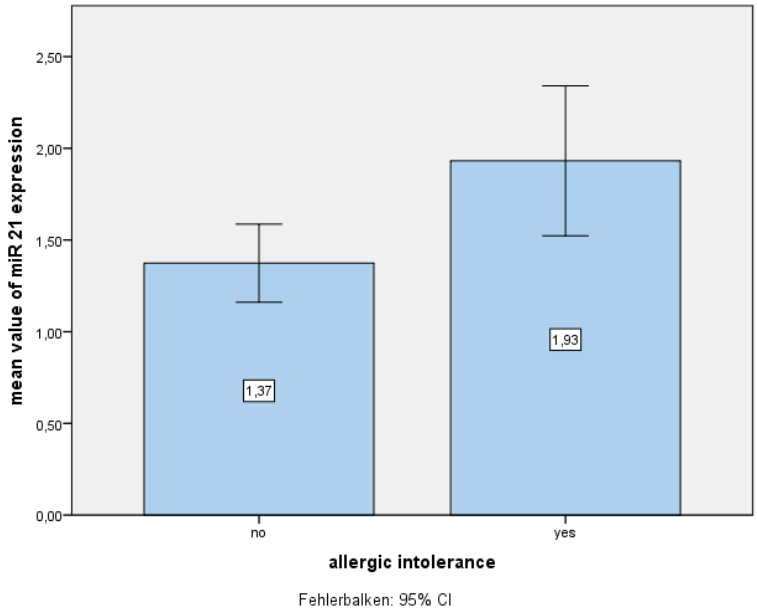

Figure 6. MiRNA expression between subjects with and without allergic intolerance

Here the Student's T-test revealed that individuals with allergic intolerance exhibit higher expression of miR-21 than healthy subjects. 80 participants were calculatd for this test of which 22 mentioned to have allergic intolerance. (N (no)=58, Mean value= 1,37353443, SD= 0.809825842, N(yes)=22, Mean value= 1.93229004, SD= 0.922463112) (p= 0.010, M differences= -0.558755611, F= 0.010, Std Error Differences= 0.210738406)

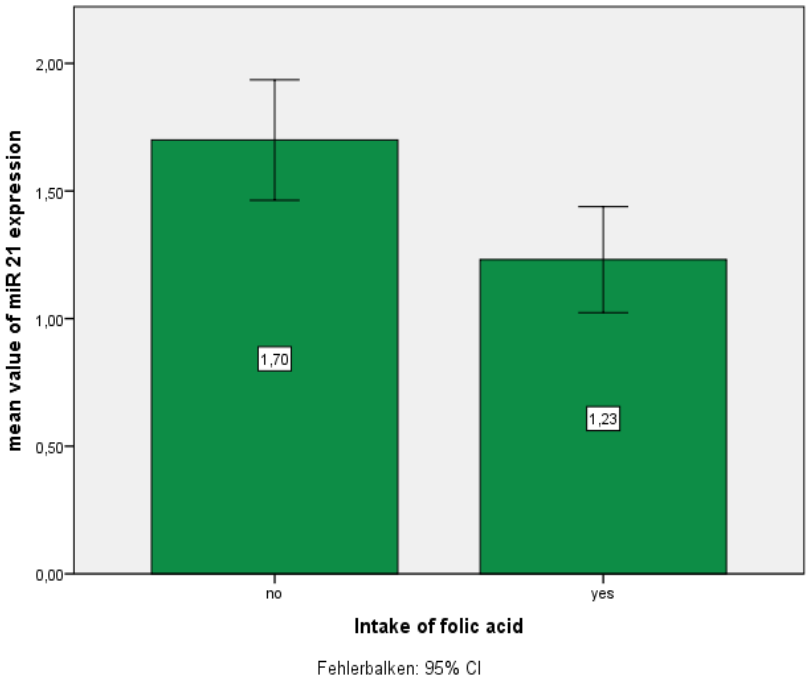

Figure 7. MiRNA 21 expression between intake with and without folic acid.

Subjects with folic acid intake have lower expression of miR-21 than those without such intake. 31 participants revealed taking folic acid supplementations which were compared with 63 individuals without folic acid intake. This comparison had a strong significant association which was calculated through mean values of T-test. (p=0.012, mean difference= 0.468613643, Std. error difference=0.183176141, F=5.180)

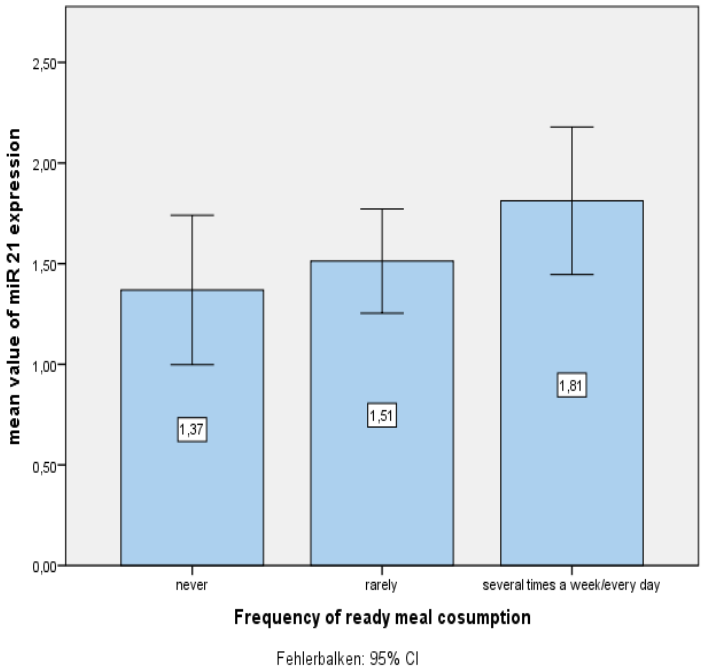

Figure 8. MiRNA 21 expression and frequency of ready meal consumptions.

Here in this depiction shows a higher expression of miR-21 with higher consumptions of ready meals but Spearman's Correlations test did not indicate a significant correlation. (p=0.069)

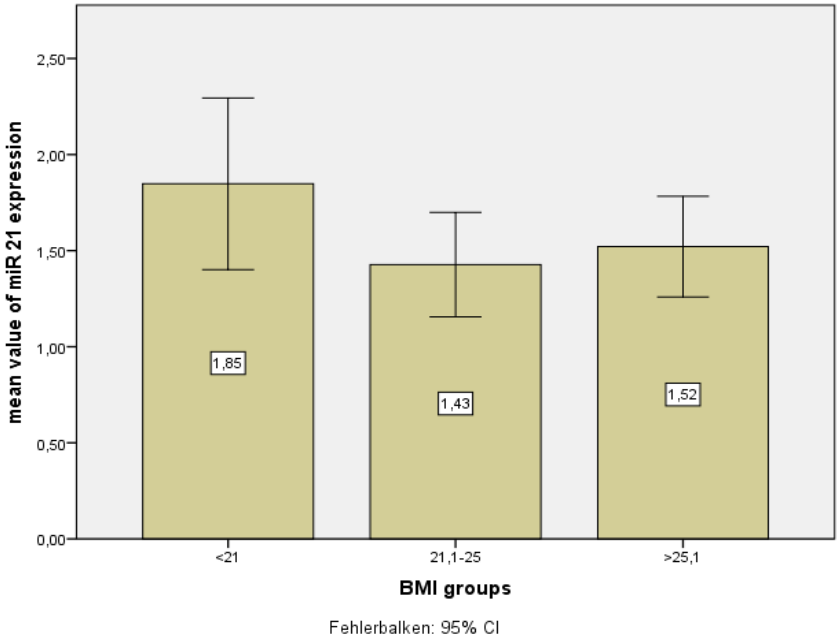

Figure 9. MiRNA 21 expression and BMI groups.

Pearson's Correlations Test nor Student's T-test indicated any significances between the three BMI groups "<21","21,1-25" and ">25". (Pearson Correlation Test: N=118, p=0.891 and T-test between BMI <21 and BMI 21,1-25: N= 60, p=0.086)

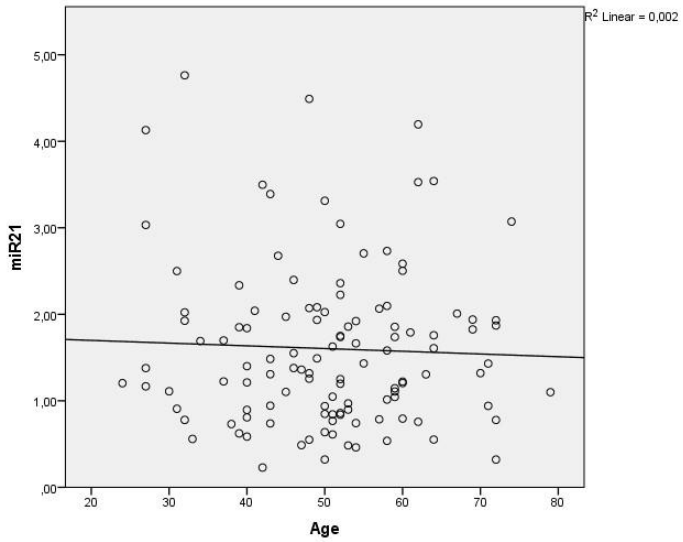

Figure 10. MiRNA 21 expression and age.

No significant correlations between expression of miR-21 with BMI and with age were found after statistical examination with Student's T-test and Pearson Correlation, respectively. (N= 118, BMI: p=0.891, Age: p=0.664) However no significant association is determined between two BMI groups <21 BMI and 21-25 BMI. (P=0.86, N=60)

4.2.2. MiR-34a

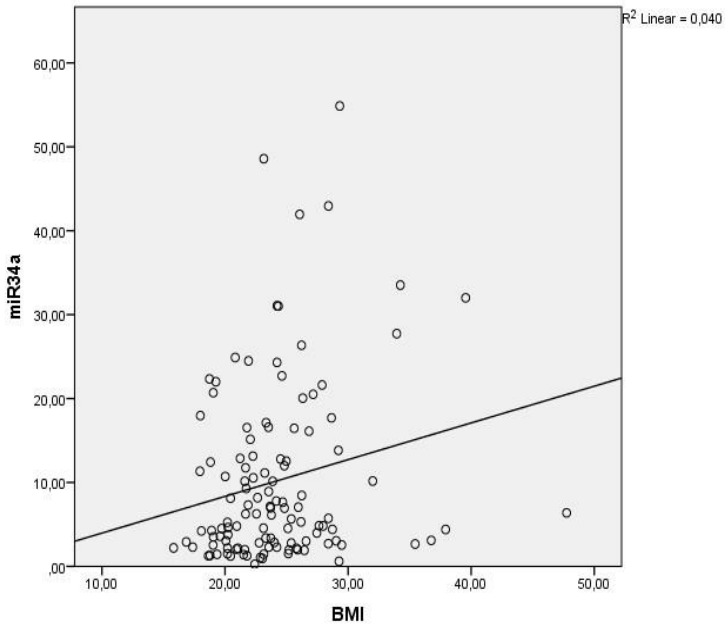

Figure 11. MiRNA 34a expression and BMI.

There is a significant positive association between BMI and expression of miR-34a. (R^2= 0.040, p=0.033, N= 117, Correlation Coefficient= 0.198)

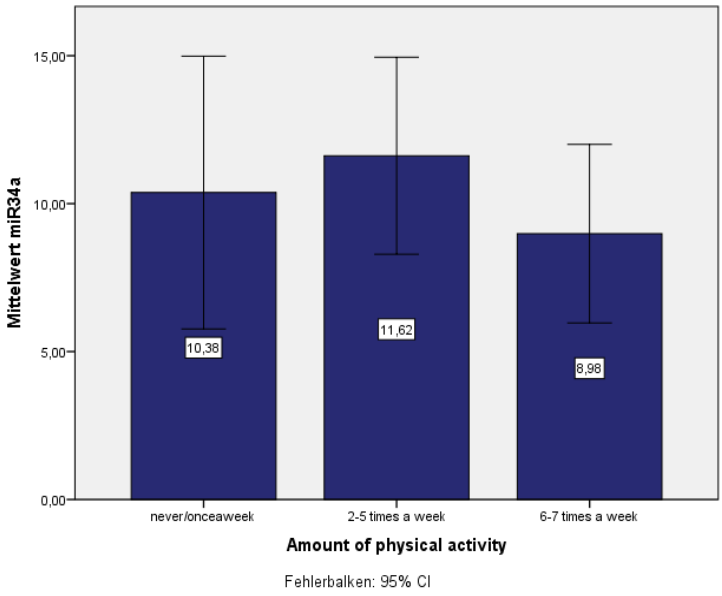

Figure 12. MiRNA 34a expression and amount of physical activity.

The diagram is depicting higher expression at 2-5 times a week of physical activity and at 6-7 times a week a lower expression compared to no/once a week of physical activity. Spearman's Correlation showed a negative association between miR-34a and amount of physical activity. (Correlation Coefficient= -0.215, p= 0.021, N=116)

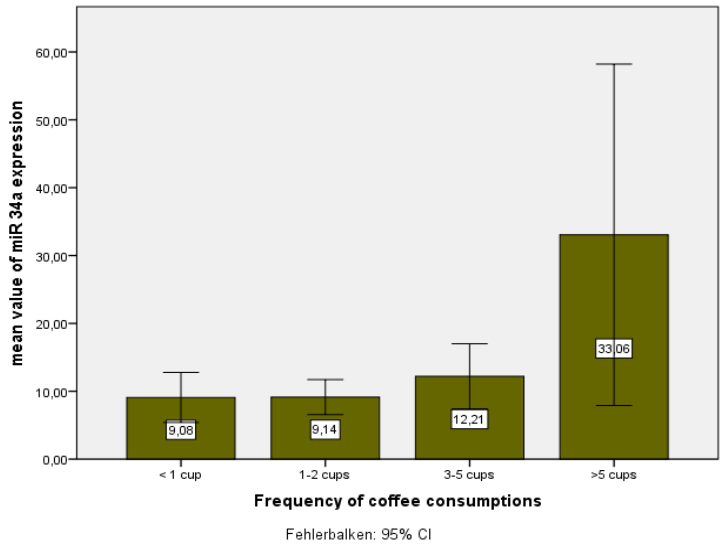

Figure 13. MiRNA 34a expression and frequency of coffee consumtions.

