DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„The effects of periodic fasting/ fasting mimetics on gut microbiota and epigenetic regulation, especially sirtuins, with the aspect of healthy aging"

verfasst von / submitted by

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5. Abbreviations

ß- Hydroxybutyrate (BHB)

Adenosine monophosphate-activated protein kinase (AMPK)

Mechanistic target of rapamycin (mTOR)

MutL homolog 1 (MLH1)

Caloric restriction (CR)

Epigallocatechin-3-gallate (EGCG)

Mitochondrial DNA (mtDNA)

Reactive oxygen species (ROS)

Adenosine triphosphate (ATP)

Nicotinamidadenindinucleotide (NAD+)

Histone deacetylases (HDAC)

Sirtuin (SIRT)

Long Interspersed Nuclear Elements (LINE)

DNA methyltransferases (DNMT)

Micro-RNA (miRNA)

Small inhibitory RNA (siRNA)

MicroRNA induced silencing complex (miRISC)

Histone acetyltransferases (HAT)

Nicotinamide (NAM)

Nicotinamide phosphoribosyltransferase (Nampt)

Peroxisome proliferator-activated receptors (PPARs)

PPARg coactivator (PGC1-alpha)

Uncoupling protein 2 (UCP2)

Forkhead box, subgroup O (FOXO)

Nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB)

Brain-derived neurotrophic factor (BDNF)

Manganese superoxide dismutase (MnSOD)

Isocitrate dehydrogenase 2 (IDH2)

Glutathione (GSH)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Pyruvate kinase M2 (PKM2)

Tumor necrosis factor (TNF)- alpha

Glucose transporter type 1 (GLUT1)

Cyclin-dependent kinase1a (CDKN1a)

Cyclin-dependent kinase2a (CDKN2a)

Senescence associated secretory phenotype (SASP)

Interleukin- 6 (IL6)

Matrix- metalloproteinase (MMP)

Gastrointestinal tract (GI)

Short-chain fatty acids (SCFAs)

Gut-associated lymphoid tissue (GALT)

G-protein coupled receptor (GPR)

NOD, LRR and pyrin domain-containing protein 3 (NLRP3)

Insulin-like growth factor 1 (IGF1)

Adenosine monophosphate (AMP)

Sirtuin-activating compounds (STACs)

quantitative polymerase chain reaction (qPCR)

High-resolution melt (qPCR- HRM)

Reverse transcriptase (RT-qPCR)

BHB dehydrogenase (BHD1)

HMG-CoA synthase (HMGCS2)

Fibroblast growth factor 21 (FGF21)

Retinoid x receptor (RXR)

Toll-like receptor 4 (TLR4)

Dehydrogenase kinase isoform 4 (PDK4)

Bromodeoxyuridine (BrdU)

Nuclear factor erythroid 2–related factor 2 (NFR2)

Beginning of the intervention (T1)

End of the intervention (T2)

6. Abstract

Aging is not just the passing of time, it is defined by different hallmarks, including mitochondrial dysfunction, telomere shortening, epigenetic alterations, DNA damage etc. To retard the early onset of aging or age-related diseases, fasting and fasting mimetic therapies are under investigation.

Fasting leads to a switch in cellular molecule generations, e.g, increased AMP and ADP, instead of ATP, thus activates a cascade of enzymes, involved to be protective against premature aging. One of this mechanism is the activation of sirtuins (SIRTs). SIRTs exhibit several functions, namely cell cycle regulation, regulation of glucose and fat metabolism, DNA repair, antioxidative capacity, autophagy and more.

Secondary plant ingredients have attended high attention regarding their fasting mimicking effects via modulating AMPK activation and epigenetic activity, due to SIRT modulation, anti-inflammatory, anticancer, neuroprotective and weight maintenance properties. They also modulate senescent cells, which accumulation can be detrimental in adipose tissue leading to tissue dysfunction, insulin resistance and type two diabetes. We conducted this study to investigate the SIRT activating effects of a fasting intervention compared to the fasting mimicking effects of secondary plant ingredients. In addition, we asked if SIRTs interfere with the different hallmarks of aging and with the gut microbiota, which was differently modulated by these interventions.

Indeed in vitro we found EGCG as the most potent bioactive substance to reverse proinflammatory SASP via *SIRT3* in preadipocytes. Activation of SIRT3 inhibits low grade inflammation, with its further consequences of premature aging and age-related disorders. For both interventions in vivo, namely periodic fasting versus fasting mimicking supplement, we saw a significant elevation in gene expression, namely *FOXO1*, *SIRT1*, *PDK4 and MLH1. SIRT3* increased but was not statistically significant and *SIRT6* only raised slightly in the fasting group. The supplementation group showed no *SIRT3* changes and *SIRT6* even slightly decreased. *MiR125b-5p, miR93-5p, miR16- 5p* and *miR21-5p* increased and *miR34a-5p* decreased significantly after fasting. Beside *miR125b-5p*, which slightly decreased after the three months intervention the same miRNA expression patterns were observed for the supplement group.

Due to fasting we observed significant higher *mtDNA* levels compared to the control group. The same result was obtained for the supplementation group, although only a strong trend could be seen. No changes in telomer length and LINE1 methylation were seen before and after the interventions. Nevertheless, telomere length negatively correlated with age. At the fasting baseline, telomere length positively correlated with *SIRT1* expression, and with *SIRT6* and *miR125b-5p* expression at the supplementation baseline.

The gut microbiota itself is not defined as a hallmark of aging. Nevertheless, it influences gene expression and human host health, contributing to increased health span. Both fasting and bioactive compounds deriving from plants have been reported to modulate gut microbiota composition and its metabolites, like SCFAs. Fasting changed the gut composition. The most interesting results considered the increase in *Verrucomicrobia* and C*hristensenella*, in addition the elevation of α- diversity and butyrate, although not all significantly tested. Butyrate showed a trend for the correlation with higher *mtDNA* content. *Christensenella* positively correlated with *SIRT3* expression and negatively with age. *Verrucomicrobia* also significantly correlated with *SIRT6* expression in a positive extend but decreased with age. In the supplementation intervention, elevation of *Veillonellaceae* and *Bifidobacterium* were observed. The expression of *SIRT3* correlated with *Actinobacteri*a, namely *Bifidobacterium* and *Veillonellaceae* positively correlated with *MLH1* expression, belonging to mismatch repair mechanism.

Our results suggest that both, fasting and fasting mimetics, address SIRT expression and modulation of different hallmarks of aging. In addition, we showed evidence that the gut microbiota has a major impact on host gene expression and should be considered as a hallmark for healthy aging. Thus, a dysbiosis is linked with aging and its pathologies. Some specific species, namely *Verrucomicrobia* and *Christensenella* protect against adiposity, inflammation, and retard the development of metabolic and cognitive dysfunction.

Healthspan differs between countries and indicates the importance of lifestyle factors and the need to work on all characteristics of aging prevention to lower hallmarks of aging. In our study we demonstrate a cost effective and holistic intervention for prolonged health. Periodic fasting clearly showed to retards some hallmarks of aging and increases human's health span. For those with low fasting compliance or contraindications the supplement would be a great alterative to beneficially impact their health.

7. Introduction

7.1 Fasting and the hallmarks of aging

Human's lifespan has been doubled since the 18th century [1]. Aging is a physiological process dependent on biological and genetic mechanisms determining lifespan and health span [2]. Human lifespan is consistently elevating but health span is not accompanying in the similar way [2]. Aging is defined as a time-dependent increase of cellular damage which declines physiological integrity, thus impaired physiological functions and leading to age-related diseases, like cardiovascular diseases, musculoskeletal disorders, neurological diseases and cancer [2][3].

Different hallmarks, namely genomic instability, telomere shortening, epigenetic alterations, loss of proteostasis, impaired nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication have been defined by following these criteria: 1.) manifestation during aging and its modification in experiments show 2.) an acceleration or 3.) reversal in aging [2][3].

Thus, aging is an intrinsic factor determining our health and lifespan and the greatest risk to suffer from age-related diseases [4]. Medicines improved lifespan via fighting age-associated diseases, like hypertension, cancer, obesity etc., but health-span is not accompanying in the same space [4]. Interventions have been investigated in the aspect to diminish aging hallmarks, like RD, exercise and the use of secondary plant ingredients or synthetic bioactive medicines, like metformin [4].

Caloric restriction (CR) is defined as a daily caloric reduction of 30%, up to a total caloric uptake of 250kcal per day, as practiced during periodic fasting, without malnutrition [4][5][6]. During fasting, which is a ritual in many cultures, a shift in energy production occurs [4]. After glycogen depletion lipolysis and gluconeogenesis from glucogenic amino acids and glycerol are activated [4]. Free fatty acids are converted into acetyl-CoA further into ketone bodies, especially ß-hydroxybutyrate (BHB) which serves as an alternative energy source [7][4][6]. BHB itself regulates gene expression and cellular signaling, acting anti-inflammatory by binding to GPR109A or by inhibiting the NOD, LRR and pyrin domain-containing protein 3 (NLRP3) inflammasome [6] [8]. Additionally, RD reduces the release of insulin and insulin-like growth factor 1 (IGF1), inhibits mechanistic target of rapamycin (mTOR) pathway and decreases cholesterol levels and blood pressure, which

minimizes the risk for age-associated disorders, like neurodegenerative diseases, cancer and metabolic syndrome [4]. Nutrition depletion triggers cellular hormesis, meaning a low induced stress that activates the longevity associated Forkhead box, subgroup O3 (FOXO3) and upregulates antioxidative pathways [9]. Moreover, RD reduces the size of proinflammatory cytokine-producing monocytes improving chronic inflammation [8].

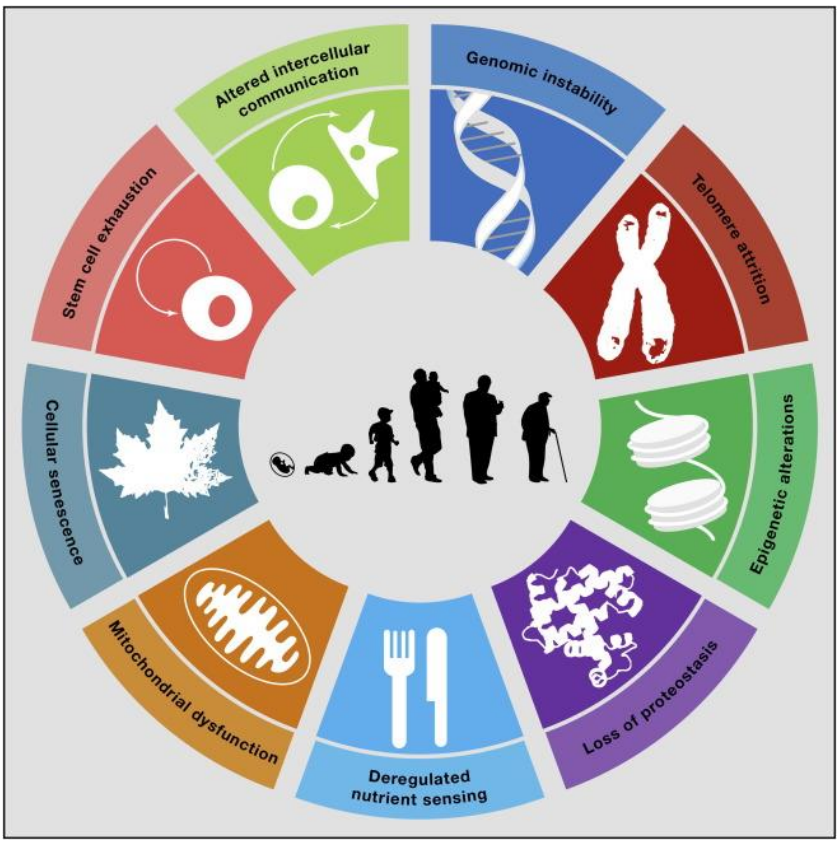

Figure 1: Hallmarks of aging [3]

7.1.1 Genomic instability

DNA damage accumulates due to exogenous and endogenous factors, namely extrinsic physicals, chemical and biological agents [3]. Intrinsic factors include a defect in nuclear lamina, point mutations, chromosomal abnormalities, telomere shortening, gene disruption caused by impaired DNA replication, hydrolytic reactions, and excess generation of reactive oxygen species (ROS) [3]. The human body developed several mechanisms to repair damaged DNA, which declines within age [2][3]. Base mismatches occurring during DNA replication are corrected by DNA mismatch repair mechanism, like the MutL family proteins, such as MutL homolog 1 (MLH1) [10]. MLH1 activates enzymes

needed for the DNA mismatch repair to replace the damaged DNA region through the action of DNA polymerase δ and DNA ligase I [10]. Deficiency in the mismatch repair system is associated with telomere shortening, senescence, aging and age-related diseases [10]. Thus, the consistency of altered DNA sequences leads to unstable cell signaling pathways, as well as cell homeostasis, due to attenuated DNA accuracy [10].