It is noticeable that miR-34a expression increases as more often coffee is consumed. Both data are normal distributed therefore a Pearson-Test was done and showed a significant positive correlation. (p=0.012, N=114)

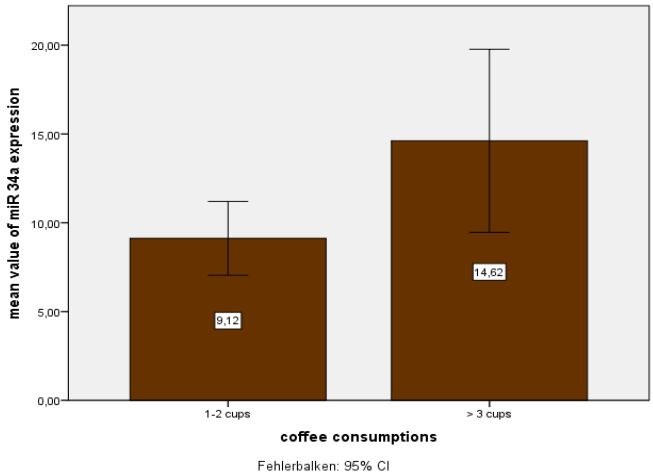

Figure 14. MiRNA 34a expression and two groups of coffee consumptions.

The expression of miR-34a is higher in the group with consumption above 3 cups daily whereas the group drinking 1-2 cups daily showed a decreased expression of miR-34a. By the mean value of Mann-Whitney Test a significance of P=0.046 was shown. (N for 1-2 cups=88, N for >3 cups= 26)

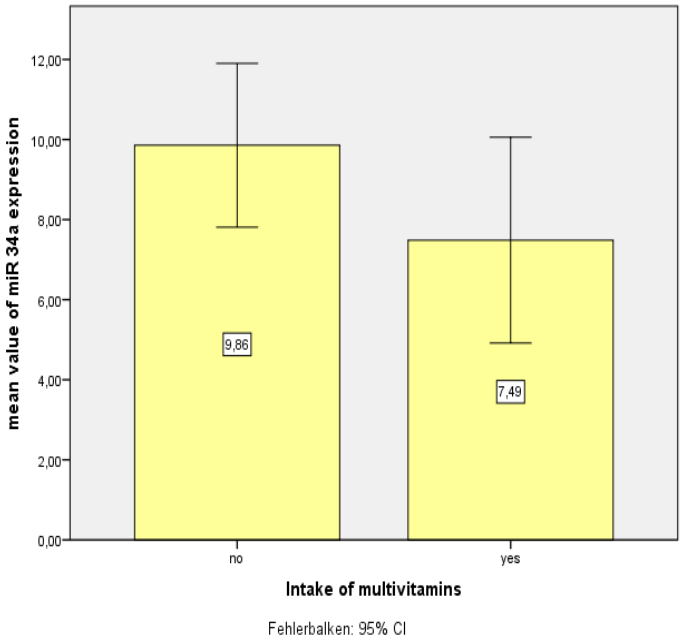

Figure 15. Level of miRNA 34a expression and intake of multivitamins.

Individuals with multivitamin intakes have reduced expression of miR-34a compared without multivitamin intake. The statistical evaluation was done with Mann- Whitney U Test resulting a significance of p=0.032. (N with vitamins intake= 58, N without vitamins intake= 41)

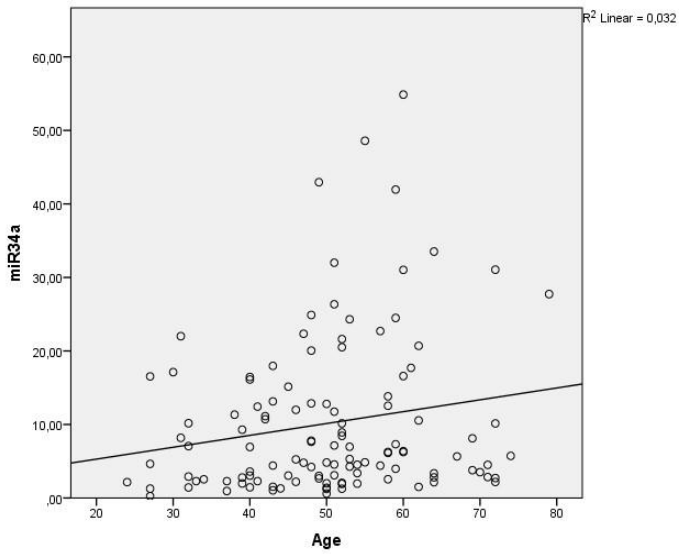

Figure 16. Correlation between expression of miRNA 34a and age.

Spearman's Correlation Test of 117 samples revealed a positive association between age and expression of miR-34a but it was not significant.

4.2.3. MiR-92a

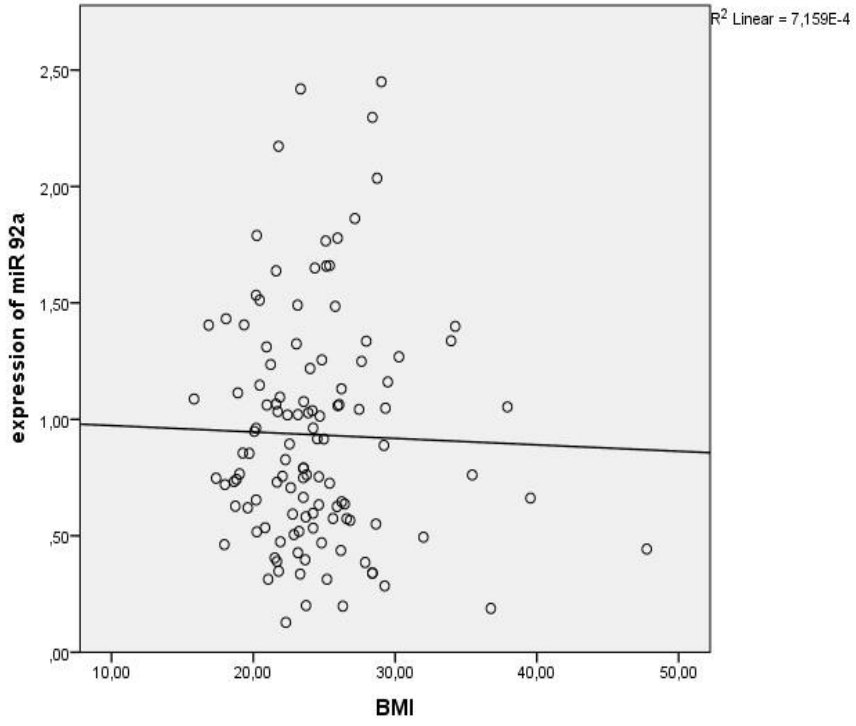

Figure 17. Correlation between expression of miRNA 92a and BMI.

A slightly negative association between expression of miR-92a and Body Mass Index was shown by examining with Spearman Correlation Test but the result did not show any significances. (p=0.797, N= 118)

4.2.4. MiR- 106b

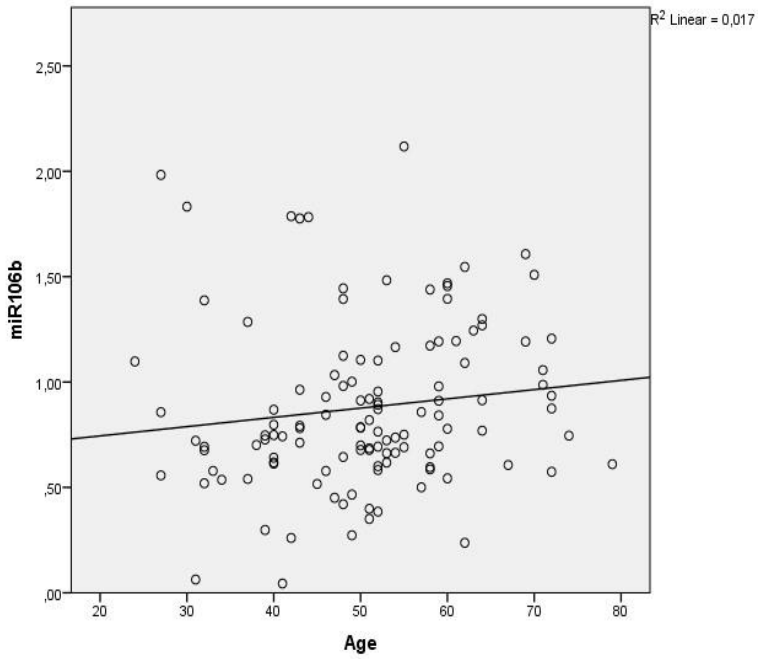

Figure 18. Correlations between expression of 106b and age.

A positive associations is indicated through Spearman's Correlation Test between both metric variables expression of miR-106b and age. (N=120, p=0.015 and Correlation Coefficient= 0.221) The linear regression analysis exhibit higher miRNA 106b expression in older subjects.

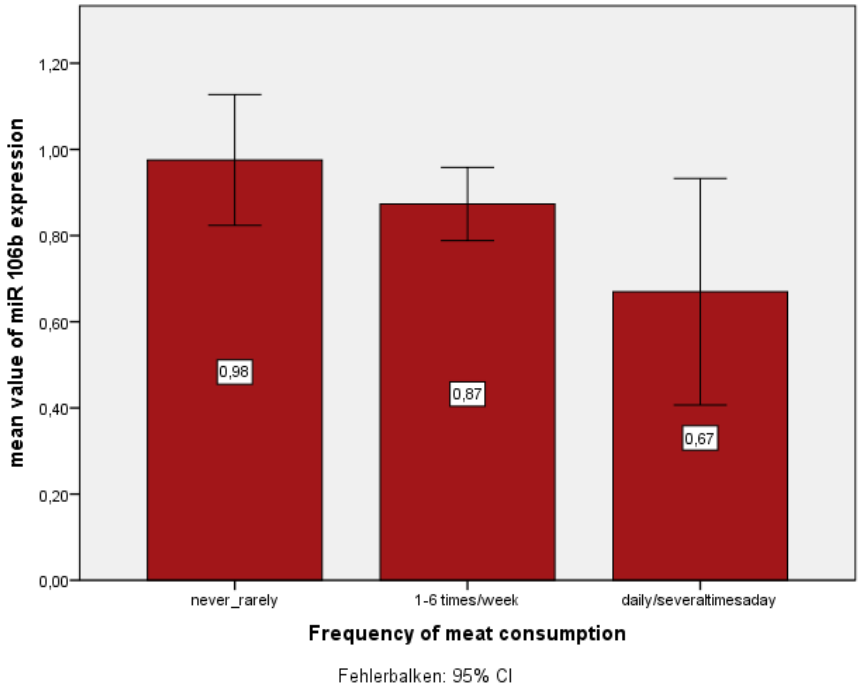

Figure 19. Frequency of meat consumption and miRNA 106b expression.

There was a significant negative association between miR-106b expression and frequency of meat consumtions. (N=119, p=0.025, Pearson Correlation= -0.205) It showed that this expression decreased if meat is consumed often.

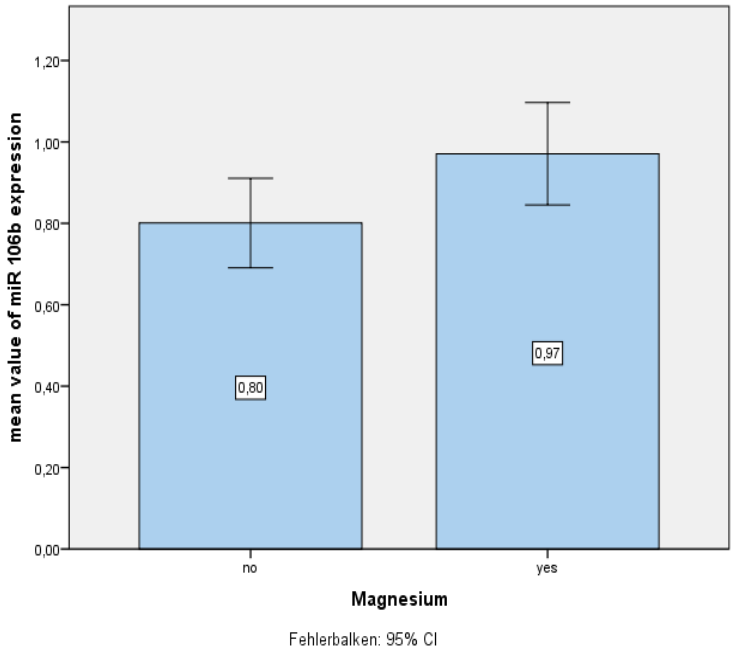

Figure 20. Magnesium intake and expression of miRNA 106b.