Restrictive diets (RD), like CR)are well-established interventions, which can prevent the onset of cancer and can delay its progression [11]. Additionally, CR activates stress response genes, DNA repair pathways and reduces DNA damage in both nuclear and mitochondrial DNA [11]. Moreover, polyphenols, like epigallocatechin-3-gallate (EGCG) in green tea are linked to reactivate expression of MLH1 in hypermethylated cancer cells, thus increase DNA repair mechanism [12].

7.1.2 Nuclear DNA

DNA damage, chromosomal gain or loss and copy number variations, somatic mutations alter gene transcription and impair the function of cells, which is associated with aging [3]. If the damaged cells are not sent to apoptosis or senescence, they impair the homeostasis of the organism, which has a big relevance for stem cells and their tissue renewal [3]. Cell homeostasis is a balance between cell proliferation and cell death, whereas the latter is a protective mechanism for accumulating damaged organelles, which can be increased by CR or secondary plant ingredients [13][14].

7.1.3 Telomeres

Telomeres, the protective ends of chromosomes, are prone to DNA damage [3]. A special DNA polymerase, telomerase, replicates the linear DNA up to the terminal end [3]. Telomerase is not expressed by most mammalian cells, thus leading to telomere shortening, observed during natural aging in humans and mice [2][3]. DNA damage at telomeres is invisible to DNA repair mechanisms due to shelterin, as specialized complex formation [3]. This persistent damage leads to senescence or apoptosis and is associated with aging, but can be revered by genetical telomerase activation, without increasing cancer incidences, at least in animal models [2][3]. In stem cell models, nutrition depletion increased the percentage of stem cells with longer telomeres due to mTOR inhibition [15].

7.1.4 Epigenetic alterations

Epigenetic mechanisms and their alteration affect DNA methylation, non-coding RNAs, histone modification, and chromatin remodeling [3]. These heritable modifications in gene expression can silence or activate gene expression without changing the DNA sequence [16]. Epigenetic has been associated with inflammation, obesity, insulin resistance, type 2 diabetes, cardiovascular and neurodegenerative disorders and immune system, all factors contributing to age-related diseases [16] [17]. Epigenetic modifications can be altered by intrinsic (mutations, hormones, immune system) and extrinsic factors (nutrition, lifestyle, toxins) [16]. Nutrition and bioactive food compounds are able to modify gene expression thus epigenetic mechanisms., [16][18].

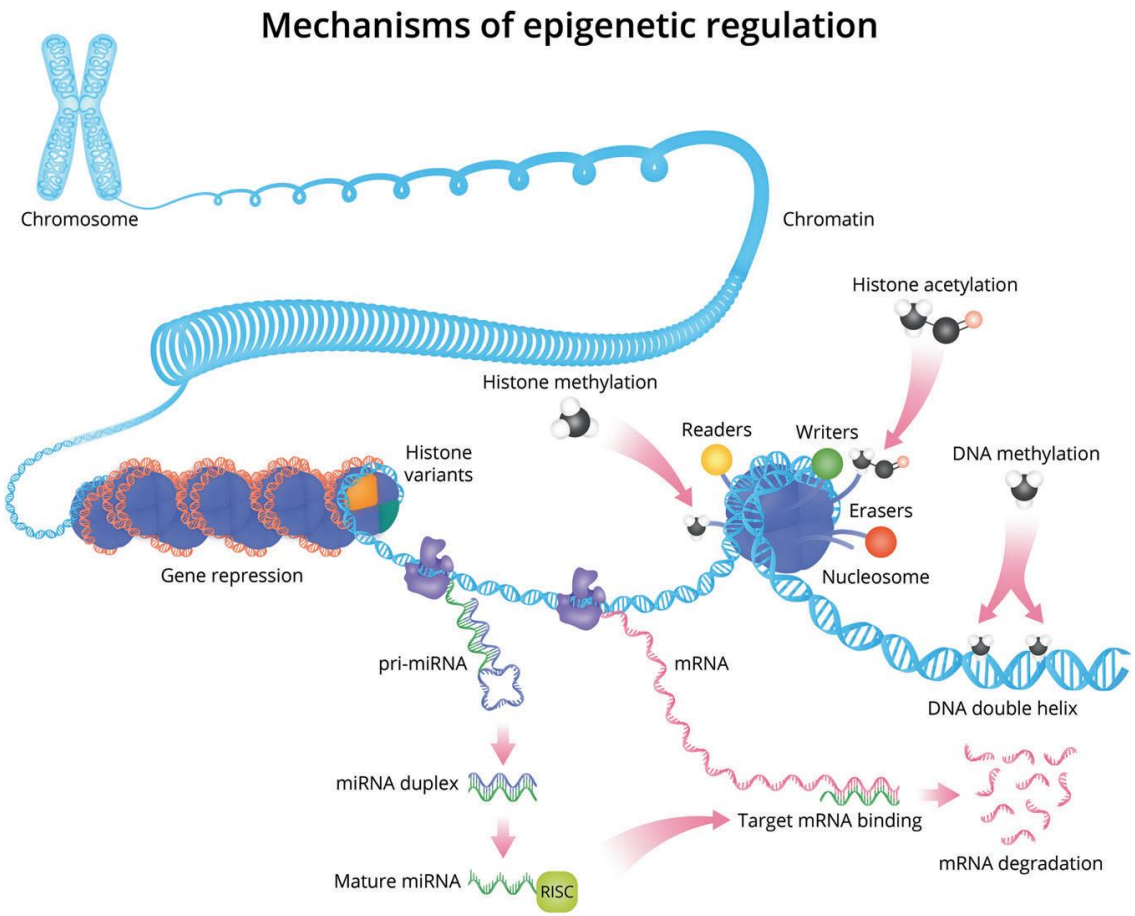

Figure 2: Epigenetic mechanism [19]

7.1.4.1 DNA methylation

DNA methylation is linked with aging and longevity [20]. This epigenetic mechanism leads to silencing gene expression, originally developed as a host defense and protection of the

genome stability [21]. The methylation status in several genes, like Long Interspersed Nuclear Elements (LINE) has been associated with aging and age-related pathologies like cancer or Parkinson's disease [21][20]. LINE1 is a mobile DNA element in humans and contributes to genome stability. Its hypomethylation and in general a decrease in methylcytosine is common with older age [21].

DNA methylation is catalyzed by DNA methyltransferases (DNMT) and a methyl group is added to the 5` position of the cytosine base at the CpG dinucleotide, resulting in 5-methylcytosine [16][20]. DNA methylation is balanced by methylation and demethylation. DNMT1 maintains methylation during the cell cycle and DNMT3 attaches a methyl group onto unmethylated DNA sides [22]. S-adenosylmethionine, which is a methyl donor and metabolized to S-adenosylhomocysteine, a methyltransferases inhibitor. Both are metabolites in the 1-carbon metabolism for DNA and histone methylation and can be modulated by bioactive compounds [16].

The water-soluble B-vitamin folate carries a methyl group and delivers the carbon metabolism for the synthesis of S-adenosylmethionine. Choline, betaine and vitamin B12 are other methyl donors but the latter serves as a cofactor for methionine synthase, a methyl donor and precursor for S-adenosylmethionine [16]. Deficiencies in Vitamin B12, folate and methionine are associated with global hypomethylation [16]. Certain secondary plant ingredients, like polyphenols and isothiocyanates, modulate DNA methylation, thus gene expression via DNMT1 inhibition [16][23]. Aging is associated with decreased DNMT1 expression and global hypomethylation [16][22].

7.1.4.2 Small non-coding RNA based modification

Small non-coding RNAs silence gene expression and consist of dicer-dependent micro-RNA (miRNA) and small inhibitory RNA (siRNA) [24]. Only 1-5 % of the human's genome are miRNAs but regulate at least 30% of coding genes. Up to now, 940 miRNAs have been identified [25]. MiRNAs are 22 nucleotides long, evolutionary conserved, single-stranded, non-coding RNA molecules [25][26]. They are transcribed by RNA polymerase II [26]. In the nucleus, primary miRNA are processed into precursor miRNAs by RNase III Drosha and DiGeorge Syndrome Critical Region 8 complex [26] [27]. Exportin5 transports the immature miRNA with the hairpin shape into the cytoplasm, where the functional miRNAs duplex are generated via RNase III Dicer and Argonaut protein which unwinds the double

standard RNA [25][26][27]. Two functional miRNAs are generated, namely 5p and 3p originated from the directionality of the pre-miRNA stem-loop. MiRNAs target their mRNA for degradation or silences its translation via incorporating into the microRNA-induced silencing complex (miRISC) [25][26][27].

MiRNAs are interesting epigenetic targets to study healthy aging regarding its interaction in multiple pathologies [28].

7.1.4.3 Histone modification- Sirtuins

The chromatin consists of a nucleosome and histone proteins [16]. Regulation of transcriptional processes occurs due to modification of DNA or histones [16]. Histone modifications are methylation, acetylation, phosphorylation, biotinylation, ubiquitination, sumoylation, ADP-ribosylation, occurring at the histone tails at the lysin residue at H3 and H4 subunits [16]. Histone acetylation is reversible and balanced by Histone acetyltransferases (HAT) and histone deacetylases (HDAC) [16]. Acetyl-CoA is the cofactor for HAT and binds an acetyl group to the histone side chain, which opens the chromatin structure and genes can be transcribed. HDACs reverse the lysine acetylation, thus suppress gene transcription [29]. In humans, HDACs are divided into four different classes. Class I consists of HDAC1, 2, 3 and 8, class II of HDAC4, 5, 6, 7, 9, 10, class IV of HDAC11, and class III the SIRT family [30]. Class I, II and IV are zinc-dependent and the SIRT require (nicotinamidadenindinucleotide) NAD+ as a cofactor [30]. HAT and HDACs have been implicated as therapeutic strategies to support healthy aging, especially SIRTs have been intensively studied [30][31].

SIRTs belong to the epigenetically active histone deacetylases class III and consist of a NAD+ binding catalytic domain [1][32]. NAD+ is required for the enzymatic activity of SIRTs, which increased during metabolic stress, like periodic fasting, CR and exercise [32]. Moreover, stress, like observed during RD, can produce HAT and HDACs, especially SIRTs [1]. NAD+ is then cleaved to nicotinamide (NAM) and acetyl, generating O-acetyl- ADP ribose during deacetylation of histones or proteins [32][33]. NAM is generated via nicotinamide phosphoribosyltransferase (Nampt) to NAD+ [32].

In mammals, seven SIRTs are known, which not only target histones but also proteins in the cytoplasm and mitochondria [32]. The seven SIRT members differ in their cellular location, tissue distribution but also gene expression and biological function [1]. SIRTs are

divided into four different classes [32]. SIRT1, 2 and 6 exhibit deacetylase activity, characteristic for class I SIRTs. ADP-ribosylation activity belongs to class II, thus SIRT4 [32]. SIRT5 belongs to class III which exhibits demolonylation and desuccinylation [32]. SIRT6 and 7 are clustered in class IV. SIRT6 has both class I and II activities both none were reported for SIRT7, although conversely reported [32][34].

The energy status during fasting is reflected by an increased ratio of adenosine monophosphate (AMP) to ATP, in addition to adenosine monophosphate-activated protein kinase (AMPK) activation and an elevation of NAD+ [6]. AMPK regulates the blood circulating monocytes pool thus having anti-inflammatory properties [8]. In addition, AMPK triggers the activation of several transcriptions namely increased expression of SIRTs, PGC1a, and FOXO [35]. This leads to a cascade of physiological events namely autophagy, reduction of senescence thus inflammation, elevation of glucose tolerance, mitochondrial biogenesis, brain-derived neurotrophic factor and prevents age-associated disorders [35].

Bioactive compounds, derived from plants, interfere in the mitochondrial adenosine triphosphate (ATP) synthase, by blocking mitochondrial complex IV and inhibits ATP generation, leading to an increasing AMP/ATP ratio and activation of AMPK with its further mechanism [36][37]. This in turn activates NAD+ and SIRTs, although secondary plant ingredients can directly activate SIRT [38]. Additionally, they target NAMPT, for NAD salvage leading to an increased NAD+/NADH ratio and SIRT activation [39][40].

The potent SIRT-activating compounds (STACs) include flavones, stilbenes, chalcones, and anthocyanidins, and exhibiting antioxidant, anti-inflammatory, anti-cancer, anti-viral, anti-obesity, cardi and neuroprotective properties [38][41].

SIRT expression can be regulated by miRNAs modulated by secondary plant ingredients [42][43]. *MiR34a- 5p* is involved in senescence and associated with obesity [42][43][44]. Its upregulation silences SIRT1 expression [42][43][44].

Table 1. Mass, activity and localization of mammalian sirtuins.

	Molecular Mass (kDa)	Enzymatic Activity	Sub-cellular Localization
SIRT1	81.7	Deacetylase	Nuclear, cytoplasmic
SIRT2	43.2	Deacetylase	Cytoplasmic, nuclear
SIRT3	43.6	Deacetylase	Mitochondrial
SIRT4	35.2	ADP-ribosyltransferase	Mitochondrial
SIRT5	33.9	Deacetylase Demalonylase Desuccinylase	Mitochondrial
SIRT6	39.1	ADP-ribosyltransferase Deacetylase	Nuclear
SIRT7	44.8	Deacetylase	Nucleolar

Table1: SIRTs and their enzymatic activity and location [45]

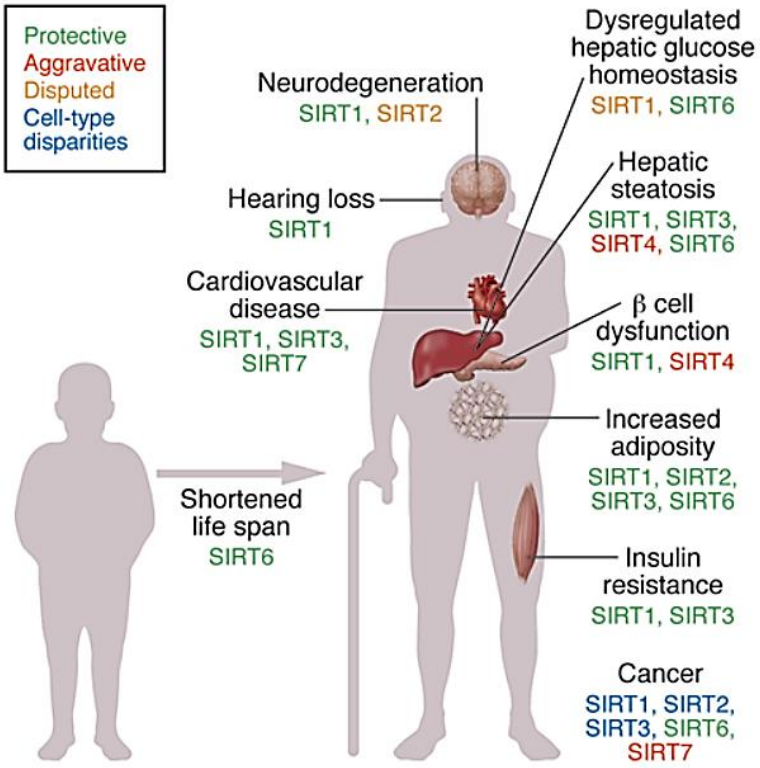

Figure 3: Tissue dependent activities of SIRT1-7 in humans [1]

7.1.4.3.1 SIRT1

SIRT1 is predominantly found in the nucleus and is the most extensively studied SIRT in the role of healthy aging [1]. SIRT1 is linked to a broad range in physiological processes thus targeting multiple transcription factors [33]. SIRT1 regulates peroxisome proliferator-

activated receptors (PPARs) and its cofactor PPARg coactivator (PGC1-alpha), thus SIRT1 is involved in lipid homeostasis and prevents obesity-related metabolic disorders, namely hepatic stenosis, cholesterol homeostasis and diabetes [33]. SIRT1 also plays an important repair mechanism in DNA damage, protection against chromosome aberrations and tumor suppressor, whereas the latter is controversially discussed [33]. SIRT1 stimulates fat loss in adipocytes via PGC1-alpha mediated ß-oxidation in mitochondria accompanied with mitochondrial biogenesis via this transcription factor and AMPK [33] [45]. Additionally, uncoupling protein 2 (UCP2) expression is silenced, a negative regulator for insulin secretion leading to a more effective mitochondrial respiratory chain reaction [33]. Other transcription factors targeted by SIRT1 are FOXO and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) [45]. Latter is suppressed and leasing to anti-inflammatory responses [45]. FOXO is involved in multiple mechanisms, beneficial for glucose metabolism, DNA repair, cell cycle, and neurological health via upregulating brain-derived neurotrophic factor (BDNF) [45].

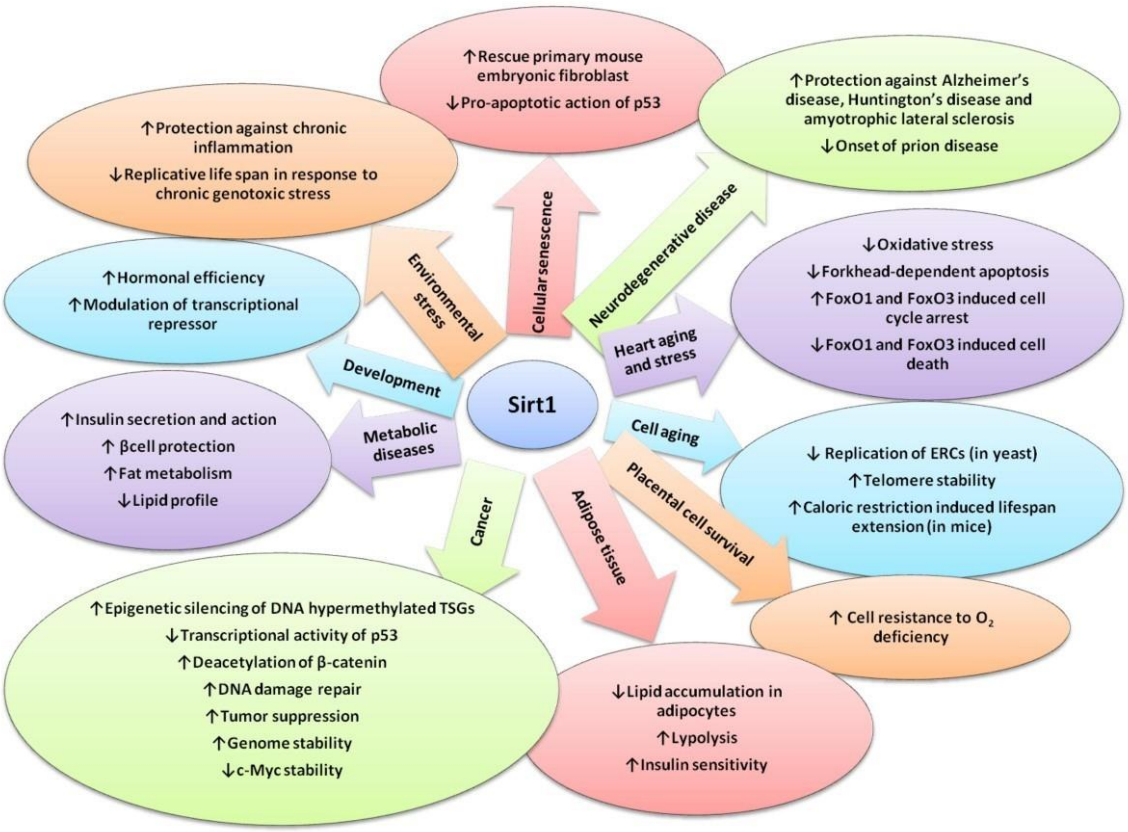

Figure 4: SIRT1 functions [33]

7.1.4.3.2 SIRT2

SIRT2 is predominantly found in the cytosol but can be shuttled into the nucleus during G2/M phase within cell cycle [45]. SIRT2 targets FOXO1 and 3, is involved in DNA repair, cell cycle, apoptosis, metabolism and can activate autophagy, dependent on SIRT2 activation, via nutrition depletion, thus oxidative stress [45]. SIRT2 silences NF-kB, a proinflammatory cytokine producer associated with neurodegenerative disorders and in general inflammation [45].

7.1.4.3.3 SIRT3

The mitochondrial SIRT has attended big attraction regarding healthy aging, with the highest expression in metabolically active tissue [32]. SIRT3 activates enzymes involved in mitochondrial metabolism, namely ß-oxidation, oxidative phosphorylation and antioxidative responses [32]. Mitochondria are dynamic energy-producing organelles, whereas the process is balanced between energy production and demand [32]. During energy production in the mitochondria, ROS is generated as a byproduct [46][33]. At higher concentration, ROS are toxic and cause oxidative damage, consequently, contributing to aging, cancer and neuronal disorders [33]. At lower levels, ROS maintains a cellular redox state, and regulates the proliferation of cells [33].