A mean value T-test showed a significant difference in expression of miRNA 106b with and without magnesium intake. Those taking magnesium daily revealed higher expression of miRNA 106b. Analysis with K-S test indicated that miRNA 106b had a normal distribution whereas magnesium was not normally distributed. Therefore student t-test and Mann-Whitney test were used of which both showed a significant result. (N for no= 56, N for yes= 40, Mann-Withney test p = 0.028)

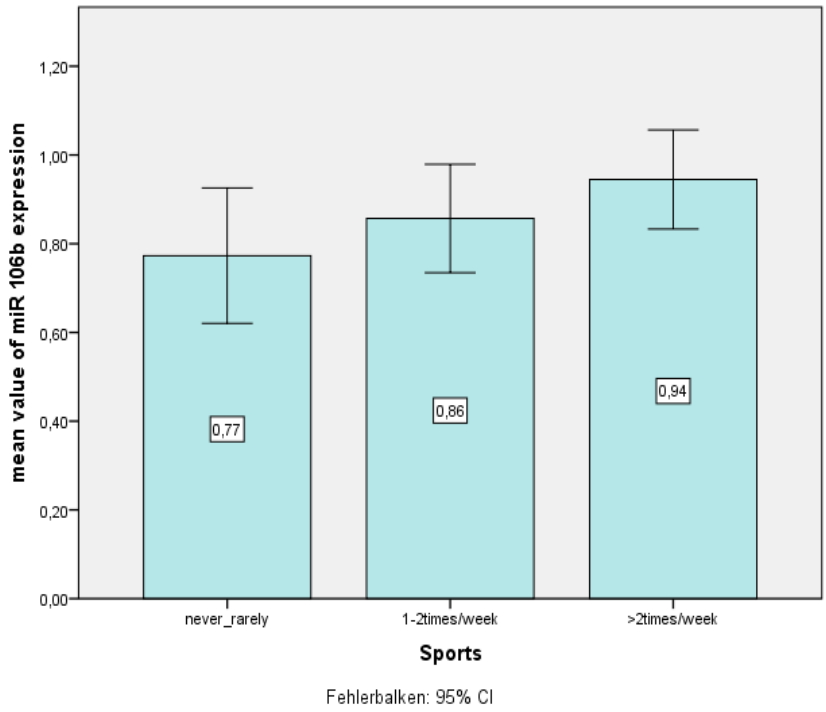

Figure 21. Sports and expression of miRNA 106b.

There was a noticeable trend between frequency of sport weekly and expression of miR-106b but when it was tested statistically it showed no significance. (N=119, p=0.081, Spearman's Correlation=0.160)

Also the sport groups were stratisfied into 2 groups one with subjects who never or rarely do sport compared with subjects who do sport more than twice a week. In this mean value t-test results a better significance than the previous examination with all 3 groups. (p=0.075, F=0.081, N for never/rarely=24 and N for over 2 times a week=50)

4.2.5. MiR- 151a

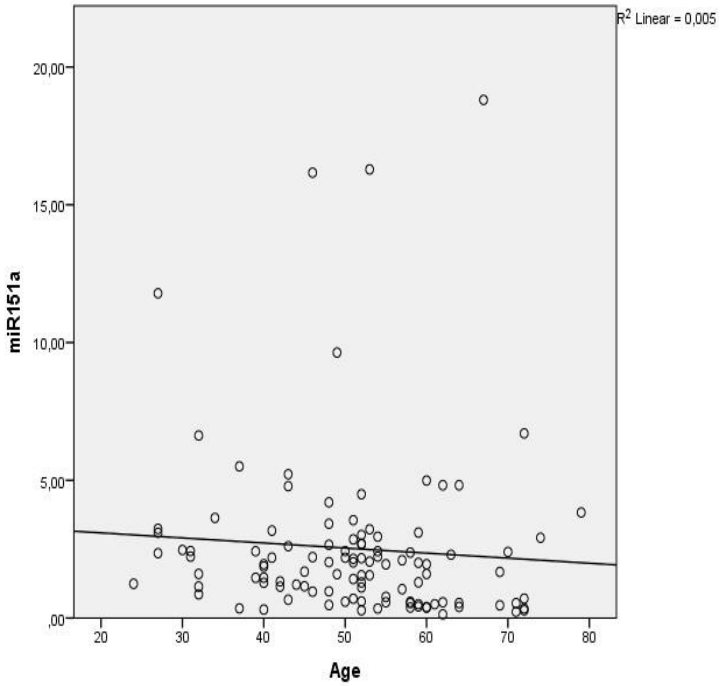

Figure 22. Expression of miRNA 151a and age.

Analysis with Spearman's Correlation showed a statistically significant association between age and expression of miR-151a. Increased age was associated with lower expression of miR-151a. (N=110, p=0.016, Correlation Coefficient=-0.229)

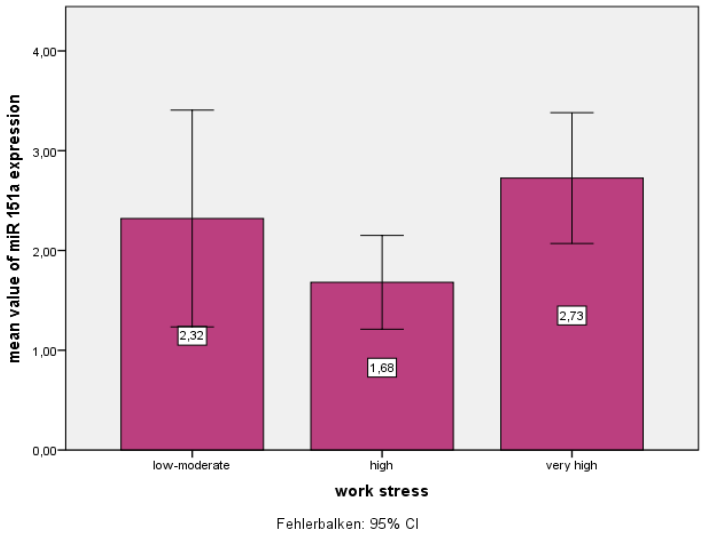

Figure 23. Expression of miRNA 151a and level work stress.

Here results from Spearman's correlations indicate a strong significant U-shaped association between expression of miR-151a and level of work stress in 3 groups low-moderate, high and very high. (N=103, p=0.005, Correlation Coefficient=0.274) The mean value t-test between the group with high and very high level of work stress showed a significance of p=0.013 (N for high work stress=31 and N for very high work stress=37, F=1.048)

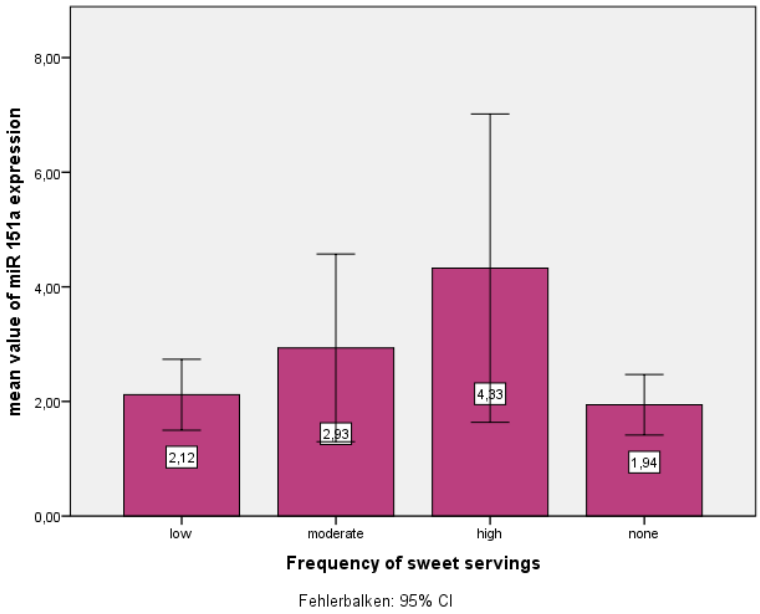

Figure 24. Expression of miRNA 151a and frequency of sweet servings.

Evaluation with Spearman's Correlation test between sweet servings and expression of miR-151a revealed a significant positive association. Meaning that as much sweets are consumed higher expression of this miRNA is expected. It has to be noted that also the group avoiding sweets have the lowest miRNA expression therefore this result showed a clear enhanced correlations from low to high consumption. (N=105, p=0.015, Correlation Coefficient=0.236)

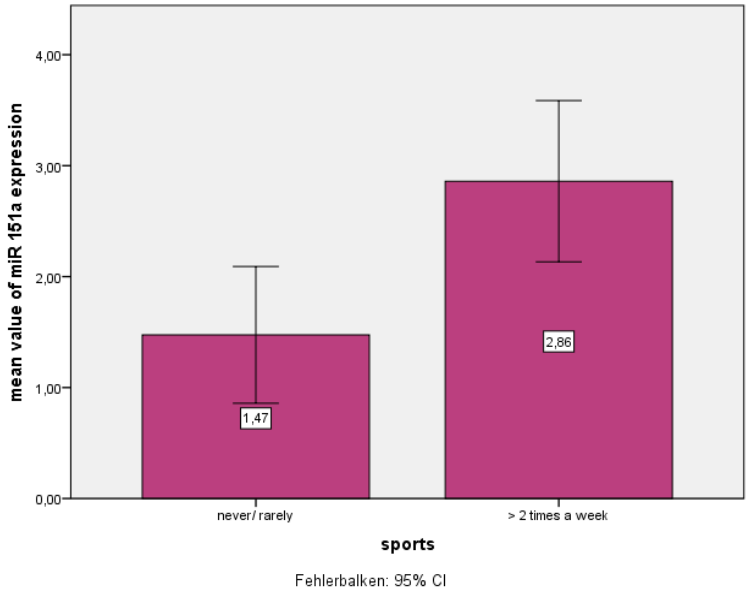

Figure 25 Expression of miRNA 151a and frequency of sports.

There is also a strong significance between frequency of sport and expression of miR-151a resulting a low expression in the group who never or rarely do sport and higher expression in the group who do over 2 times a week sport. The group who mentioned doing rarely or never sport counts for 25 subjects and the other group contains 84 subjects. Mann-Whitney-U test showed a significance of p=0.003.

4.2.6.MiR-155

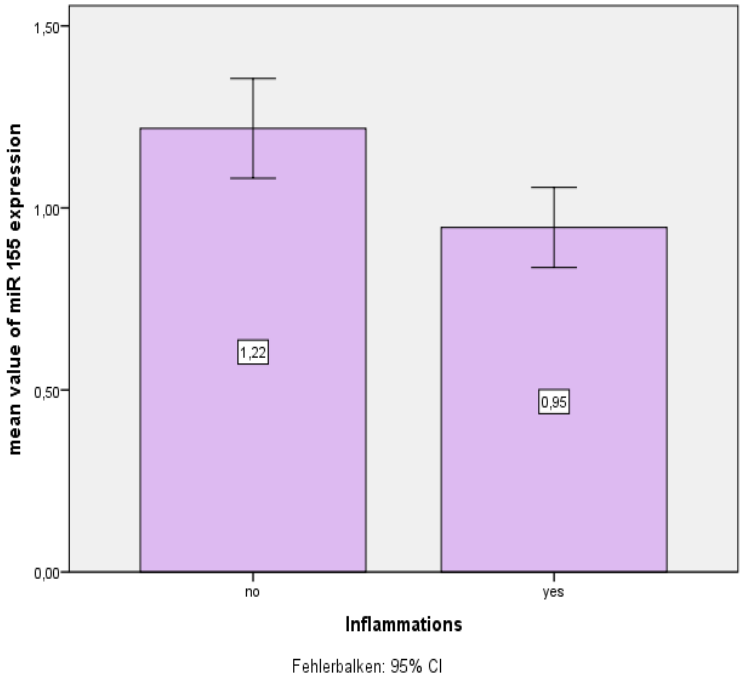

Figure 26. Association between inflammation and expression of miRNA 155.

The data are normally distributed therefore independent sample t-test is used to analyze the expression of miR-155 between subjects with and without inflammation. Standard deviations of both groups are almost similar and is significance is p=0.005. (N with inflammation= 35, N without inflammation= 52, t=2,884). The result indicates that subjects without inflammation had higher expression of miR-155 compared to the group with inflammation.

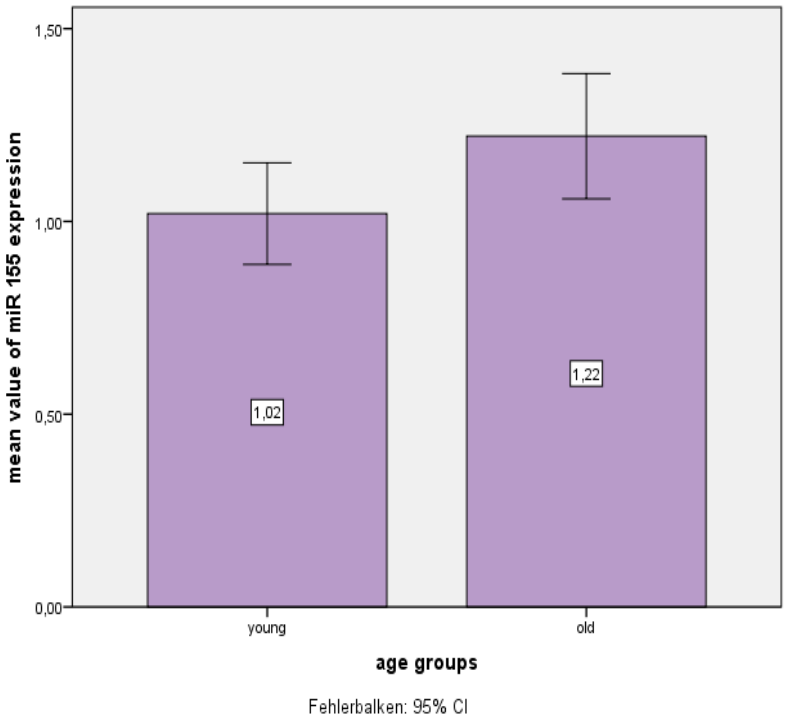

Figure 27. Association between age and expression of miRNA 155

Altough Spearman Correlation Test showed no significance, still results revealed by Idependent Sample T-Test that there is an almost statistically significant association in expression of miR-155 and age after it is grouped within old and young subjects. (N for old= 35, N for young= 35, p=0.005, t=-1,954) In old individuals resulted into higher expression compared to their younger counterpart.