SIRT3 can regulate ROS generation by activating antioxidative defense mechanisms, which are attenuated at higher age [32]. Additionally, SIRT3 levels fluctuate due to different stimuli. RD and exercise increase its expression in the liver, skeletal muscle and adipose tissue, whereas a high fat diet and aging suppress SIRT3 expression [32]. PGC1- alpha regulates mitochondrial enzymes for mitochondrial biogenesis, ROS suppression and SIRT3 expression[32]. SIRT3 regulates PGC1 alpha due to a positive feedback loop via AMPK [32]. Via an AMPK dependent pathway, FOXO3a migrates to the mitochondria, accompanying SIRT3, both upregulate all 13 mitochondrial encoded genes [32]. SIRT3 alone interferes with mitochondrial complex I and II proteins, namely NDUFA9 and succinate dehydrogenase alpha [46].

Moreover, SIRT3 expression activates manganese superoxide dismutase (MnSOD), a key mitochondrial antioxidant enzyme, that dampens ROS [32]. Isocitrate dehydrogenase 2 (IDH2), an enzyme in the Krebs cycle is activated by SIRT3-mediated deacetylation [32]. IDH2 produces NADPH, which in turn activates the NADPH-dependent glutathione

reductase, which facilitates oxidized glutathione to the antioxidative substance glutathione (GSH), diminished with aging [32]. All these mechanisms are essential for healthy neurons, thus preventing neurodegenerative diseases. Due to the ROS by-production, neurons that require highly ATP , are predisposed for DNA damage, excitotoxicity and oxidative stress [32].

7.1.4.3.4 SIRT4

The mitochondrial SIRT4 exhibits ADP-ribosyltransferase, lipoamidase and deacylase activities, but varies within the tissue [7]. In pancreatic cells, SIRT4 regulates insulin sensitivity via insulin secretion inhibition by reducing glutamate dehydrogenase activity [7]. Latter is necessary for glutamine metabolism, ATP production and insulin secretion [7].

In liver and muscle tissue and fat cells, SIRT4 represses fat oxidation via inhibition of mitochondrial malonyl-CoA decarboxylase, leading to elevated malonyl-CoA levels [7]. Latter supports both fat synthesis and repress fat catabolism [7]. Malonyl-CoA is converted to acetyl-CoA, which can be converted back to malonyl-CoA via the AMPK dependent acetyl-CoA carboxylase [7]. During fasting malonyl-CoA levels are attenuated, thus ß-oxidation is increased [7]. The opposite metabolism occurs during the fed state [7]. Thus, SIRT4 regulates ATP homeostasis and mitochondrial biogenesis [7]. Depletion of SIRT4 decreased ATP levels, leading to AMPK activation and increased ß- oxidation [7]. Nevertheless, SIRT4 exhibits tumor-suppressive effects due to glutamine synthesis inhibition. Thus, it inhibits damaged cells to proliferate [7].

7.1.4.3.5 SIRT5

SIRT5 located in the mitochondria belongs to the class III SIRTs and has a broad tissue distribution, namely brain, heart, liver, kidney and muscle [47]. SIRT3 activates glycolysis via demalonylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and desuccinylation pyruvate kinase M2 (PKM2) to generate pyruvate [47]. Latter enters the Krebs cycle, when converted to acetyl-CoA, which is accelerated by SIRT5 and leading to increased ATP production [47]. SIRT5 activates IDH2 to generate NAPDH [47]. Thus promoting NAPDH generation SIRT5 is involved in ROS detoxification [47]. SIRT5 interacts

with the mitochondrial protein complexes I, II and cytochrome C, whereas controversy discussed [47]. SIRT5 protects against DNA damage via inhibition of acyl-CoA oxidase 1, a major H2O2 contributor [47]. Additionally, SIRT5 exhibits beneficial roles in cardiac health and stress response [47].

7.1.4.3.6 SIRT6

SIRT6 is located in the nucleolus and has a promising effect on life extension [1]. Mice lacking SIRT6 expression show metabolic disorders and decreased lifespan [1][48]. Compared to other SIRT members SIRT6 deacetylase activity is around 1000 times slower, but still the most described enzymatic activity for this SIRT [49]. Researchers found that SIRT6 catalyzes long-chain fatty deacylation and that free fatty acids, such as myristic acid, oleic acid and linoleic acid elevate SIRT6 activity up to 35 fold, at least in vitro [49]. In addition, SIRT6 regulates tumor necrosis factor (TNF)- alpha secretion, Poly (ADP-ribose)-Polymerase 1 dependent DNA repair, gene expression, telomere maintenance and integrity, and cell cycle [49]. SIRT6 acts as a tumor suppressor and silences LINE1 expression, which is linked to genomic instability, leading to DNA damage [49].

Metabolic homeostasis is one main function of SIRT6, thus regulating gene expression regarding glucose and fat metabolism [49]. SIRT6 increases ß-oxidation and silences glycolytic genes like glucose transporter type 1 (GLUT1), phosphoinositide-dependent kinase1 and lactate dehydrogenase A [49]. Thus, pyruvate is transported into the mitochondria for energy production and not converted into lactate [49]. This means SIRT6 suppresses tumorgenesis and acute inflammation, which need gluconeogenesis [49]. Although beneficial effects, suppressing GLUT1, which attenuates glucose uptake may be contrary for diabetes patients [49]. SIRT6 inhibition in mice led to increased transporter expression thus improved glucose tolerance [49]. Simultaneously, it would lead to decreased ß oxidation in the liver with elevated triglyceride levels [49].

Additionally, overexpression of this SIRT, reduces insulin growth factor 1 and delayed senescence due to silencing NF-kB, induces autophagy [49]. Mechanisms which are known to prolong health-span [49].

7.1.4.3.7 SIRT7

SIRT7 is found in the nucleus and belongs to class IV SIRTs [32]. SIRT7 is involved in protein translation by forming part of the RNA Polymerase I complex [34]. Resistant to several cellular stressors, SIRT7 exhibits important roles in cell cycle and survival, genomic stability, DNA damage signaling and repair [34]. SIRT7 relieves mitochondrial protein folding stress and endoplasmic reticulum stress, latter attenuating false protein folding [34]. Additionally, glucose, fat and mitochondrial metabolism are regulated by SIRT7, although lipid metabolism is conflicting [34].

7.1.5 Mitochondrial DNA (mtDNA)

Mitochondrial mutations and a decline in mitochondrial function are associated with aging and contribute to age-related diseases [50]. MtDNA alterations contribute to aging, due to the oxidative environment, lack of protective histones in the mtDNA, diminished DNA repair effectiveness [3]. The causality of mtDNA mutation contributing to aging is controversial due to its heteroplasmy, thus have to reach a threshold value within a given tissue [3][50]. Nevertheless, in aged cells the mutational load becomes dominant to the normal genome, attaining the state of homoplasmy [3][50]. Additionally, there is evidence that these mutations occur due to replication errors with a lack of mitochondrial DNA polymerase gamma rather than oxidative damage leading to reduced life span [3]. Nevertheless, there is evidence that decline in mitochondrial enzyme activity, respiratory chain reaction capacity, muscle mass and increase ROS production occurs during aging [51]. A normal weighed human's body consists of 40-50% muscles [52]. A loss in strength and mass is observed during aging and pathologies like cancer and diseases accompanied by a retarded catabolic conditions, function and content of the mitochondria [52].

ROS generations occurring mainly in the complex I and III in the respiratory chain reaction [46]. During oxidative phosphorylation electrons derived from NADH and succinate passes a from I to IV protein complexes, following a transmembrane proton gradient to generate energy, adenosine triphosphate (ATP) at the last complex V, the ATP synthase [46].

Mitochondrial metabolites generated during metabolism, namely alpha-ketoglutarate and NAD+ are important transcriptional and epigenetic regulators [53]. Latter declines within increased age [53]. Sirtuin3 (SIRT3), an NAD+ dependent HDAC, located in the mitochondria protects cells from oxidative damage [53][46]. ROS below or above its

homeostasis, which is generated to a higher extent in older and damaged mitochondria, contribute to oxidative damage, cellular function and accelerate aging pathologies [46]. Thus SIRT3 regulates ROS production and detoxification via antioxidative enzyme activation [46]. SIRT3 can be expressed upon fasting, CR and bioactive substances, like the main flavonoid Epigallocatechin gallate (EGCG) in green tea [54][55][32][56].

7.1.6 Loss of proteostasis

Protein aggregation and altered protein turnover are impairments of protein homeostasis and contribute to aging [2]. Autophagy is not only a recycling process to balance energy during nutrition depletion, it also removes misfolded or aggregated proteins but declines with aging [2][57]. During physiological processes, namely cellular stress, apoptosis and immune system, autophagy is involved [58]. Several synthetic and natural bioactive substances activate autophagy via AMPK and/or SIRTs [58][59].

7.1.7 Senescence

Senescence is defined as an irreversible growth arrest of cells in response to stressors, like telomere aberrations, DNA, damage, oxidative stress, or oncogenes [45][46]. These cells show phenotypic alterations, including a flat morphology, thus only In vitro, increased expression of cell cycle inhibitor cyclin-dependent kinase1a and 2a (CDKN1a, CDKN2a), increased DNA damage, increased ROS, and lysosomal ß-galactosidase production [45][46]. Although arrested for proliferation, senescent cells stay metabolically active [45]. The senescence secretome, known as senescence-associated secretory phenotype (SASP) releases cytokines, chemokines, and growth factors [45][46]. Additionally, senescent cells show decreased mitophagy contributing to metabolic abnormalities, due to defective mitochondrial metabolism [46]. Already 20% of senescent cells within a tissue the SASP can spread to neighbored cells and contribute to inflammation and tissue dysfunction, observed in adipose tissue [45]. Senescence is needed for tissue homeostasis and is a defense mechanism against damaged or tumorigenic cells, to inhibit the incorporation of their genome [45][46]. Nevertheless, an impaired removal of senescent cell via apoptosis or the immune system promotes tumor progression due to the SASP [45][47]. Accumulation of the growth-arrested cells occurs because of chronic diseases of

infections, oxidative stress, hormonal disbalances, exposures to radiation or chemotherapeutics [45]. The secreted proinflammatory cytokines, Interleukin- 6 (IL6), IL8, TNF- alpha and NF-kB and other secretomes, like matrix- metalloproteinase (MMP) contribute several disorders, namely pulmonary fibrosis, atherosclerosis, liver cirrhosis, diabetes type 2, osteoarthritis, glaucoma, sarcopenia, Alzheimer disease and impaired immune system, thus aging [46] [47].

Clearance of senescent cells has become a broad research to investigate different age-related pathologies and aging itself [45]. Bioactive substances are under investigation regarding SIRT activation, having senescence suppressive effects, their senolytic effects, thus sending senescence cells into apoptosis, modulating SASP and decreasing inflammation or support immune system [8][48][49][50].

7.2 Gut microbiota

Although the gut microbiota is not defined as a hallmark of aging, causal relationships have been observed between the microbiome and age [3][60]. The human gut microbiome contains 10^{13} to 10^{14} microorganisms composed of bacteria, archaea and eukaryotes, whereas human and bacterial cells show a 1:1 ratio [60][61]. Bacterial abundance and distribution differ within the gastrointestinal tract (GI) and their physiological functions [61][62]. The largest inner organ has different physiological functions, namely nutrient utilization, metabolism, pathogen defense, immune modulating, vitamin synthesis, and shaping the intestinal epithelium [60][61].