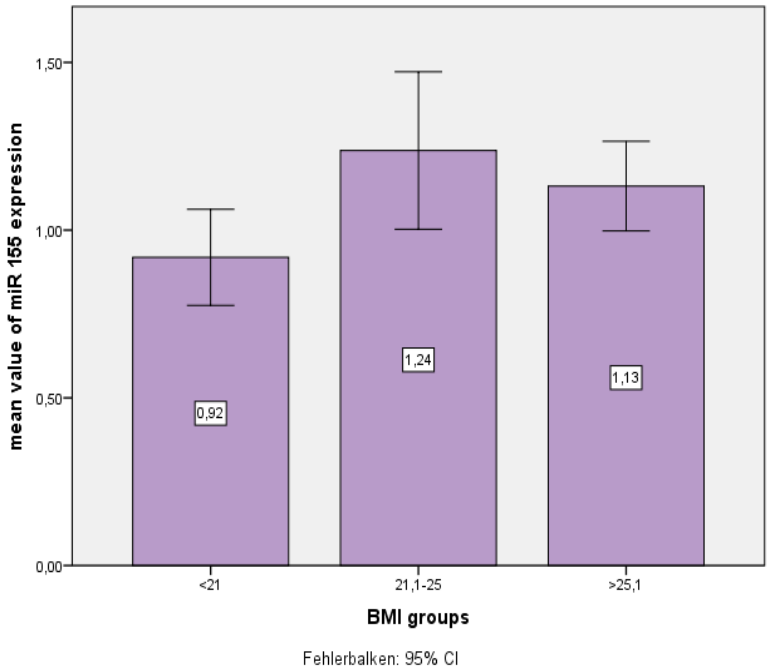

Figure 28. Expression of three BMI groups and expression level of miRNA 155

In case of BMI groups no significant correlation is exhibited by Spearman's Correlation Test. (N=70, p=0.177) Nevertheless other tests revealed significant association between BMI <21 and >25,1 showing that BMI > 25,1 had higher expression of miR-155 than the group with lower BMI. (N for BMI <21= 16, N for >25,1=29, p=0.042, t=-2,099) Also between BMI <21 and BMI 21,1-25 showed a significant result even though the standard deviations of both groups had greater distance. (St.dev. =0.2689 and 0.5685) (N for BMI 21,1-25= 25, p=0.43, t=-2.090)

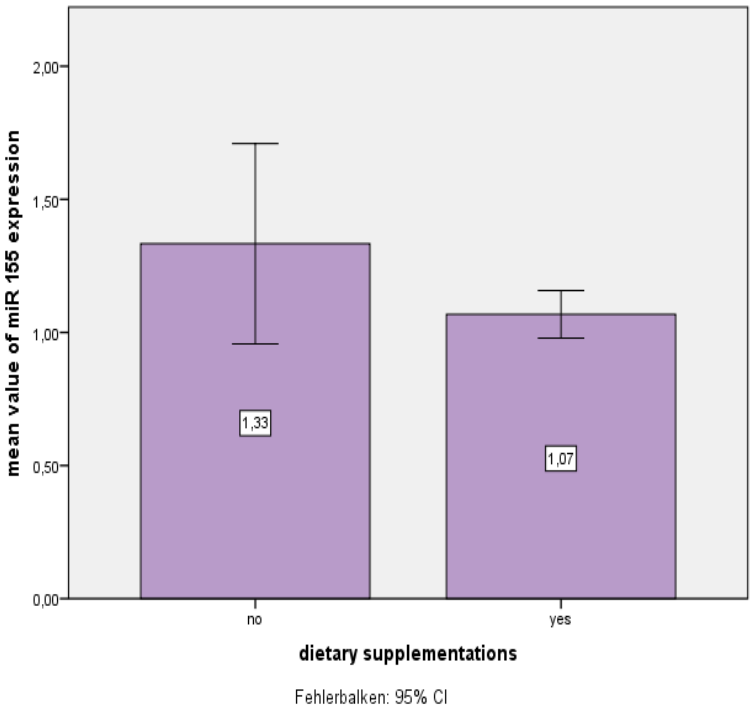

Figure 29. Association between dietary supplementations and expression of miRNA 155.

To analyze dietary supplementation intake, 81 subjects mentioned their status but only 16 individuals did not take any supplementations which were compared with 65 subjects that did. An independent sample t-test still provided a significant result indicating higher expression in individuals without dietary supplementation. (p=0.037 when standard deviations between both groups are similar). However in this case variance of standard deviations (Std. dev. without supplementation =0.70665 and Std. dev. with supplementation= 0.36069) are huge and it resulted in no significant association. (p=0.163)

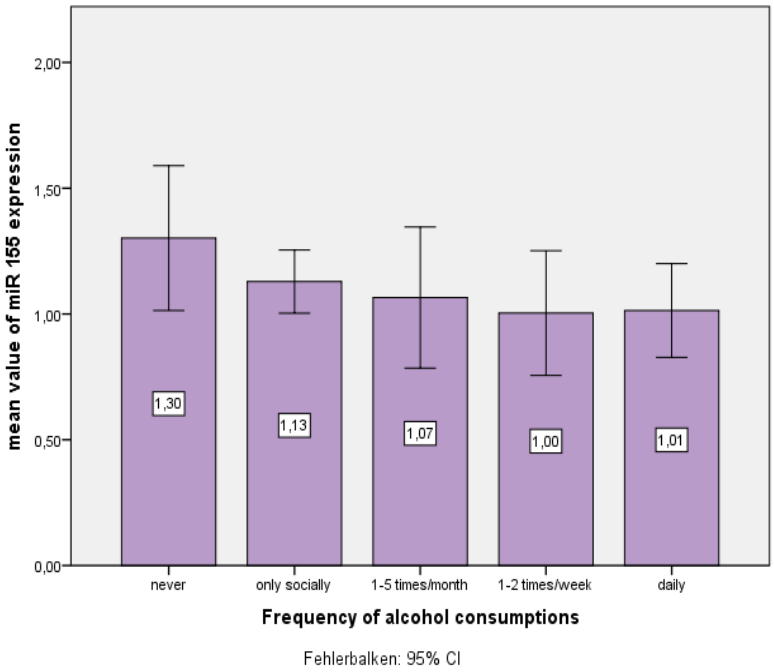

Figure 30. Correlation of alcohol consumptions and expression level of miRNA 155.

Spearman's Test showed a significant negative correlation between miR-155 expression and alcohol consumption which means that higher consumption of alcohol was associated with lower expression of miR-155. (b=-0.258, p=0.018, N=84)

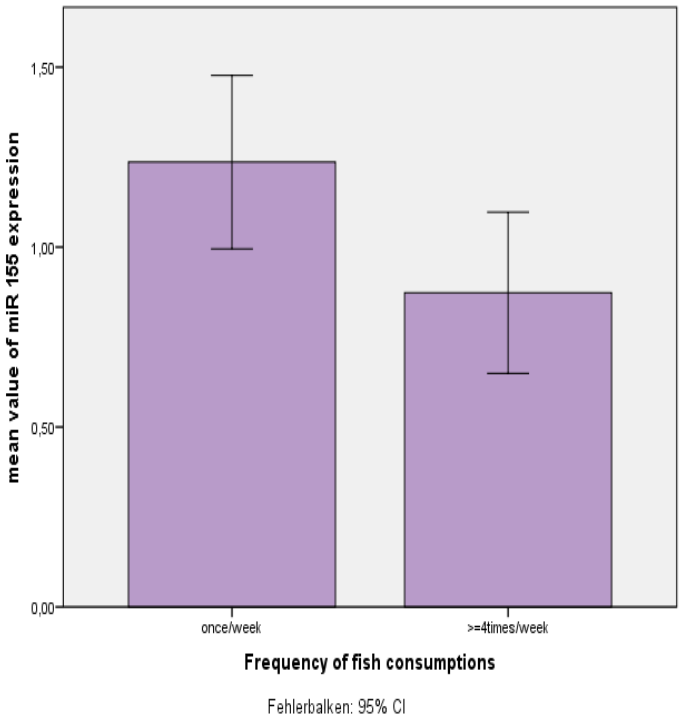

Figure 31. Association between frequency of fish consumptions and expression of miRNA 155.

It is noticeable that if fish is eaten more often lower expression of miR-155 is expected. It is examined with independent sample test with 36 subjects which showed a significant result. (N for the group with fish consumption of once a week= 23, N for fish consumption over 4 times a week=13, p=0.044,t=2.093)

4.3. Correlations between miRNAs

4.3.1. Significant positive correlations

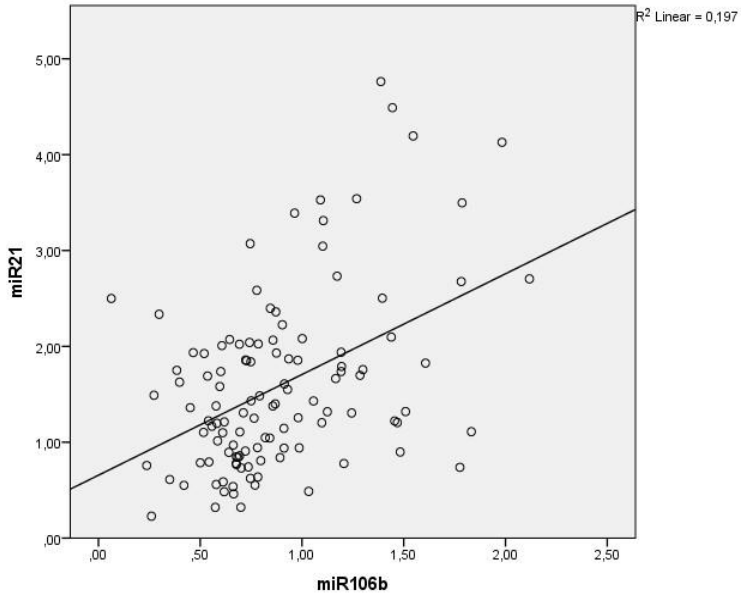

Figure 32. Correlation between expression levels of miRNA 106b and miRNA 21.

Data of both miRNAs 21 and 106b are normally distributed therefore Pearson Test was done and it demonstrated a significant and strong correlation between both variables. (N=155, p=0.000, Pearson Correlation=0.444, R^2=0.197)

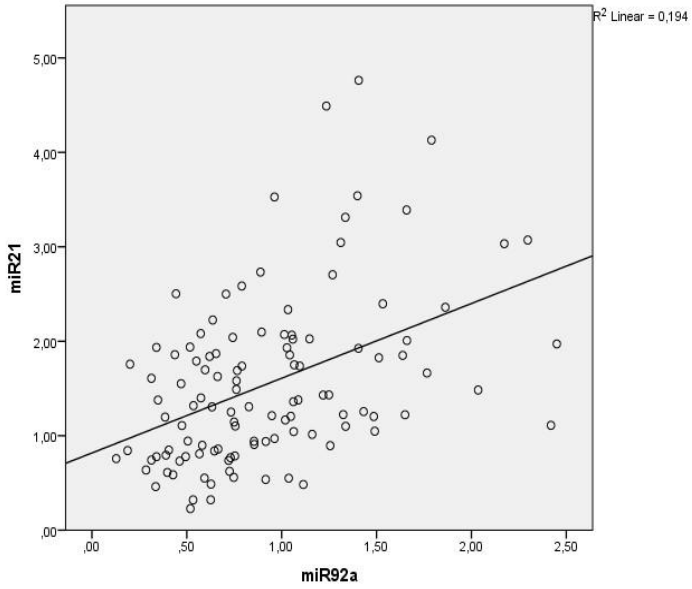

Figure 33. Correlation between expression levels of miRNA 92a and miRNA 21.

Here the results and the statistical test are very similar to the figure above. (N=113, Pearson Correlation=0.440, p=0.000, R^2=0.194)

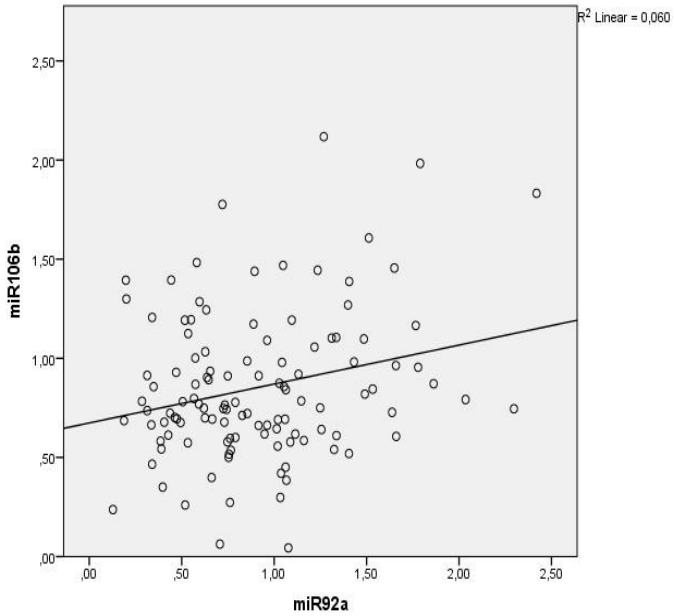

Figure 34. Correlation between expression levels of miRNA 92a and miRNA 106b.

The same goes for the correlation between miR- 92a and miR-106b. It appeared a strong positive correlation. (N=116, p=0.008, Pearson Correlation= 0.245, R^2= 0.060)

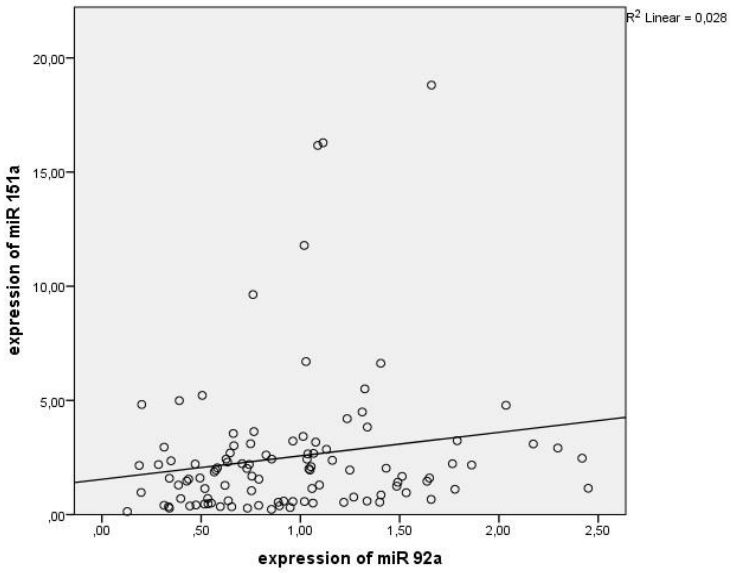

Figure 35. Correlation between expression levels of miRNA 92a and miRNA 151a.

Data for miR-151a are not normally distributed therefore Spearman Test was chosen for the statistical analysis. Between miR-92a and miR-151a a significant positive correlation was found but not as strong as the other three results indicated above. (N=105, p=0.014, b=0.240, R^2= 0.028)

4.3.2. Significant negative correlations

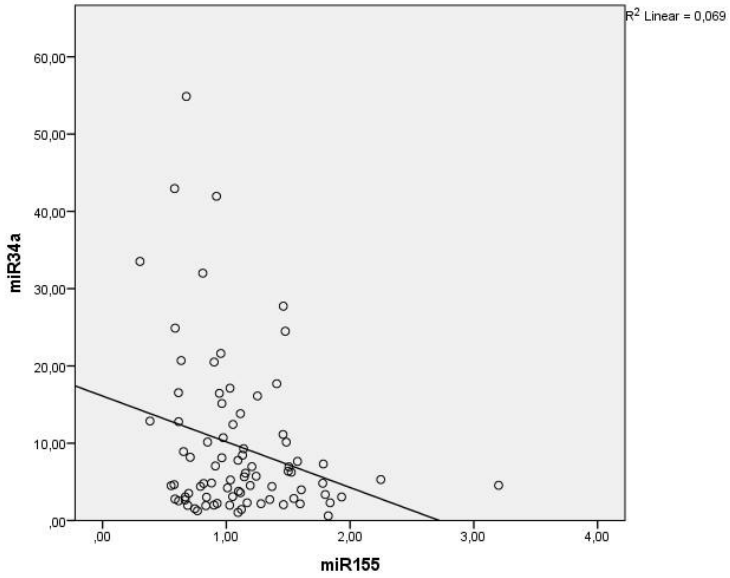

Figure 36. Correlation between expression levels of miRNA 34a and miRNA 155.

A significant negative correlation was found between the expression levels of miR-34a and miR-155. (N=82, b=-0.262, p=0.017, R^2= 0.069)

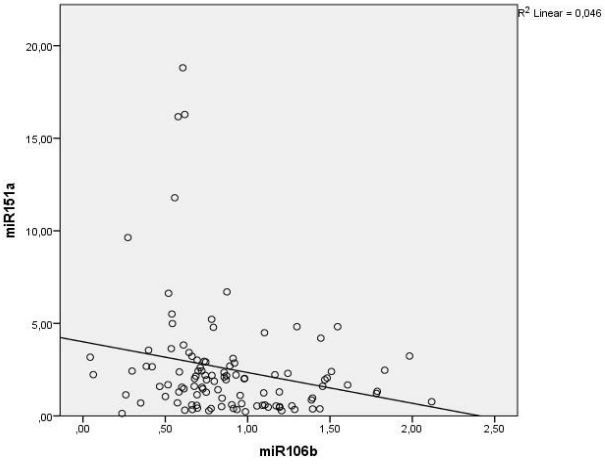

Figure 37. Correlation between expression levels of miRNA 151a and miRNA 106b.

The linear regression done with Spearman test revealed a very strong negative correlation between expression levels of miR-151a and miR-106b. (N=107, b=-0.257, p=0.007, R^2=0.046)

5. Discussion

5.1. MiR-21

5.1.1. Allergic intolerance

Our results regarding allergic intolerance showed that participants with such disease experience a significantly higher expression level of miR-21. The interaction between miR-21 and allergy have been a new investigative field these recent years therefore not many studies are done yet.

One study observed an overexpression of miR-21 in induced IL-13 transgenic mice compared to control mice. [Lu X. T. et al., 2009] This type of interleucine plays a role in allergic airway inflammation but noticeably this study did not mention any investigations with Ig-E which is an important contributor for allergies. Seemingly miR-21 regulates various immunological and developmental processes still some inconsitencies between previous studies exist. [Kumarswamy R. et. al., 2011] Even tough one current study on miR-21 and allergy demonstrated that indeed there is a positive association between expression of miR-21 and the diseases asthma and eosinophilic esophagitis but no relation between miR-21 and atopy thus associated with allergen-specific Ig-E could be found. [Sawant DV et al., 2015] Futhermore the given question about allergic reactions and intolerance is not precise enough to gether informations whether participants have allergy or intolerance only. In scientific research there are clear differences between the two pathological conditions. As this questionnaire were self-reported without recieving any accurate explainations to questions before filling them out thereby limitations can occure.

5.1.2. Inflammation

We also revealed similar results in expression levels of miR-21 concerning inflammation. It is evaluated that individuals with inflammations have a significantly higher expression of miR-21. According to other studies an overexpression of this miRNA is related to oxidative stress and inflammation which endorses our findings.

[Rüegger S and Großhans H, 2012] Moreover several studies support the observation of a positive correlation between serum levels of miR-21 and C-reactive proteins. [Bye A et al., 2013] Interestingly our examination had a small sample size conducted with 34 participants with inflammation and 80 without inflammation that was still enough to show a significant association.

5.1.3. Folic acid intake

In general as we expected from results above an overexpression of miR-21 is tought to be a pathological condition whereas its downregulation is equivalent to good health. In our case the examination of folat status with 94 participants of which 31 taking folat supplementation showed a lower expression and therefore such supplementation may have a positive effect on maintaining great health condition in both genders. On the other hand it can also have a negative impact depending on the current health condition of ther person shortly before taking folat supplementation.

A study by Beckett at al. investigated the correlation between miR-21 and folate status but with special emphasize on colorectal cancer. Serum miR-21 expression was related to occurrence of adenomatous polyps only in females. Additionally the stimulation of different CRC cell lines with excess folic acid increased expression of miR-21. It must be taken into account that dietary components, nutritional and genetic status may affect cancer progression by non coding RNA modulation but also need to be taken care on the timing of assessing the value of these molecules as possible biomarkers. [Beckett E L et al., 2015] A more similar investigation to our study discussed that folate status may alter the expression of miRNAs and furthermore change the severity of fatty liver disease. The adverse effect induced by folate-deficient diet in mice dysregulated MiR-34a. But no informations could be found in association with miR-21. [Lee J H et al., 2014]

5.1.4. BMI

In our investigation we could not find any significant association between miRNA and Body Mass Index. Statistical examination still showed an almost significant association

of miR- 21 levels between BMI < 21 and BMI 21-25. A couple of studies could provide insights in expression of miR-21 and obesity and support our result. Both in our case and in those studies obese individuals tend to have increased expression of miR-21, partly due to existing inflammations. Despite our findings showed higher expression at BMI >25 statistical methods showed no significance. Other study by Guglielmi V et. al., however, exhibited miR-21 expression that was two fold greater in adipose tissue in patients with T2D. They also did another experiment realizing that miR-21 expression increased after 24-h exposure to high glucose and insulin in primary cultures of adipocytes from non diabetic overweight subjects. [Guglielmi V et. al., 2017] In the same year more accurate pathway of molecular mechanisms is described revealing that miR-21 expression is up-regulated during adipogenic differentiation of ADSCs, which leads to silencing TGFβ receptor-2. The study concluded that miRNAs are able to inhibit adipogenesis. [Brandão B B et. al., 2017] Also it is suggested that unsaturated acids trigger steatosis by inducing NF-κB signaling and consistently activates miR-21 which directly suppress the expression of the phosphatase and tensin homolog gene (PTEN).[Lee J H et al., 2014] One explenation why our result didn't provide any significance is that the participants where mostly healthy and did not have any secondary pathological condition such as diabetes.

5.1.5. Nutrition

No associations could be found regarding the nutrition but an trend towards a correlation was observed with ready meal consumptions. High consumption of ready meals may lead to higher expression of miR-21. The sample size was small for evaluating 3 different consumptions groups of ready meals, "never", "rarely" and " twice a week".

5.1.6. Age

There are many studies on miRNAs and aging but especially for miR-21 results are inconsistent. It is recognized that it is more probable to find significant positive correlations when the young group is between approximately 20-65 years and the old

group is aged around 66-95 years. Studies which stratisfied age groups into young ~30 years and old ~64 years were not able to find any significant correlations. [Noren Hooten N et al., 2013] Likewise it is shown that our age groups had no significant associations because the range of age is not wide enough to reach a significance. Moreover, it is detected that big amount of miRNAs are found in chronic age-related diseases due to altered DNA damage response and inflammations. [Harries L W, 2014] This may explain that our old individuals seem not to develop inflammations yet or DNA damage leading to changes in expression of miR-21 is not expected.

5.2. MiR-34a

5.2.1. BMI

The expression of miR-34a was elevated in obesity indicating its potential role to inhibit the process of fat browning and weight loss. [Yang Z et al., 2016] It is also discovered that miR-34a which is involved in the hepatic cellular level has the ability to directly target and decrease SIRT1 expression. [Choi S E et al., 2013] Also in animal models such in obese and aging mice the level of miR-34a was overexpressed. [Rottiers V and Näär A M, 2012] Moreover, miR-34a, a regulator of apoptosis, was enourmously upregulated in the livers of mice receiving a methyl-deficient diet. A similar perturbance in expression of MiR-34a has been reported in NASH of humans. [Pogribny I P et al.,2010] This miRNA is a direct regulator of PPARγ and RXRα expression and also directly targets NAD-dependent deacetylase Sirtuin-1 which affects hepatic lipid metabolism. Sirt1 is a histone deacetylase that is capable to compact the chromatin structure. [Portius D et al., 2017] These findings could be confirmed in this study pointing out a significant association between miR-34a and BMI.

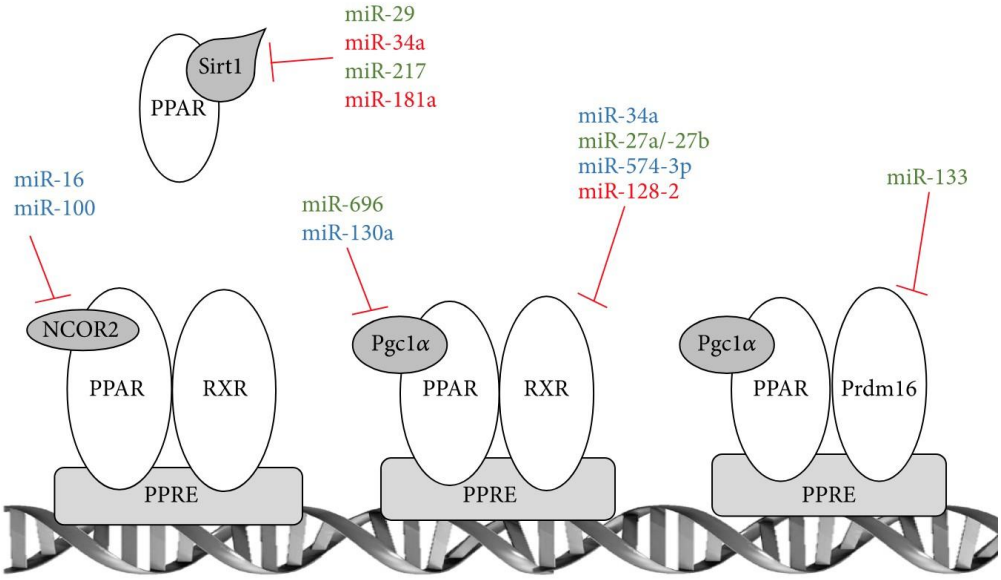

Figure 38. Interactions between PPARs and miRNAs [Portius D et al., 2017]

5.2.2. Physical Activity

Analysis of physical activity correlated with miR-34a indicated a significant negative correlation depicting downregulated expression in individuals who do sports 6-7 times a week. Still futher research is needed to clarify the present result figuring out where the expression of miR-34a stands after regular physical activity. So far no study could be found on this topic but only one illustrated that circulating inflammiR-34a was increased immediately after a marathon and returned to basal level after 24 hours. [Gonzalo-Calvo D et al., 2015] Futhermore studies on physical activity and miRNA differs depending on the type of sport like endurance or strength training as well as the dependence on regularity and intensity. Also the expression levels can change during and after the training thereby researchers took particular attention when the sampling is done. Even differences in gender specifity and age are observed. [Polakovičová M et. al., 2016]

5.2.3. Supplementations

As well as no studies exist on evaluating multivitamin supplementations and expression levels of miRNAs but there are some literatures on single supplementations and specific miRNAs. For instance, a study by Liu C M et al. featured an upregulation in zinc deficiency and after zinc intake contributed to a downregulation of different types of miRNAs. [Liu C M et al., 2017] Another recent study by Geoffroy et al. illustrated an increased level of miR-34a during a folat deficiency and with folic acid supplementation the expression was restored back to baseline which led to a reduction of structural and functional defects taking place during the perinatal period. [A Geoffroy et al., 2016] Similar statements revealed in an article by Tome-Carneiro et al. that in peripheral blood miRNAs could be used to monitor the beneficial effect of specific supplementations. They exhibited a lower expression of miR- 34a in type 2 diabetic patients receiving supplementation with resveratrol-containing grape extract for one year. [Tome-Carneiro et al. 2013]

Our results indicated similar miRNA expression patterns with multivitamin supplementation. Still further research should be done to understand whether only subjects with multivitamin deficiency had advantage on such supplementation or the expression is altered even in healthy individuals. In our case participants did not undergo any exact blood test on multivitamin status thereby their status was not known.

5.2.4. Coffee consumption

No study was conducted on coffee consumptions and expression of miRNAs hence no comparision can be done with our significant positive correlation. Drinking more than 3 cups of coffee a day resulted in significant higher expression of miR-34a compared to drinking up to 2 cups a day. Besides other studies did some investigation on alteration of miR-34a after treatment with some nutritional components. To date most of these studies were done in cell culture or animal models, more precisly on cancer cells or rat models. For example, high expression of miR-34a in pancreatic and non-small cell lung cancer cells could inhibit cell growth and induce apoptosis after treatment with Genistein, a compound found in some plants including soybean and coffee. [Xia J et al.,

2012] Hui C et al. stated that the flavonoid compound 3, 6-dihydroxyflavone leads to enhanced expression of miR-34a which mediated a reduction of MNU-induced breast carcinogenesis in rats. [Hui C et al., 2012] Upregulation of miR-34a was also observed after administration of difluorinated curcumin on colorectal cancer cells [Roy et al., 2012]. These studies would give insights for our study presenting higher expression of miR- 34a after oral administration of specific nutritional compounds. As it turned out this happens with healthy subjects and not only on pathological cells and animal models. Still further and more accurate research need to be done with different types of coffee to understand the effects by quality and quantity and the occuring molecular mechanism behind it.