2172 species have been isolated and identified in GI, contributing to 12 distinguished phyla [61]. 93.5% of microorganisms contain of *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* [61]. Although a relatively stable gut microbiota composition is observed in adults, shifts are linked with diet, health, antibiotics and infections [61][62]. Inter-individual changes are determined by genetic, age, diet, health and geographic origin [62].

GI microbiota ferments complex carbohydrates and fibers to generate short-chain fatty acids (SCFAs), namely propionate, butyrate, and acetate with a ratio of 1:1:3 [61]. These metabolites exhibit anti-inflammatory, anti-tumorigenesis properties, regulate the immune system, appetite, and affect glucose and lipid metabolism [61]. Butyrate acts as an energy substrate for colonocytes, beneficially affect barrier function, including tight

junctions and mucin generation [61]. Diminished SCFAs production is linked to inflammation, cardiovascular disease, vitamin D deficiency, reduced appetite, fragility, cognitive and immune system impairments [63][64].

The gut-associated lymphoid tissue (GALT) harbors 70% of the bodies immunocytes, forming the Peyer's patch. Latter acts as an immune sensor and pathogen defense, tightly interacting with pathogen recognition receptors. *Bacteroides* and *Bifidobacterium* contribute recruiting Th17 and dendritic cells, which recruit B cells to differentiate to antibody-producing plasma cells in the lamina propria [63]. Nevertheless, overstimulation of Th17 may lead to auto immune disorders [54] [55]

Butyrate acts anti-inflammatory via G-protein coupled receptor (GPR) dependent activation of NLRP3 inflammasome, activation immune response [65]. Further proinflammatory cytokine secretion at the first step of the immune system activates the adaptive immune system [64]. Additionally, butyrate is epigenetically active and silences gene expression via HDAC inhibition [61].

Gut microbial diversity and produced SCFAs acids decrease within age, originated by factors like senescence, immune system impairment, different lifestyles, namely diet, lower mobility, medications, and hospitalization [63]. In older people, *Bifidobacterium, Bacteroides* and *Lactobacilli* attenuate and *Enterobacteria* elevates, whereas inter-individual changes are observed [63]. Alterations in the gut microorganism and its metabolites, mediated e.g by malnutrition or medications, are linked with increased cytokine levels and leaky gut representing a senescent gut, leading to the progression of age-related diseases [63][66].

Gut composition differences have been investigated between obese and lean individuals [65]. In obesity elevated abundance of *Eubacterium ventriosum*, *Roseburia intestinalis* and attenuated abundance of *Bacteroides thetaiotaomicron*, whereas in lean higher levels of *Oscillospira spp., Methanobrevibacter smithii* and lower *Firmicutes/Bacteroidetes* ratio were found [65]. Nevertheless, gut microbiota composition can be shifted with a more fiber-rich diet, thus promote health-span [63].

Fasting not only affects gene expression but also modulates the gut microbiota. After periodic fasting an increase in butyrate-producing *Faecalibacterium prausnitzii*, an increase in the overall diversity and a decrease in *Firmicutes/Bacteroidetes* ratio have been observed [6][5][67].

Additionally, secondary plant ingredients harbor prebiotic effects due to modulating gut microbiota and favor the generation of SCFAs production [68]. Comparing the effects of SCFAs, the generated metabolites during secondary plant ingredients degradation in the human body, exhibit broader physiological activities [69]. Due to their heterogeneity and originally found in combination in nature, secondary plant ingredients show synergistic effects, with a higher biological activity than the sum of the individual ones [41].

Resveratrol and quercetin increase the amount of *Bacteroidetes* and decrease *Firmicutes*, thus prevent weight gain [68]. The apple compounds increase *Bifidobacterium, Akkermansia* and *Lactobacillus*, keeping the gut barrier healthy and decreasing inflammasomes [68]. Moreover, the gut microbiota has been linked with higher levels of SIRTs, which can also be expressed in the gut epithelial cells in humans [70].

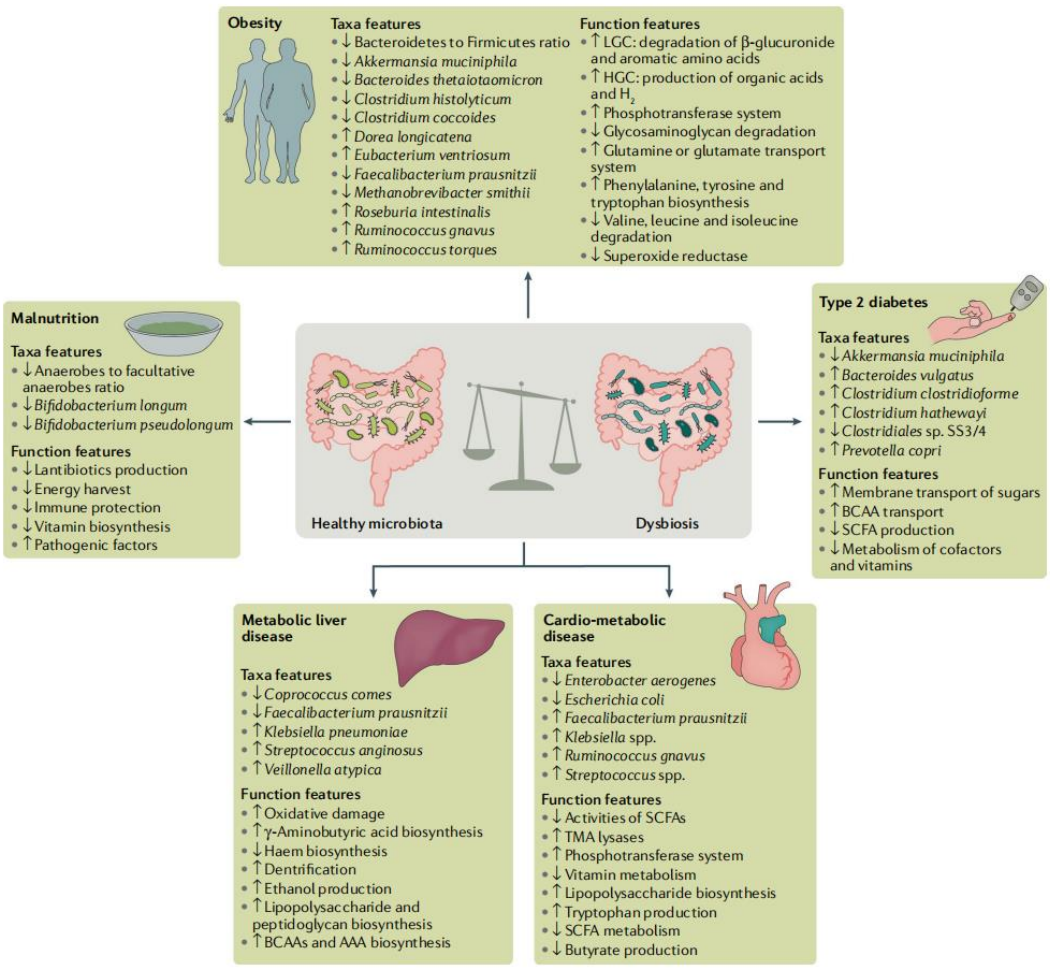

Figure 5: Impact of gut microbiota in human's health [65]

8. Hypothesis and objectives

Genetic, epigenetic and host's microbiome contribute to the genotype and phenotype of aging. Periodic fasting, but also secondary plant ingredients are known for their beneficial effects on human host health. Both interventions modulate mechanism and gut microbiota and act anti-inflammatory, thus protect against health consequences related to aging, which are, hypertension, dyslipidemia, cardiovascular diseases, diabetes, cancer, and Alzheimer's disease.

Fasting or secondary plant ingredients supplementation may act as potential therapeutics for those whose compliance for nutrition depletion is low and/or count to the contra-indicator group.

In vitro cell culture and in vivo dried blood spots and stool samples were analyzed by molecular methods, including qPCR (quantitative polymerase chain reaction), qPCR- HRM (High-resolution melt), RT-qPCR (reverse transcriptase) and Illumnia sequencing for detecting several Hallmarks of aging.

The following main objectives have been addressed:

1. Investigation of whether periodic fasting address SIRT expression in human.
2. Investigation of whether periodic fasting address hallmarks of aging in human.
3. Investigation of whether periodic fasting leads to a switch in the gut microbiota.
4. Investigation of whether the gut microbiota interferes with the other selected markers, especially SIRT.
5. Investigation of whether secondary plant ingredients modulate the similar effects as periodic fasting.
6. Investigation of whether secondary plat ingredients modulate senescence in preadipocytes by targeting SIRT3 (in vitro).

9. Results and Discussion

In Austria, the number of years that a person is expected to continue to live without limitation in function and without disability is 57 years with a life expectancy of 82 years [71]. Compared to Swedes, Austrians lose 16 healthy years of their lives [71]. That shows the importance of lifestyle factors and the need to work on all characteristics of aging prevention to lower hallmarks of aging. In our study we demonstrate a cost effective and holistic intervention for prolonged health [71].

The highlights of our study were the identification of the potent SIRT activator EGCG in vitro, thus reversing the proinflammatory SASP in adipose tissue, which would lead to tissue dysfunction and ageing. Further we clearly can show that fasting but also the combination of STACs addresses genes beneficial to increase health span, especially SIRTs. The most interesting and unexpected result was obtained after the fasting period were an increase in *Christensenella* was observed, which has been found to be more abundant in centenarians and Mediterranean lifestyle. These results indicate promising health effects after periodic fasting. In the supplementation group other beneficial shifts in the gut microbiota were seen. Furthermore, we demonstrate the fine-tuning network between gene expression and gut microbiota for both interventions, which are reported below.

These results will play an important role in the new field of precision medicine and precision nutrition. Identifying biomarkers to identify individual needs to support the body with either a caloric restriction or bioactive components is the basis for health prevention. Our results are very important in order to act preventively before symptoms arise and you can only react instead of acting.

9.1 Effects of fasting and fasting mimetic on ketogenesis and body weight

In our paper **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021,** 20 participants followed a Buchinger fasting intervention for five consecutive days and 30 subjects were enrolled for a non-fasting control group. Ketogenesis was monitored with BHB with the GK Dual blood glucose and ketone meter (swiss point of care). The BHB levels significantly increased from 0.2 mM to 5.7mM, which reflects the results from the literature on amounts of BHB during fasting [72][73]. Due to nutrition depletion, a mean weight loss of 4.26kg was recorded.

BHB generation occurs mostly in the liver mitochondria, via the BHB dehydrogenase (BHD1) and HMG-CoA synthase (HMGCS2) [74]. The delivered fatty acids for BHB synthesis are controlled by insulin and glucagon [74]. HMGCS2 expression is controlled by SIRT-mediated FOXO family activation and mTOR inhibition [74]. Latter activates PPARα and fibroblast growth factor 21 (FGF21), both required for BHB production [74].

BHB acts as an HDAC class I inhibitor and binds to GPR109 and GPR41 [74]. Inhibition of HDACs belonging to class I leads to upregulation of BDNF, which acts neuroprotective [74]. GPR109 and GPR41 exhibit several functions, like downregulation of lipolysis in dysregulated fat cells to protect against insulin resistance [74]. This receptor can be found in immune cells and gut epithelial cells, inducing anti-inflammatory enzymes, thus acting neuroprotective and improves mitochondrial health [74][75].

In the supplementation group (N= 100), described in the paper **"Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020,** BHB levels were not measured due to the maintenance of eating habits without any CR [5]. A mean body weight loss of 0.5 kg was observed, as we did in the control group.

9.2 Effects of fasting and fasting mimetics on gene expression, especially SIRTs

One of our main hypotheses was to investigate whether fasting increases SIRT expression in humans. As reported in our paper **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021** and **"Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020,** the expression levels of our selected genes changed. After five consecutive fasting days the expression levels of *FoxO1*, *SIRT1*, dehydrogenase kinase isoform 4 (*PDK4) and MLH1* increased significantly. Although *SIRT3* elevated, it was not statistically significant and *SIRT6* only raised slightly.

Thus, SIRT3 interacts with ketone body production via deacetylation of HMGCS2, which was individual, thus some showed a maximum production of 1mM BHB in blood after the intervention [32]. If feeling faint, participants were encouraged to consume a spoon of honey. Considering this aspect, *SIRT3* increases accompanying BHB levels. This is reflected in our results, where non-fasting controls had significantly lower *SIRT3* expression

compared to fasting subjects. Shimazu et al. [76] reported that mice lacking *SIRT3* showed decreased BHB levels during fasting. Ketogenesis was also verified via expression of *PDK4*, which positively correlated with BHB levels at the end of the intervention. The gene is involved in the inhibition of the pyruvate dehydrogenase complex. Thus acetyl- CoA generation is not supplied by pyruvate but instead by increased lipolysis, usually increased during fasting [77].

In addition, elevated BHB levels upregulate *FOXO1* expression in fasting due to HDAC inhibitory properties already at levels of 1mM [73]. FOXO1 activates antioxidative enzymes and regulates autophagy, thus prevents accumulation of damaged organelles [73]. SIRT3 not only contributes to ketone body production, but also improves mitochondrial quality by increasing biogenesis due to activation of antioxidative enzymes, like FOXO1 and superoxide dismutase [78].

FOXO1 is also regulated by *SIRT1*. SIRT1 exhibits multiple beneficial effects, namely DNA repair, mitochondrial biogenesis, antioxidative thus protect against age-associated disorders [79]. Both interventions increased SIRT1 significantly. Glucose deprivation, like in fasting leads to a switch in AMP/ATP and increased NAD+ concentrations, subsequently activating AMPK and sirtuins [39]. Bioactive substances target sirtuins, by targeting nicotinamide phosphoribosyltransferase (NAMPT), an enzyme in the NAD salvage pathway, which leads to an increased NAD+/NADH ratio [39]. Polyphenols can target AMPK, by blocking the F0F1- ATPase/ATP synthase, the complex IV in the respiratory chain reaction in mitochondria, leading to a switch in the AMP/ATP ratio and elevation on NAD+ in the cell [39].

Compared to the non-fasting control group, the fasting group had higher levels in *FOXO1*, *SIRT1*, *SIRT3, PDK4* but same *SIRT6* levels due to the intervention. Although SIRT6 is linked with healthy aging, its deacetylases activity is low [80][81].

Fasting not only impacts histone modulations, but also regulates miRNA expression, which in addition influences SIRT expression. *MiR34a* downregulates *SIRT1* and acts senescence mediating due to inflammasome induction, thus increasing the risk of cardiovascular diseases [82]. Higher expression of *miRNA34a* was observed in type 2 diabetes subjects by Fomison-Nurse et al [83]. According the literature, we observed an increased *miR34a* expression with age but did not see correlations with *SIRT1* [84].

Cannataro et al. [85] observed increased *miRlet7b-5p* levels due to ketogenic diet, which is usually decreased in cardiovascular diseases [86][87]. An overexpression of *miRlet7b-5p* leads to lower levels of triglycerides and insulin secretion by targeting the retinoid x receptor (RXR) and insulin receptor substrate, thus impacting adipogenesis and glucose metabolism [85]. Nevertheless, a correlation of *miRlet7b-5p* and BHB was not observed.
MiR125b-5p, miR93-5p, miR16- 5p and *miR21-5p* increased significantly after fasting. *MiR125b- 5p* acts as a tumor suppressor and is elevated via downregulation of mTOR, conducted by RD [88][89]. Thus, *miR125- 5p* has been observed to be upregulated due to RD, as we did in our study [90]. Elevation of *miR21-5p* has been linked with inflammation and cancerogenesis [91]. At the same time, *miR21-5p* protects cardiomyocytes against oxidative stress and dampens cytokine secretion mediated by toll-like receptor 4 (TLR4) [92][93]. *miR16- 5p* declines with age, observed by Kim et al. in mice [94]. Additionally, in human patients with systemic sclerosis *miR16-5p* was identified to be diminished, but upregulated in postmenopausal osteoporosis patients [95][96].
Meder et al. [97] found a negative correlation between miR93-5p and age, as did Ameling et al.[98], but not in our study. [97][98]. *MiR93-5p* acts as a tumor suppressor and improves chemosensitivity in breast cancer [99]. Thus, upregulation of *miR93- 5p* would assume a beneficial impact on aging.

Compared to the fasting intervention we observed if secondary plant ingredients in combination with a prebiotic can mimic fasting effects and was described in our papers **"Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020** and "**Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin- activating drink" Bioactive Compounds in Health and Disease 2021.** The same miRNA expression patterns were observed for the supplement group, besides *miR125b-5p*, which slightly decreased after the three months intervention. Bioactive plant ingredients are able to modulate *miR16-5*p expression, thus regulating gut health [100][101]. *MiR16-5p* interferes with claudin 2 [102]. Claudin2 is linked with leaky gut and bowel dysfunction [102].
In addition, we observed the same expression pattern for *FOXO1, SIRT1, PDK4* and *MLH1* as in the fasting group. No changes were obtained for *SIRT3* gene expression and mRNA

levels of *SIRT6* slightly decreased. Comparing to the control group, the supplementation group had higher *SIRT1* and *SIRT3* expression, although not statistically significant. Nevertheless, polyphenols, like EGCG, resveratrol, phloretin, but as well butyrate show increasing evidence to enhance the function of immune system, modulate gene expression via epigenetic modifications, namely histones, methylation and miRNAs [103][104]. One of these facts is reflected by the elevation of SIRTs. As in the fasting group we also found an upregulation of *PDK4* in this group, resulting in increased ß oxidation. This effect can be mediated by FOXO1/SIRT1 pathway, whereas the additionally PDK4 axis is not yet fully understood [105]. Interactions with miRNAs were observed, namely overexpression of *miR34a-5p* correlates with decreased *SIRT1* expression at the baseline, although only a trend.

9.3 Effects of SIRTs expression of hallmarks of aging (LINE1 methylation, mtDNA, telomere)

Thus, aging is determined by hallmarks of aging, we investigated whether the different interventions affect some hallmarks of aging and whether these interfere with the observed elevated SIRT expression.

Regarding mitochondria as a hallmark of aging, we measured relative *mtDNA* content in blood and described the results in our papers paper **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021** and **"Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin-activating drink" Bioactive Compounds in Health and Disease 2021.** Due to fasting, we observed significant higher *mtDNA* levels compared to the control group, assuming ameliorating SIRT mediated mitochondrial biogenesis du to RD, according to the literature [106]. The same result was obtained for the supplementation group, although only a strong trend. SIRTs are linked with increased mitochondrial biogenesis and reducing inflammation and limit oxidative damage in tissues via SIRT3/SOD pathway in the mitochondria [41][53][46][58][59]. Our results on telomeres and methylation have been described in the paper **"Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin-activating drink" Bioactive Compounds in Health and Disease 2021.** Telomere length

negatively correlated with age including participants from both interventions and the supplementation group only but not the fasting intervention. At the fasting baseline, telomere length positively correlated with *SIRT1* expression, and with *SIRT6* and *miR125b-5p* expression at the supplementation baseline. SIRT1 and SIRT6 are known to act telomere protective via modulation of 5-hydroxymethylcytosine, the primary oxidative product in the demethylation of 5- methylcytosine [40][107]. Knockout of these two genes induced contrary results leading to senescence [32]

According methylation, *LINE1* acts as a biomarker for global methylation and response to extrinsic factors [108]. Higher methylation status has been linked with BMI and overweight [108]. Whether fasting nor the supplementation led to a change in *LINE1* methylation, but methylation status correlated with body weight for the supplementation group, according to the literature [109]. Additionally, literature reports a link between *LINE1* hypermethylation and lower body fat, contrary to our results [110]. We did not measure body fat mass, but we observed in our results that increased *LINE1* methylation levels negatively correlated with telomere length at the end of the supplementation intervention but not for the fasting group. This means higher *LINE1* methylation is seen in participants with higher weight, maybe obesity resulting in shorter telomeres, thus premature aging.

9.4 Secondary plant ingredients and their effect on senescence

Premature aging, seen in obesity, and how secondary plant ingredients may reverse senescence in adipose tissue was addressed in our publication **"Epigallocatechin Gallate Effectively Affects Senescence and Anti-SASP via SIRT3 in 3T3-L1 Preadipocytes in Comparison with Other Bioactive Substances" Hindawi Oxidative Medicine and Cellular Longevity Volume 2020.** 3T3-L1 preadipocytes were incubated with bromodeoxyuridine (BrdU) for 8 days to induce senescence, which was verified with increased β-galactosidase activity, colored cells in blue and cell cycle inhibition via increased *CDKN1a* expression was observed. After BrdU treatment, cells were treated with different bioactive substances like EGCG, anthocyanidin, resveratrol, spermidine, butyrate and BHB. Additionally, we incubated the cells with a mixture of EGCG, resveratrol and spermidine, thus secondary plant ingredients have been reported to have synergistic effects [41]. Our results demonstrate that almost all substances decreased β-galactosidase activity to a higher or

lower extend. This was also seen in the repression of *CDKN1a*, whereas here the endogenous substances, namely BHB, spermidine and butyrate were not as effective. Only BHB at a concentration of 4mM, which reflects concentrations usually observed after several days of nutrition depletion could reverse senescence via *CDKN1* repression [72][73].

Senescence in adipose tissue can be detrimental [111]. Consistency of proinflammatory senescent cells leading to tissue dysfunction and insulin resistance [111]. Disturbed glucose and fat metabolism lead to the onset of age-related disorders [111].

The proinflammatory status of senescent cells was determined via IL6 secretion using an ELISA. Only anthocyanidin, phloretin, EGCG, the mix and the positive control roxithromycin exhibit anti-inflammatory effects, with the highest effect of EGCG.

Nuclear factor erythroid 2–related factor 2 (NFR2) and SIRT3 are under therapeutic investigation to reduce low grade inflammation in adipose tissue [112]. *NRF2* can active *SIRT3 e*xpression via antioxidant response element (ARE) in its enhancer region, both necessary to protect against oxidative stress via induction of antioxidative enzymes [112][113]. Therefore, we investigated which substances interfere with *NRF2* and *SIRT3* expression and observed that the minor was able to induce its expression. *NRF2* expression increased after anthocyanidin, resveratrol and roxithromycin incubation, but not with the other substances. Furthermore anthocyanidin, EGCG, phloretin and the positive control exhibit *SIRT3* activating properties in different extend. IL6 negatively correlated with *SIRT3* expression in our study but not with *NRF*2, assuming anti- SASP effect is rather due to *SIRT3* activation than *NRF2*. Downregulated *SIRT3* expression is also linked with metabolic syndrome, hyperlipidemia and diabetes [114][115]. These are all pathologies linked with accumulated senescence cells [116][111].