5.2.5. Age

A previous study by Li et al. noticed an upregulation of miR- 34a in older individuals. [Li et al., 2011] In this study we, however, could not discover a significant correlation between age and miR-34a. But possibly a greater sample size could show some significance in age.

5.3. MiR-92a

5.3.1. BMI

Literature by Tarallo et al. indicated that higher BMI was associated with decreased miR-92a in both plasma and stool. [Tarallo et. al. 2014] The abudance of serum exosomal miR-92a was significantly lower in the high BAT group compared with the low BAT group. [Chen Y et al., 2016] Indeed, our results revealed that BMI was inversely correlated with miR-92a but didn’t show any significance. Above all its linear regression depicted only very slight negative correlation. The sample size of 118 participants would be sufficient to examine whether the results are significant or not.

5.4. MiR-106b

5.4.1. Age

Aging induced a downregulation of miR-106b through activation of the CDKN2A-ARF and p53 components of the DNA damage checkpoint. The members of the miR-106b cluster have been associated with cellular senescence [Harries L W, 2014] Also miRNA Base pointed out that in T cells of eldery is decreased by 40,4%. [miRbase.org] Contrary to this our results with blood samples showed an increase with age and this does not match with previous studies. But it is of importance to gather more literature in the future to find possible explanation for this occurance.

Another molecular mechanism of 106b is the ability to repress the expression of MICA and MICB functioning as stress-activated ligands recognized by receptor NKG2D. They are involved in the removal of tumor cells and invading virus particles. The exposure to heat shock, oxidative stress, viral infection and DNA damage induce upregulated MICA and MICB expression. Both proteins need to be kept on a low level by the repression to permit their rapid acute activation during times of emergencies. [Liang R et. al, 2009] In older individuals it is often documented having higher DNA damage and therefore such an acute activation to repress MICA and MICB with the expression of miR-106b would be meaningful.

5.4.2. Nutrition

So far no literatures about the direct connection between miR-106b and meat consumption are published. But publications on meat consumptions, miRNA and pathological conditions were available. Our present study found a negative association between miR- 106b and meat consumptions but most studies done on pathological conditions and inflammation indicated a higher expression of miR-106b. Still it depends on the type of the pathological conditions, for example MiR-106b and miR-92 are known for oncogenic properties in various malignancies which were highly expressed in gastric cancer tissue whereas in gastric mucosa with H. pylori infection were significantly decreased. [Matsushima K et. al., 2010] Due to different expressions

of miR-106b depending on the type of diseases no conclusion on our present study with miR- 106b can be done yet.
Researchers from the study by Humphreys KJ et al. did an interesting intervention study with small sample size following High Red Meat diet (300 g/day lean red meat) and an HRM.HAMSB diet (HRM with 40 g/day butyrylated high amylose maize starch) preceded by an entry diet and separated by a washout. Levels of oncogenic mature miR17–92 cluster miRNAs and miR-21 increased in the rectal mucosa with the HRM diet, whereas the HRM.HAMSB diet restored miR17– 92 miRNAs to baseline but not miR-21.[Humphreys KJ et al., 2014]

5.4.3. Supplementations

Interestingly, our results indicated increased expression of miR-106b in subjects with magnesium intake. A cross sectional study from 2009 examined serum and intramononuclear magnesium in patients with metabolic syndrome without diabetes and correlated them with cardiovascular risk factors. Hypomagnesemia (SMg < 1.7 mg/dL) and intracellular depletion were seen in 23.2% and 36.1% of the patients, respectively. SMg and MMg means were significantly lower in patients than in controls. Notably, inverse correlation was observed between SMg and MMg with BMI and only SMg with systolic blood pressure and waist circumference in women. Patients with insulin resistance, low HDL levels and with moderate and severe hepatic steatosis showed lower MgM means. Taken all together magnesium depletion in serum and mononuclear cells is common in obese people with metabolic syndrome. But studies on miRNA and magnesium are missing. [Lima Mde L et. al., 2009]

5.5. MiR-151a

5.5.1. Age

Our present findings on age and expression level of miR-151a demonstrated a significant negative correlation. It matches with previous results that stated a decreased expression in age-related phenotypes and age-related diseases. This miRNA regulate

inflammatory responses and may contribute to elevated proinflammatory mechanisms in aging. [Noren Hooten N et al., 2013]

5.5.2. Physical Activity

Individuals who specified doing physical activity more than twice a week indicated higher expression of miR-151a than those who never does sports. Other studies figured out that miR-151 may act as biomarker for exercise level while miR-151a can show an altered expression on caloric restriction. Apparently our results with miR-151a had a significant altered expression in active individuals and not only depending on calorie intake. [Rome S, 2015]

5.5.3. Nutrition

The bar diagram from statistical analysis depicted an explicit ascending expression levels of miR-151a when more sweets were consumed. To our knowledge this is the first study explored that the sweet consumptions altered certain miRNA patterns.

5.5.4. Stress

An u-shaped correlation was seen between expression of miR-151a and stress at work. This can be explained by this same phenomenon which previously was discovered by other studies in physical activity depending on its degree of difficulty. [Boccatonda A et al., 2016] In our case moderate stress may have physiological advantages whereas exceeding its limit individuals may experience higher expression similar to those without stress at work.

5.6. MiR-155

5.6.1. Inflammation

MiR-155 has pro- and anti-inflammatory properties. [Xiaoyi Li, 2016] Various investigations have exhibited that miR-155 has opposite effects depending on different cell types, pathological stages and animal models of AS. Some studies stated that elevated miR-155 relieves chronic inflammation by a negative feedback loop and inhibits the formation of atherosclerosis associated foam cell via the miR- 155–CARHSP1–TNF-α pathway. Its anti-inflammatory properties happens trough targeting MAP3K10 in macrophages. NF-κB upregulates the transcription of miR-155 along with other inflammatory responsive genes through a signaling cascade in macrophages.
The duration and timing of acute stress responses is an important factor to direct inflammation to continue developing or reverse it. [Leung A K L and Sharp P A, 2010] After exposure to LPS or TNF-α and activating JNK signaling pathway miR-155 expression is elevated and seem to take part in innate immunity. [Corral-Fernández N E et al., 2013] In our case participants with inflammation showed a lower expression of miR-155.

5.6.2. Age

The examination of our present study on age does not match with previous results that was expected to indicate lower expression in older individuals. [Noren Hooten N et. al., 2010] A low expression is also seen in another study in 45 year old male patients with type 2 diabetes. [Corral-Fernández N E et al., 2013] It is known that miR-155 is a multifuctional regulator and its series of complexicity need further research to obtain more knowledge regarding the reciprocal effects of miR-155 with other molecular substances.

5.6.3. BMI

The expression level in subjects with BMI 21,1-25 was significantly higher than the group with a BMI under 21 and similar significant results turned out for the third group presenting a BMI over 25,1. But overall between all three groups it was not significant.

One of the first in vivo studies made a comparison in effects of diet-induced obesity (DIO) vs. caloric restriction (CR) on colon carcinogenesis and investigated energy balance interventions and miRNA expression in a murine colon cancer model which showed an upregulation of miR-155. Diet-induced obesity altered several biological pathways and consequently increased the development of colon tumor while the calorie restriction had an opposite effect. In addition, other underlying molecular mechanisms in DIO mice were high levels of insulin-like growth factor-1, tumor necrosis factor and interleukin-6. Such cytokines are known to activate NF-kB 8, nuclear factor kappa-light-chain enhancer of activated B cells. [Gavrilas L I et. al., 2016] MiR-155 expression is induced in adipocytes and adipose tissues which are involved in inflammatory conditions of obesity in murine and human models and participate at the pro-inflammatory loop by targeting PPAR γ mRNA 3'UTR. The adipose tissue in obese subjects indicated an increased expression of miR-155 stimultanously an increased inflammatory state in adipocytes. In summary a gain and loss of function of miR-155 showed its effect on adipocyte function. [Karkeni E et al., 2016] Interestingly a study proved that adipose tissue gains the ability to expend excess energy which consequently improved the protection against obesity. The deletion of miR-155 in female mice prevents diet-induced obesity. The body weight between wild-type and miR-155 knockout mice didn't differ after feeding with control diet but after high fat diet miR-155 knockout mice had 56% less body weight and 74% less gonadal white adipose tissue compared to wild type mice. Furthermore miR-155 KO mice on HFD emitted 21% more heat than wild type mice indicating an enhanced WAT thermogenic potentional.

Some genes from brown and white adipose and from glucose metabolism are upregulated when miR-155 is eliminated and thereby inhibiting HFD-induced adipocyte hypertrophy and WAT inflammation. (Ucp1, Cidea, Pparg, Fabp4, Pnpla2, AdipoQ,

Fasn, Glut4, Irs1). [Gaudet AD et. al., 2016]

Contradicted results were detected in another work showing negative associations between expression of miR- 155 with several biochemical parameters including glucose, HbA1c, BMI and triglycerides. But they observed altered distributions of the miRNAs associated with metabolic control and body mass index only in the group of T2D patients. In our case mostly we investigated samples from healthy subjects only very few had diabetes. [Corral-Fernández N E et. al., 2013] An upregulation of miR-155 is found in livers of obese mice and was primarily detected in CD11b+ macrophage that were surrounded by inflammatory mediators like LPS and TNFα. Another critical mediatior is the diet-induced activation of NF-κB. The study by Miller et al. stated that miR-155 promoter of mouse and human has multiple binding sites for LXR/RXR heterodimers, which could have been directly induced by oxysterols from HFD. Although miR-155 is generally considered a pro-inflammatory miRNA in macrophages during chronic inflammatory diseases but as well the increased expression in obese liver macrophage has a protective negative regulatory feedback mechanism and slows down disease progression by preventing an excessive lipid accumulation in the liver. [Miller A M et. al., 2013]

So far many studies concluded that miRNAs are implemented in several aspects of cell metabolism including glycolysis and mitochandial TCA cycle which are pathways also known to take part in Warburg's effect. MiR-155 is contributed in glycolysis more precisly in hexokinase 2. [Wong CC et al., 2017]

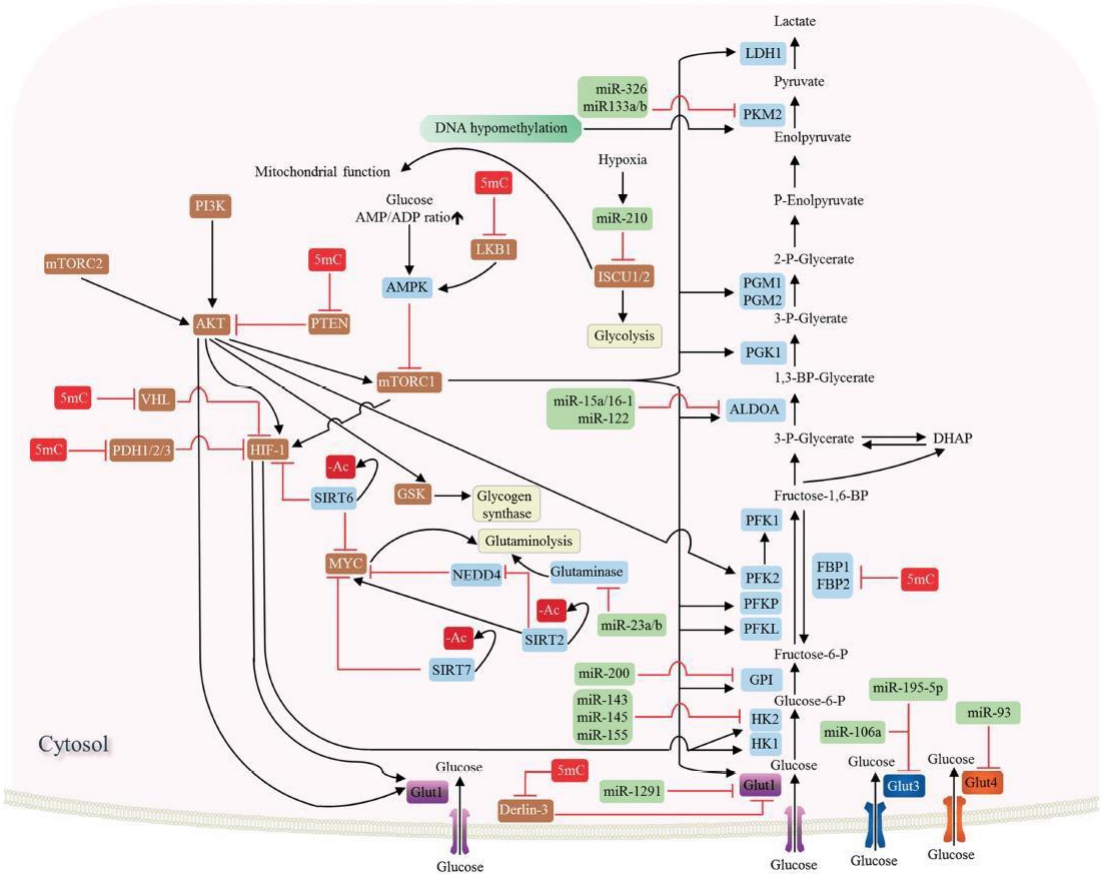

Figure 39. Expression of metabolic enzymes through DNA hypermethylation, histone deacetylation and microRNA in pathways of glycolysis and glutaminolysis. [Wong CC et al., 2017]

In mice given a methyl-deficient diet exhibited an increase in miR-155 and consequently a decrease in C/ebp-b a transcription factor located in the liver, and Socs1 proteins, a tumor suppressor, at the same time. Also Socs1 plays a crucial role in regulating cytokines negatively and mediating cellular oxidative stress in which methionine adenosyltransferase 1a and catalase are involved. They showed to be downregulated and regulated by C/ebp-b in NASH. [Pogribny J P et al., 2010] An overexpression of miR-155 is also presented in diet-induced NASH models through NF-κB signaling. [Lee J H et al., 2014]

5.6.4. Dietary supplementations

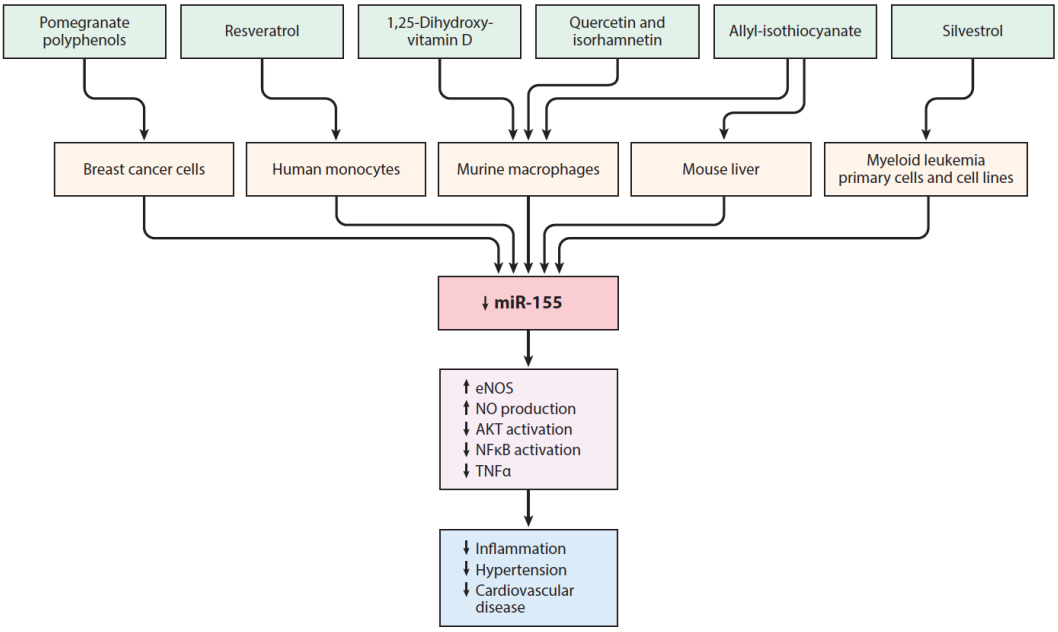

Figure 40. Different dietary supplementations may alter the expression of miRNA 155. [Ross S A and Davis C D, 2014]

So this miRNA is able to modulate the expression of genes correlated with inflammation in various cell types in vitro and atherogenesis in vivo. However, many different dietary components have been shown to be able to mediate the level of miR-155 expression. For example in hypertensive patients with coronary artery disease a supplementation with a grape extract containing resveratrol for 12 months downregulated miR-155 through regulation of NF-κB and TLR signaling as well as the expression of TNFα and IL1β. In summary, several types of experiments indicated the ability of many different dietary components to downregulate miR-155 expression in breast cancer cells, human monocytes, murine macrophages, mouse liver, myeloid leukemia cells among others. This consequently leads to reduced inflammation, hypertension and cardiovascular disease. [Ross S A and Davis C D, 2014] In our questionnaire participants were asked if they take any kind of dietary supplementations regardless of its content.

5.6.5. Fish consumption

A current study has examined the effects of fish oil on the expression of hepatic miRNAs in male rats that were fed with a lab chow, high-fat high-cholesterol diet and WD supplemented with fish oil.

A total of 79 miRNAs were identified as differentially expressed miRNAs of which miR-155 did not appear in their list. The most differently expressed between FOH and WD groups were rno-miR-29c-3p, rno-miR-30d-5p, rno-miR-33-5p, rno-miR-34a, and rno-miR-328a-3p. It has also been reported that elevated expression of miR-34a due to high fat and high caloric diet reduced the expression of sirtuin 1 in the liver of obese mice. Fish oil feeding diminished the high expressed rnomiR- 33-5p and rno-miR-34a-5p in Western style diet-induced NAFLD in rats indicating the protective effects of fish oil on hepatic triglyceride and cholesterol metabolic disorder. Notably other studies mentioned that certain miRNAs take part in the metabolic disorders especially in NAFLD such as miR-122, miR-33a/b and miR-34a and their circulating fragments are considered as biomarkers of this disease. [Wang H et al., 2017] A clinical study examined the circulating miRNAs changes of 192 common miRNAs in healthy women before and after an 8-week high PUFA diet intake and identified miR-106a, miR-130b and miR-221 which were correlated with diet-induced changes. [Ortega FJ et al., 2015]

5.6.6. Alcohol

MiR-155 knockout mice also maintained a protection from alcohol-induced steatosis and inflammation by reducing alcohol-induced fat accumulation. The molecular regulators in these states are increased PPRE and PPARa binding and decreased MCP1 production. Treatment with a miR-155 inhibitor increased PPAR expression in naive and alcohol treated RAW macrophages. So a deficiency in miR-155 attenuates chronic alcohol-induced steatosis, liver injury and oxidative stress in the liver. [Bala S et al., 2016] Our findings indicated a negative correlation between miR-155 and alcohol consumption presenting a lower expression in daily consumption of alcohol. As nutritional sience experts discussed alcohol having a positive physiological impact when drinking 30g for men and 20g for women daily. Furthermore a chronic alcohol

treatment in vitro resulted in a time-dependent increase of miR-155 in macrophages. They also found a linear correlation between alcohol-induced increase in miR-155 and TNFalpha induction in a mouse model with alcohol liver disease, more precisely in Kupffer cells. [Bala S et al., 2010] Lipopolysaccharide a component of the cell wall of gram-negative bacteria is responsible for TNF-a release by Kupffer cells. One of the madiatiors for LPS signaling in Kupffer cells, hepatocytes and hepatic stellate cells are miRNA. After exposure to ethanol an overexpression of LPS signaling and upregulation of miR-155 and miR-21 were indicated. Many studies identified that miR- 155 regulates inflammatory agent secretion in the liver. [McDaniel K et al., 2014] A reason why our data does not match with previous studies might be that subjects have not developed fatty liver or steatosis yet to show an overexpression of miRNA-155 by ethanol exposure or long-term fatty diet. Therefore a physiological protection against excessive alcohol consumption was not necessary. Also it warrants further investigation with human samples on alocohol consumptions to recieve a more secure results and observe ethanol exposure on humans.

6. Conclusion

Evaluating epigenetic mechanisms of miRNAs is a promising techniuqe to obtain accurate biomarkers of specific disease such as metabolic syndrome and eventually showing ways for better treatment. Many studies repeated the importance of several miRNAs for diagnosis, prevention and treatment but still it needs deeper investigations to understand the broad range of miRNA network. Most of the miRNAs showed multifactoral functions therefore it can influence many different molecular pathways which may exhibit an amelioration on one disease but at the same time induce a negative impact on other physiological function. In addition, many lifestyle factors such as nutrition, stress, age and weight are able to modulate the levels of miRNAs which can make the investigation even more challenging. In our present study with miRNA we attempt to obtaindeeper insights in such modulations by various lifestyle factors with special emphasize on inflammation and metabolic syndrome. Finally it is aknowledge that epigenetic mechanisms including the miRNA machinary gain attention as biomarker of diseases which are often choosen specifically to be relied on certain physiological and pathophyioslogical conditions. Of great advantage is that these small molecules are able to be detected with basic methods of molecularbiologoy. Generally the expression of a single miRNA can vary in each sample materials therefore it is suggestet to compare miRNAs within onesample materials.

Interestingly, miR-34a, miR-155 and miR-21 are involved in energy homeostasis and regulate lipid metabolism orginated from the liver via common genes and protein targets such as PPAR α, PPAR γ, RXR α, and SIRT1. It is often described that miR-21 has great impact on various inflammations and is involved in PTEN as well while miR-34a and miR-155 regulated particular pathways in PPAR, SIRT1, glycolysis and proteins such as C/ebp-b and Socs1. These three miRNAs seem to be involved in the molecular pathway of NF-κB and may target similar yet distinct signaling pathways important for the weight control. Despite many studies on synergistic interactions between DNA methylation and miRNA as epigenetic regulators on transcriptomic changes which may result in specific clinical outcomes are still unexplored.

Bioinformatics on miRNA interactions would be an additional useful tool to have a more clear explanation of the complexity of epigenetic regulation and solve its unanswered questions.

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8. Appendix

8.1. Lifestyle Questionnaire of Health Bio Care

Vorname / First Name

Nachname / Surname

Geburtsdatum / Date of birth

Geschlecht / Gender

Gewicht/ Weight ○ kg ○ lbs

Grösse / Height ○ cm ○ ft

Strasse / Hausnummer / Street / House No.

PLZ / Ort / Postal code / City

Telefon / Phone

Email / Email

Datum / Date

Ethnie / Ethnicity

- ○ Afrikanisch (Subsahara) oder Afro-amerikanisch / African (sub-saharan) or Afro-american
- ○ Kaukasisch ("weiß") / Caucasian ("white")
- ○ Nordasiatisch / Northern Asian
- ○ Nordostasiatisch und Arktisch / Notheast Asian and Arctic
- ○ Südasiatisch (Inkl. Südostasiatische Inseln) / Southern Asian (Incl. insular southeast Asia)
- ○ Pazifische Inseln / Pacific Islands
- ○ Neu Guinea und Australische Aboriginals / New Guinean and Australian Aboriginals
- ○ Indigene Amerikaner / Indigenous North- or South-American

2

1.a Leiden Sie oft an Entzündungen und/ oder Infektionen? Do you often suffer from inflammation and/or infections?

- ○ Ja / Yes
- ○ Nein / No
- ○ Weiß nicht / I don't know

1.b Wenn ja, welche Art von Entzündungen und wo treten diese auf? If yes, what type of inflammation and where do they occur?

- ☐ Grippale Infekte / Flu virus
- ☐ Entzündungen der Haut und oder Schleimhaut / Inflammation of the skin and or mucus
- ☐ Entzündliche Erkrankungen im Magen-/Darmbereich / Inflammatory diseases in the gastrointestinal region
- ☐ Muskel- oder Gelenksentzündungen / Muscle- or joint inflammation
- ☐ Blasenentzündung / Cystitis
- ☐ Pilzinfektionen im Genitalbereich / Fungal infections in the genital area
- ☐ Pilzinfektionen von Haut oder Füssen / Fungal infections of the skin or feet
- ☐ Sonstiges / Other

2. Was sind Ihre vordringlichsten Probleme, weshalb Sie Ihren Arzt/Therapeuten konsultieren? Welche Symptome traten auf? What are the main issues which cause you to consult a doctor or therapist?

3. Nehmen Sie derzeit Medikamente zu sich? Are you currently taking medication?

- ○ Nein / No
- ○ Ja, folgende / Yes, the following

3

4. Rauchen Sie oder haben Sie mal geraucht? Do you smoke or have you smoked in the past?

- ○ Nein / No
- ○ Früher (vor weniger als 10 Jahren) / In the past (less than 10 years ago)
- ○ Früher (vor mehr als 10 Jahren) / In the past (more than 10 years ago)
- ○ Zur Zeit weniger als eine Schachtel am Tag / Currently less than 1 pack per day
- ○ Zur Zeit mehr als eine Schachtel am Tag / Currently more than 1 pack per day

5. Halten Sie eine bestimmte Ernährungsweise oder Diät ein (z.B.: vegetarisch, low-carb)? Do you adhere to a specific long-term diet? (e.g. vegetarian, low-carb)?

6. An wie vielen Tagen in der Woche essen Sie sehr deftige Speisen oder Fertiggerichte? How many days a week do you eat processed foods or ready made meals?

- ○ Jeden Tag in der Woche / Every day
- ○ Mehrmals die Woche / Several times a week
- ○ Selten / Rarely
- ○ Nie / Never

7.a An wie vielen Tagen in der Woche essen Sie Gemüse und Obst? How many days a week do you eat vegetable and/or fruit?

- ○ Hin und wieder / Occasionally
- ○ Jeden zweiten Tag / Every second day
- ○ Jeden Tag / Every day
- ○ Mehrmals am Tag / Several times a day
- ○ Fünf Mal am Tag / Five times a day

7.b Welche Obst und Gemüsesorten essen Sie am häufigsten? Which fruit and vegetable do you eat the most?

7.c In welcher Form konsumieren Sie Ihr Gemüse hauptsächlich? In what form do you mainly consume your vegetable?
Mehrfachnennungen möglich / Multiple answers possible

- ☐ Roh / Raw
- ☐ Gekocht / Boiled in water
- ☐ Gebraten / Fried
- ☐ Gegart / Steamed
- ☐ Gedünstet / Stewed
- ☐ Frittiert / Deep-fried

8.a Vertragen Sie Milch und Milchprodukte? Do you tolerate milk and dairy products?

○ Nein / No ○ Ja / Yes ○ Weiß nicht / I don't know

8.b Wenn ja, an wie vielen Tagen in der Woche konsumieren Sie Milch und Milchprodukte? If yes, how many days per week do you consume milk or dairy products?

- ○ Nie / Never
- ○ Gelegentlich bis jeden zweiten Tag / Occasionally, up to every other day
- ○ Jeden Tag / Every day
- ○ Mehrmals am Tag / Several times per day

9. Wie oft in der Woche essen Sie Fisch? How many times a week do you eat fish?

- ○ Selten bis nie / Rarely, almost never
- ○ Einmal pro Woche / Once per week
- ○ 2-3 Mal pro Woche / 2-3 times per week
- ○ Vier und mehr Mal die Woche / Four times and more per week

10. Wie oft in der Woche essen Sie Fleisch und Wurst? How many times a week do you eat meat and processed meat products?

- ○ Selten bis nie / Rarely, almost never
- ○ 1-6 Mal pro Woche / 1-6 times per week
- ○ Täglich / Daily
- ○ Mehrmals täglich / Several times per day

11.a Vertragen Sie Weizen bzw. Gluten? Do you tolerate wheat and gluten?

○ Nein / No ○ Ja / Yes ○ Weiß nicht / I don't know

11.b Wenn ja, Wie oft in der Woche essen Sie Vollkornprodukte? If yes, how many times a week do you eat whole grain products?