9.5 Effects of fasting and fasting mimetics on modulating gut microbiota and its metabolites

In the paper **“Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans”** ***Int. J. Mol. Sci.*** **2021** and **“Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition” Functional Foods in Health and Disease 2020** stool samples were collected and analyzed via illumnia sequencing and liquid chromatography coupled to mass

spectrometry to assess gut microbial and SCFAs changes. After Fasting changes at the phylum level were seen in *Euryarchaeota, Tenericutes*, *Verrucomicrobia*, *Cyanobacteria*, *Proteobacteria*, *TM7* and *Fusobacteria.* Only the last three were significantly increased ($p < 0.05$). *Verrucomicrobia* and *Cyanobacteria* increased, *Euryarchaeota and Lentisphaerae* decreased, but no significant changes were seen for *Actinobacteria*, *Bacteroidetes*, and *Firmicutes.* Comparing to the non-fasting control, where no changes were seen, the fasting group had greater abundance in *Verrucomicrobia, Firmicutes, Actinobacteria* and *Proteobacteria* after the intervention. The phylum *Verrucomicrobia* consists of only a few species, like *Akkermansia muciniphila*. This specie has the ability for mucin degradation supporting intestinal function [117]. In older adults *Verrucomicrobia* is linked with increased motoric ability, cognition and learning and sleep quality [117]. Although *Firmicutes* and its higher ratio to *Bacteroidetes* have been associated with obesity and diabetes type 2, whereas species specific [118]. *Firmicutes* to *Bacteroidetes* ratio decreased after fasting.

Faecalibacterium prausnitzii, a butyrate producer was less abundant in humans aging 90 years or older [117]. *Faecalibacterium prausnitzii* have been controversially discussed in regard to RD [5][67]. In our results we observed, that *Faecalibacterium prausnitzii* decreased and the amount correlated with lower weight loss. *Christensenella* belongs to the phylum *Firmicutes* and might have contributed to the increased butyrate levels after periodic fasting, which we observed. *Christensenella* is also associated with lower BMI and obesity and its related disorders [118],

Although *Proteobacteria* increased after five days of fasting, which are often connected with inflammation and bowel diseases, this effect is strain-specific and they are more abundant in centenarians [5] [117].

Additionally, in the paper **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021,** shannon diversity index was used to calculate α- diversity. Although a higher diversity for all significantly changed microbiota was observed in the fasting group, the overall diversity was not statistically significant tested. The dataset was further subjected to principal coordinates analysis PCoA based on Bray–Curtis dissimilarity index and showed significant different clusters for fasting and non-fasting group at the end of the intervention. Compared to the fasting group, the fasting mimetic group showed no changes in α-

diversity at any timepoint compared to a placebo control group and fasting group (latter data not jet published).

Aging and obesity are associated with lower bacterial diversities and altered metabolic pathways, which are involved in nutrient harvesting and energy production [6]. After PF, we observed a more diverse composition of gut microbiota at the species level, which was distinctive from the composition of the control group. Together, these findings indicate that PF increases the expression of genes and the diversity of gut microbiota relevant for longevity.

At the genus level changes in the abundance of *Actinomyces*, *Granulicatella*, *Roseburia*, *Rothia*, *Rominococcus*, *Eggerthella* and *Christensenella* were observed after fasting and described in the paper **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021.** *Unspecific Firmicutes* and *Bifidobacteriaceae* correlated the increased butyrate levels after fasting. The higher amount of butyrate showed a trend for correlation with higher *mtDNA* and *Bifidobacteriaceae* correlated with higher levels of weight loss. *Bilophilia* is linked with aging and metabolic diseases [119][117]. At the baseline, *Bilophilia* abundance correlated with body weight. *Bifidobacteriaceae*, is an important butyrate producer, which declines with aging and is associated with lower BMI and higher weight loss, all of which we observed in our fasting study [120] [121].

Comparing the effects of a three-months supplementation intervention, the results were described in the publications **"Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020** and **"Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin- activating drink" Bioactive Compounds in Health and Disease 2021.** An increase in *Tenericutes, Lentisphaerae, Bacteroidetes, Euryarchaeota,* and *Actinobacteria* and decreased *Cyanobacteria, Verrcoumicrobia, Proteobacteria, TM7*, *Elusimicrobia,* and *Fusobacteria* but not in a significant manner were observed after three months. Nevertheless, *Firmicutes* reduced and a significant elevation in *Actinobacteria* was obtained. *Tenericutes*

positively correlated with butyrate, although it only slightly increased after the intervention. Consistent with the literature, prebiotics like GOS and bioactive plant ingredients exhibit prebiotic effects and increase *Bifidobacterium,* belonging to the *Actinobacteria* phyla [69][122][123]. The butyrate producing *Bifidobacterium* declines with age and obesity, diminishing its gut integrity, pathogen protection, immune and hormone modulating, probiotic, activity [63][121][124].

In addition, the amount of *Veillonellaceae* increased after the supplementation which metabolizes lactate, produced by *Bifidobacterium* [125][126]. Lactate contributes to fatigue during exercise [125][126].

As observed in the fasting *Firmicutes* to *Bacteroidetes* ratio decreased and significant correlations are seen for *Firmicutes/Bacteroidete*s ratio with BMI and weight in the total population size, including fasting and the supplementation group and the latter intervention only, but not for fasting only.

9.6 Interactions of gene expression and microbiota

The different observed interactions of gene expression and the gut microbiota composition for the fasting group was reported in our papers **Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020** and **"Five Days Periodic Fasting Elevated Levels of Longevity Related Christensenella and Sirtuin Expression in Humans"** ***Int. J. Mol. Sci.*** **2021.**

Christensenella was the most interesting result, which abundance increased after periodic fasting*. Christensenella* is usually more frequently found in centenarians [117]. Age negatively correlated with this bacterium at the beginning of the intervention (T1) and positively correlated with *SIRT3* expression due to the intervention. *Christensenella* correlated with lower BMI in the fasting participants, consistent with the literature [117] [118]. As a butyrate producer, *Christensenella* might have contributed to the significant generation of butyrate following PF. Butyrate exhibits favorable effects on mitochondria [127][128]. Consistent with this, we found a positive correlation, even though only a trend.

SIRT1 can also be expressed in gut epithelial cells and can be activated by *Lactobacillus brevis T2102* [129][130]. At the baseline higher amount of *Prevotella* and *Lactobacillus*

correlated with higher *SIRT1* expression. Due to the major switch in gut microbial composition after nutrition depletion, these correlations were not seen after periodic fasting. Nevertheless, after fasting *SIRT1* positively correlated with *Fusobacteria* and *Actinobacteria* and in addition negatively with *Cyanobacteria*, but only significant for *Fusobacteria*.

At the beginning of the fasting intervention *Cyanobacteria and Lentisphaerae* positively correlated with *miR34a-p5.* At the end of the intervention (T2) *Cyanobacteria* positively correlated with *miR16-5p* and negatively with *SIRT1. SIRT1* positively correlated with *Actinobacteria* at the same timepoint. *Cyanobacteria* usually found in the atmosphere, soil and water are also detected in the human's gut [131]. *Cyanobacteria* fixing nitrogen [131]. There is a big gap in literature regarding their interaction with human host health. Nevertheless*, Cyanobacteria* contribute to the synthesis of Vitamin K and B [131].

Verrucomicrobia also significantly correlated with *SIRT6* expression to a positive extend but decreases with age. *Verrucomicrobia*, is known for its anti-inflammatory and health protecting activities and is increased in very old aged patients [117].

Results from the intervention group consuming a supplement were reported in the publications **"Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition" Functional Foods in Health and Disease 2020** and **"Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin- activating drink" Bioactive Compounds in Health and Disease 2021.**

SPIs and galactooligosaccharides exhibit prebiotic effects, which have beneficial immune, metabolic and cognitive functions [41]. SCFAs produced by the gut microbiota are known to inhibit class I Histone deacetylases (HDACs) and also activate the UCP2-AMPK-acetyl-CoA carboxylase (ACC) pathway with decreased PPARγ gene expression, thus resulting in decreased lipogenesis and increased AMP: ATP ratio. Latter induces AMP-activated protein kinase (AMPK) and sirtuins (SIRT) activation, having multiple beneficial effects on human health [41].

Bacteroidetes positively correlated with *miR125b-5p, miR16-5p, miRlet7b-5p* and *MLH1*. In addition, it correlated with *SIRT1* expression, although only a trend. *miR125b-5p* and

miR16-5p are downregulated in participants with irritated bowel syndrome and can be modulated by secondary plant ingredients [41] [102]. MiR16-5p, which decreases within age is also an important regulator of the cell cycle, but also of serotonin thus regulating mood, satiety and sleep, factors protective against depression, eating disorders, and aging [41].

Bacteroides metabolizes quercetin catechins found in the supplement and low amount of this taxa is linked with inflammatory bowel disease [132][133]. In addition, *miR125-5p* and *miR16-5p* target claudin2, which is upregulated in bowel disease and the respective miRNAs are downregulated [102].

Mice fed a high-fat diet had a higher abundance of *Bilophilia* and lacking *SIRT3* expression, thus obtained retarded gut health accompanied by increased inflammation [134] [119]. Consistent with the literature we saw a negative correlation of *Bilophilia* and *SIRT3*, showing secondary plant ingredients can protect against gut inflammation and metabolic syndrome [41][135].

The expression of *SIRT3* correlated with *Actinobacteri*a, namely *Bifidobacterium*. SIRTs can be expressed in the gut and higher expression have been observed with probiotic supplementation [70].

Gut dysbiosis can release toxins and induce DNA damage. *Veillonellaceae* positively correlated with *MLH1* expression, belonging to mismatch repair mechanism and secondary pant ingredients modulating the abundance of *Veillonellaceae* [136][137] .

10. Conclusion

This study showed that fasting and the fasting mimetic increased SIRTS in vivo. Due to the activation of longevity-related enzymes both interventions interfere with hallmarks of aging, promoting human health. Although not defined as a hallmark of aging, the gut microbiota impacts human health tremendously. Thus, the gut microbiota influences host gene expression. *Verrucomicrobia, Akkermansia,* and *Christensenellaceae* are linked to protect against adiposity, inflammation, and the retarded development of metabolic and cognitive dysfunction. A diet high in secondary plant extracts, like the intensively studied Mediterranean diet, contributes to lower *Firmicutes* to *Bacteroidetes* ratio, higher abundances of butyrate, *Christensenellaceae* and *Faecalibacterium prausnitzii* and increased anti-inflammatory related genes. All of which we found in our study, beside the rise in *Faecalibacterium prausnitzii*, which is contrary discussed in the response of fasting. Thus, fasting, and fasting mimetics contribute to a healthy aging strategy. Furthermore, fasting is an inexpensive method to achieve the health promoting effects. Thus, not everyone belongs to the group who should follow consecutive fasting periods, supplementation with secondary plant ingredients might be a helpful alternative.

Thus, healthspan differs within the countries our study shows the importance of lifestyle factors and the need to work on all characteristics of aging prevention to lower hallmarks of aging. Additionally, our study reports the tight network between epigenetic regulation and gut microbiota. Inducing periodic fasting in human's life would be beneficial in retarding age related diseases. For humans with low compliance or having contraindications a fasting mimicking intervention including probiotic and SIRT activating compound would help to increase their health span.