- ○ Selten bis nie / Rarely, almost never
- ○ 1-6 Mal pro Woche / 1-6 times per week
- ○ Täglich / Daily
- ○ Mehrmals täglich / Several times per day

12. Nehmen Sie Nahrungsergänzungsmittel zu sich? Wenn Ja, welche Nahrungsergänzungsmittel haben Sie im letzten halben Jahr eingenommen? Do you take dietary supplements? If yes, what kind did you take in the last six months?

- ☐ Nein / No
- ☐ Vitamin D / Vitamin D
- ☐ L-Carnitin / L-Carnitin
- ☐ Knoblauchpräparate / Garlic powder
- ☐ Vitamin C / Vitamin C
- ☐ Kalzium / Calcium
- ☐ Ballaststoffe / Dietary fibre
- ☐ Biotin / Vitamin B7
- ☐ Folsäure / Folic acid
- ☐ Multivitamin / Multi-Vitamins
- ☐ Magnesium / Magnesium
- ☐ Cobalamin / Cobalamin
- ☐ Probiotische Kapseln / Probiotic capsules
- ☐ Sonstiges / Other _____

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

- ○ Selten bis nie / Rarely, almost never
- ○ Einmal pro Woche / Once per week
- ○ 2-5 Mal pro Woche / 2-5 times per week
- ○ 6-7 Mal die Woche / 6-7 times per week

13.b Wie oft in der Woche machen Sie Sport? (Bewegung mit Schwitzen über 30min...) How many times a week do you exercise intensively for more than 30 min.?

- ○ Selten bis nie (max. 2 Mal pro Monat) Rarely, almost never (max. twice a month)
- ○ 1-2 Mal pro Woche / 1-2 times per week
- ○ Mind. 2 Mal pro Woche / Minimum twice per week

14.a Wie viel Flüssigkeit nehmen Sie täglich zu sich? How much liquid do you drink per day?

- ○ Weniger als 1 Liter / Less than 1 liter
- ○ 1-2 Liter / 1-2 liters
- ○ 2-3 Liter / 2-3 liters
- ○ Mehr als 3 Liter / More than 3 liters

14.b Wie viele Tassen Kaffee trinken Sie täglich? How much coffee do you drink per day?

- ○ Weniger als 1 Tasse / Less than 1 cup
- ○ 1-2 Tassen / 1-2 cups
- ○ 3-5 Tassen / 3-5 cups
- ○ Mehr als 5 Tassen / More than 5 cups

14.c Wie oft trinken Sie Alkohol? How often do you drink alcohol?

- ○ Nie / Never
- ○ Nur zu Anlässen / Only socially
- ○ 1-2 mal im Monat / 1-2 times per month
- ○ 3-5 mal im Monat / 3-5 times per month
- ○ 1-2 mal die Woche / 1-2 times per week
- ○ Täglich / Daily
- ○ Mehrmals am Tag / Several times a day

14.d Welche Getränke nehmen Sie hauptsächlich zu sich? What kind of beverages do you mainly drink?
Mehrfachnennungen möglich / Multiple answers possible

- ☐ Energiegetränke / Energy drinks
- ☐ Tee (schwarz, grün,..) / Tea (black, green, ...)
- ☐ Tee gesüßt mit Zucker / Tea with sugar
- ☐ Tee (Kräuter) / Herbal Tea
- ☐ Tee (Kräuter) mit Zucker / Herbal tea with sugar
- ☐ Wasser / Water
- ☐ Limonade / Soft drinks
- ☐ Kaffee / Coffee
- ☐ Cola / Coke
- ☐ Kaffee gesüßt mit Zucker / Coffee with sugar
- ☐ Dicksaft / Syrup
- ☐ Nektar / Nectar
- ☐ Saft / Juice
- ☐ Verdünnte Säfte / Diluted juices
- ☐ Sonstige / Other _____

15. Wie viele Portionen der angegebenen Süßigkeiten/Süßspeisen konsumieren Sie wöchentlich? How many servings of the sweets/desserts indicated do you consume per week?

Eine Portion entspricht ungefähr 4 Stück Schokolade, 200g Pudding oder 1 Stück Kuchen.
A serving is about 4 pieces of chocolate , 200g pudding or 1 piece of cake.

	< 1	1-3	3-5	5-10	10-15	> 15
Schokolade / Pralinen / Chocolate	○	○	○	○	○	○
Kuchen / Gebäck / Cakes and pastries	○	○	○	○	○	○
Kekse / Biscuits	○	○	○	○	○	○
Gummibären / Gummisachen / Gummi bears / Jellybabies	○	○	○	○	○	○
Bonbon / Zuckerl / Sweets	○	○	○	○	○	○
Eis / Ice cream	○	○	○	○	○	○
Puddings / Cremes / Puddings / Cream puddings	○	○	○	○	○	○

Sonstiges / Other _____

16. Wann nehmen Sie meistens Ihre Hauptmahlzeiten zu sich? When is your main meal?

Mehrfachnennungen möglich / Multiple answers possible

- ☐ Frühstück / Breakfast
- ☐ Mittag / Lunch
- ☐ Abend / Dinner
- ☐ Irgendwann / Every so often

17.a Gab es im letzten Jahr einen oder mehrere Stoffe, auf die Sie allergisch oder mit Unverträglichkeiten reagiert haben? Were there one or more substances to which you had an allergic reaction or intolerance in the last year?

- ○ Ja / Yes
- ○ Nein / No
- ○ Weiß nicht / I don't know

17.b Wenn ja, auf welche Substanzen reagierten Sie allergisch? Auch nicht vom Arzt überprüfte Substanzen
If yes, which substances are you allergic to? includes also substances not checked by a doctor

Folgende: / The following:

18. Kennen Sie Ihre Cholesterinwerte? Do you know your cholesterol level?

- ○ Nein / No
- ○ Ja, folgende / Yes, the following

HDL		mg/dl
LDL		mg/dl
Gesamt Cholesterin / Total cholesterol level		mg/dl

19. Haben Sie eine der folgenden Stoffwechselkrankheiten?
Do you have one of the following metabolic diseases?

- ☐ Diabetes mellitus Typ 1 / Diabetes mellitus Type 1
- ☐ Diabetes mellitus Typ 2 / Diabetes mellitus Type 2
- ☐ Schilddrüsendysfunktion / Thyroid dysfunction
- ☐ Pankreasleiden / Disease of the pancreas
- ☐ Gicht / Gout
- ☐ Nein / No

Gibt es diesbezügliche Befunde?
Are there any referring medical findings?

20. Wie hoch würden Sie Ihre derzeitige Stressbelastung einschätzen?
How high would you rate your current stress level?

- ○ Gering / Low
- ○ Mäßig / Moderate
- ○ Hoch / High
- ○ Sehr hoch / Very high

21. Versuchen Sie den ursächlichen Anteil bei der Entstehung Ihres Stressproblems in Prozent zu schätzen. z.B. Arbeit 45%, Freizeit 20%, Familie 35%
Try to estimate the cause of your stress level in percent. e.g. Work 45% , leisure 20% , family 35%

Freizeit / Leisure		%
Familie / Partner / Family / Partner		%
Arbeit / Work		%

Abschicken / Send

Drucken / Print

Formular Zurücksetzen / Reset Form

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8.2. TaqMan® Fast Advanced miRNA Assays

TaqMan® Advanced miRNA Assays

USER GUIDE

Single-tube assays

for use with:
TaqMan® Advanced miRNA cDNA Synthesis Kit

Catalog Number A25576
Publication Number 100027897
Revision C

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Perform the poly(A) tailing reaction

1. Thaw samples and cDNA synthesis reagents on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.

   Note: Keep the assays in storage until ready for use.

   IMPORTANT! The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (next section).

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

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
10X Poly(A) Buffer	0.5 µL	2.2 µL	5.5 µL
ATP	0.5 µL	2.2 µL	5.5 µL
Poly(A) Enzyme	0.3 µL	1.3 µL	3.3 µL
RNase-free water	1.7 µL	7.5 µL	18.7 µL
Total Poly(A) Reaction Mix volume	**3.0 µL**	**13.2 µL**	**33 µL**

[1] Volumes include 10% overage.

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

4. Add 2 µL of sample to each well of a reaction plate or each reaction tube, then transfer 3 µL of Poly(A) Reaction Mix to each well or tube.

   Note: (*Optional*) Before adding the sample to the reaction plate or tube, add RNase Inhibitor Protein to each sample to minimize the effects of RNase contamination. For detailed instructions, see the documentation provided by the RNase Inhibitor Protein manufacturer.

   The total volume should be 5 µL per well or tube.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

6. Centrifuge the reaction plate or tubes briefly to spin down the contents and eliminate air bubbles.

7. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

Step	Temperature	Time
Polyadenylation	37°C	45 minutes
Stop reaction	65°C	10 minutes
Hold	4°C	Hold

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

Perform the adaptor ligation reaction

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

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X DNA Ligase Buffer	3 µL	13.2 µL	33 µL
50% PEG 8000[2]	4.5 µL	19.8 µL	49.5 µL
25X Ligation Adaptor	0.6 µL	2.6 µL	6.6 µL
RNA Ligase	1.5 µL	6.6 µL	16.5 µL
RNase-free water	0.4 µL	1.8 µL	4.4 µL
Total Ligation Reaction Mix volume	**10 µL**	**44 µL**	**110 µL**

[1] Volumes include 10% overage.
[2] 50% PEG 8000 is very viscous, follow the Important statement below to ensure accurate pipetting.

IMPORTANT! For accurate pipetting of 50% PEG 8000:

- Use 50% PEG 8000 at room temperature.
- Aspirate and dispense solution slowly.
  a. Hold the pipette tip in the solution for ~10 seconds after releasing the plunger during aspiration. This action allows the solution to be fully drawn into the pipette tip.
  b. Keep the plunger depressed for ~10 seconds to allow the solution to be fully dispensed into the Ligation Reaction Mix.

2. Vortex the Ligation Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 10 µL of the Ligation Reaction Mix to each well of the reaction plate or each reaction tube containing the poly(A) tailing reaction product.
   The total volume should be 15 µL per well or tube.

4. Seal the reaction plate or tubes, then vortex briefly or shake (1,900 rpm for 1 minute with an Eppendorf™ MixMate™) to thoroughly mix the contents.

IMPORTANT! Watch for a swirling motion of the adaptor ligation reaction to ensure proper mixing, which is necessary for efficient ligation.

5. Centrifuge the reaction plate or tubes briefly to spin down the contents.

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

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

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

Perform the reverse transcription (RT) reaction

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

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X RT Buffer	6 µL	26.4 µL	66 µL
dNTP Mix (25 mM each)	1.2 µL	5.3 µL	13.2 µL
20X Universal RT Primer	1.5 µL	6.6 µL	16.5 µL
10X RT Enzyme Mix	3 µL	13.2 µL	33 µL
RNase-free water	3.3 µL	14.5 µL	36.3 µL
Total RT Reaction Mix volume	**15 µL**	**66 µL**	**165 µL**

[1] Volumes include 10% overage.

2. Vortex the RT Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 15 µL of the RT Reaction Mix to each well of the reaction plate or each reaction tube containing the adaptor ligation reaction product.
   The total volume should be 30 µL per well or tube.

4. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

5. Centrifuge the reaction plate or tubes briefly to spin down the contents.

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

Step	Temperature	Time
Reverse transcription	42°C	15 minutes
Stop reaction	85°C	5 minutes
Hold	4°C	Hold

7. Proceed to the miR-Amp reaction (next section).

Store the RT reaction product at –20°C for up to 2 months.

Perform the miR-Amp reaction

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

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
2X miR-Amp Master Mix	25 µL	110 µL	275 µL
20X miR-Amp Primer Mix	2.5 µL	11 µL	27.5 µL
RNase-free water	17.5 µL	77 µL	192.5 µL
Total miR-Amp Reaction Mix volume	**45 µL**	**198 µL**	**495 µL**

[1] Volumes include 10% overage.

2. Vortex the miR-Amp Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 45 µL of the miR-Amp Reaction Mix to each well of a *new* reaction plate or reaction tube.

4. Add 5 µL of the RT reaction product to each reaction well or each reaction tube. The total volume should be 50 µL per well or tube.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

6. Centrifuge the reaction plate or tubes briefly to spin down the contents.

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

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

8. Proceed to performing the real-time PCR (next section).

Store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

Prepare PCR reaction plate

1. Thaw the assays on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.
2. Prepare 1:10 dilution of cDNA template.
   For example, add 5 µL of the miR-Amp reaction product to 45 µL 0.1X TE buffer.
3. Gently shake the bottle of TaqMan® Fast Advanced Master Mix to thoroughly mix the contents. Do not invert the bottle.
4. In a 1.5-mL microcentrifuge tube, prepare sufficient PCR Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]
TaqMan® Fast Advanced Master Mix (2X)	10 µL	44.0 µL
TaqMan® Advanced miRNA Assay (20X)	1 µL	4.4 µL
RNase-free water	4 µL	17.6 µL
Total PCR Reaction Mix volume	**15 µL**	**66 µL**

[1] Volumes include 10% overage.

5. Vortex the PCR Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
6. Transfer 15 µL of the PCR Reaction Mix to each well of a PCR reaction plate.
7. Add 5 µL of the diluted cDNA template to each reaction well of the plate.
   The total volume should be 20 µL per reaction well.
8. Seal the reaction plate with an adhesive cover, then vortex briefly to thoroughly mix the contents.
9. Centrifuge the reaction plate briefly to spin down the contents.

Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

The following thermal profiles are optimized for use with TaqMan® Fast Advanced Master Mix and can be used with Fast or Standard reaction plates and the corresponding instrument block configurations.

1. Load the reaction plate in the real-time PCR instrument.
2. Set the appropriate experiment settings and PCR thermal cycling conditions for your instrument. Select the fast cycling mode for all instruments.

Table 7 StepOnePlus™, ViiA™ 7, and QuantStudio™ systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

Table 8 7500 and 7500 Fast systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	3 seconds	40
Anneal / Extend	60°C	30 seconds	

3. Set the reaction volume appropriate for the reaction plate.
4. Start the run.