These results will play an important role in the new field of precision medicine and precision nutrition. Our results are very important in order to act preventively before symptoms arise and you can only react instead of acting.

11. Zusammenfassung

Altern wird anhand verschiedener Kennzeichen definiert, wie mitochondriale Dysfunktion, Telomerverkürzung, epigenetische Veränderungen, DNA-Schäden etc. Um das frühzeitige Altern und altersbedingte Erkrankungen zu verzögern, wurden in dieser Studie Fasteninterventionen und Fastenmimetika untersucht.

Da es beim Fasten zu einer Veränderung im zellulären Molekülaufbau kommt, z. B vermehrt AMP und ADP, anstatt ATP, werden eine Kaskade an Genen und Mechanismen aktiviert, die vor frühzeitigem Altern schützen.

Einer dieser Mechanismen ist die Aktivierung von Sirtuinen (SIRTs). SIRTs sind an vielen Funktionen beteiligt: Regulation des Zellzyklus, Kohlenhydrat und Fettstoffwechsel, DNA-Reparatur, antioxidative Signalwege, Induzierung der Autophagie und Vielen mehr.

Sekundäre Pflanzeninhaltsstoffe haben große Aufmerksamkeit bekommen bezüglich der Fasten nachahmenden Effekte. Sie beeinflussen die AMPK Aktivierung, die epigenetische Aktivität (SIRT Modulierung), wirken antientzündlich, antikanzerogen, neuroprotektiven und Gewichts stabilisierend.

Sie modulieren seneszente Zellen, deren Ansammlung im Fettgewebe verheerend sein kann, da dies zu Gewebsdysfunktionen, Insulinresistenz und Typ 2 Diabetes führen kann.

In unserer Studie zeigte EGCG die potenteste Wirkung unter den verwendeten bioaktiven Substanzen. EGCG reversierte das proentzündliche SASP via SIRT3. Dieses wirkt antientzündlich besonders bei geringgradigen Entzündungen und wirkt so frühzeitigem Altern und altersbedingten Erkrankungen entgegen.

Das Mikrobiom selbst ist nicht als Kennzeichen des Alterns definiert. Nichtsdestotrotz beeinflussen sie die Genexpression und die Gesundheit des Wirtes und steuern zu einer verbesserten Gesundheitsspanne bei. Fasten als auch bioaktive Substanzen aus Pflanzen sind bekannt dafür bakterielle Darmzusammensetzung und ihre Metabolite, wie kurzkettige Fettsäuren, zu beeinflussen.

In dieser Studie habe wir die SIRT aktivierenden Effekte des Fastens untersucht und diese mit den fastenimitierenden Effekten von Pflanzeninhaltsstoffen vergleichen. Zusätzlich haben wir untersucht, ob die SIRTs auch mit anderen Kennzeichen des Alterns und dem Mikrobiom intervenieren, wobei es bei Letzterem zu unterschiedlichen Ergebnissen gekommen ist.

In beiden Interventionen sahen wir eine signifikante Erhöhung in der Genexpression von *FOXO1*, *SIRT1*, *PDK4 and MLH1. SIRT3* Expression stieg an aber nicht signifikant und *SIRT6* erhöhte sich nur leicht in der Fastengruppe. In der Supplementen Gruppe gab es keine Veränderungen in der *SIRT3* Expression, und *SIRT6* Expression ging sogar ein wenig zurück *MiR125b-5p, miR93-5p, miR16- 5p* und *miR21-5p* Expressionen stiegen an und *miR34a-5p* sank signifikant nach dem Fasten. Nach der dreimonatigen Supplementen Intervention wurde *miR125b-5p* leicht runter exprimiert, sonst blieben die Expressionsmuster stabil.
Auf Grund des Fastens kam es zu signifikant höheren *mtDNA* Mengen verglichen mit der Kontrollgruppe. Das Gleiche konnte mit dem Nahrungsergänzungsmittel erzielt werden, jedoch nur als starker Trend.
Keine Veränderungen konnten in der Telomerlänge und *LINE1* Methylierung beobachtet werden. Dennoch korrelierte die Länge der Telomere negativ mit dem Alter. Zu Beginn des Fastens korrelierte die Telomerlänge mit der Expression von *SIRT1* zudem mit *SIRT6* und *miR125b-5p* in der Supplementierung T1.
Fasten verändert die bakterielle Darmzusammensetzung. Das interessanteste Ergebnis betrifft das vermehrte Auftreten von *Verrucomicrobia* and C*hristensenella*, zusätzlich die Steigerung der α- Diversität, sowie gemessene Butyratmengen. Butyrat zeigte einen Trend mit der Menge an *mtDNA*. *Christensenella* korrelierte positiv mit der *SIRT3* Expression and negativ mit dem Alter. *Verrucomicrobia* korrelierte zudem auch signifikant mit steigender *SIRT6* Expression aber negativ mit dem Alter.
In der Nahrungsergänzungsmittel Gruppe, erhöhte sich die Menge an *Veillonellaceae* und *Bifidobacterium*. Die *SIRT3* Expression korrelierte mit *Actinobacteri*a, nämlich *Bifidobacterium. Veillonellaceae* korrelierte positiv mit der *MLH1* Expression, einem DNA-Mismatch-Reperaturmechanismus.
Unsere Ergebnisse zeigen, dass Fasten als auch Fastenmimetika, SIRT Expressionen beeinflussen als auch einige Kennzeichen des Alterns modulieren. Zusätzlich zeigen wir, dass die Darmmikrobiota einen großen Einfluss auf die Genexpression des Wirtes hat und sollte somit als ein Kennzeichen des gesunden Alters berücksichtigt werden. Eine Dysbiose der Zusammensetzung ist nämlich mit Altern und altersrelevanten Erkrankungen in Verbindung gebracht worden. Einige spezielle Spezies, nämlich *Verrucomicrobia* und *Christensenella* schützen vor Übergewicht, Entzündungen und verzögern die Entstehung von metabolischen und kognitiven Dysfunktionen

12. Appendix

12.1 Papers

12.1.1 Epigallocatechin Gallate Effectively Affects Senescence and Anti- SASP via *SIRT3* in 3T3-L1 Preadipocytes in Comparison with Other Bioactive Substances

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Aim. We investigated different bioactive compounds including epigallocatechin gallate (EGCG), anthocyanidin, resveratrol, phloretin, spermidine, butyrate, and β-hydroxybutyrate with regard to their effect on *SIRT3* via *NRF2* and modulation of the proinflammatory senescence-associated secretory phenotype (SASP) in senescence induced 3T3-L1 preadipocytes. Methods. For induction of senescence, 3T3-L1 preadipocytes were incubated with bromodeoxyuridine (BrdU) for 8 days. Cell cycle inhibition was observed, and β-galactosidase activity was measured. After BrdU treatment, cells were treated with different bioactive compounds in various concentrations for 96 h. ELISA was used for determining proinflammatory cytokine IL6 in SASP cells. Results. *CDKN1a* increased significantly after BrdU incubation compared to un-treated control (p< 0:01). All secondary plant ingredients used for treatment, but not anthocyanidin 50 μM, decrease *CDKN1a* expression (p < 0:05), whereas most endogenous substances did not attenuate *CDKN1a.* IL6 secretion positively correlated with *CDKN1a* (p < 0:01), whereas EGCG could diminish both, IL6 and *CDKN1a* with the strongest effect (p < 0:01). Although *NRF2* positively correlated with SI*RT3* activation (p < 0:05), only resveratrol (p < 0:01) and anthocyanidin (p < 0:05) could activate *NRF2* significantly. Solely anthocyanidin 50 μM (p

< 0:05) and 100 µM (p < 0:01) and EGCG 50 µM (p < 0:01) could increase *SIRT3* expression. Activation of *SIRT3* with EGCG correlated with lowered IL6 secretion significantly (p < 0:05) but not with anthocyanidin. Conclusion. Accumulation of senescent cells in adipose tissue plays an important role in obesity and age-related diseases. SIRT3, located in the mitochondria, can regulate ROS via different pathways. Thus, targeting *SIRT3* activating compounds such as EGCG may delay senescence of cells and senescence induced inflammatory processes.

Introduction

In the past decade, senescence cells have emerged as possible contributors to the pathogenesis of many age-related diseases, potentially caused by cytokines released due to metabolic stress [1, 2]. Cells gradually lose their division potential under nor-mal culture conditions, also called replicative senescence. They may, however, also enter premature senescence, a simi- lar state, induced by various stimuli, including oncogene activity, oxidative stress, and DNA damage [1]. Depending on the factors involved, different pathways are activated resulting in the activation of p53 and its downstream target CDKN1a [2]. Activation of cell cycle inhibitors, like CDKN1a, is considered as a hallmark of senescence [3]. The central purpose of cells undergoing senescence is to eliminate dam-aged cells by the immune system. Yet, if the clearance is impaired, it can lead to accumulation and tissue dysfunction. Senescence cells remain metabolically active. If they persist, they consequently alter their microenvironment and nearby cells by implementing a complex proinflammatory response, and thus acquiring the SASP. The SASP is mediated by transcription factor nuclear factor κB (NF-κB) and CCAA T/enhancer binding protein-β (CEBPβ), including the secretion of pro-inflammatory cytokines (IL6 and TNFa), chemokines, macrophage inflammatory proteins (MIP) as well as transforming growth factors (TGFβ). Latter upregulates p21 through the SMAD complex [2]. Another stimulus which provokes the development of aging phenotypes is mitochondrial dysfunction, resulting in cellular senescence in vitro and in vivo [4]. Mitochondria oxidize NADH to NAD+, which de-clines with aging. NADH is generated by the TCA cycle, but cytosolic NAD+/NADH pool is also used for oxidation to NAD+. A lower NAD+/NADH ratio induces senescence and has been shown to be associated with aging [4]. Further, mitochondrial dysfunction impairs metabolism and

redox homeostasis, which is related to several chronic diseases, like diabetes type 2, obesity, metabolic syndrome, and development of age- and diabetes-dependent liver steatosis [5, 6].

The largest internal organ in humans, the fat tissue, is strongly involved in longevity and age-related metabolic dysfunctions. Besides its main role of storing highly reactive fatty acids as triglyceride in lipid droplets, fat is important for several essential physiological processes including immune function, thermoregulation, mechanical protection, and tissue regeneration. However, in doing this, the adipose tissue is a highly active endocrine system and secretes hormones such as leptin, adiponectin, growth factors, and cytokines like IL6 [7]. Throughout life, fat distribution and its function change depending on genetic and epigenetic dis- position as well as lifestyle factors [4, 7, 8]. Towards middle age, the proportion of fat tissue begins to decrease and further declines in old age. Preadipocytes are related to macrophages and give rise to new adipocytes. These fat cell progenitors are 10fold more abundant in obese subjects, but dysdifferentiate within aging [7].

Both aging and obesity are associated with a chronic, low grade inflammation, potentially fueling the development of diabetes, hypertension, cancer, cognitive dysfunction, atherosclerosis, and thus diminishing health span [7, 8]. When it comes to aging, a class of NAD+ dependent histone deacetylases (HDACs), called sirtuins, has been frequently mentioned as they are involved in the regulation of transcription, apoptosis, stress resistance and furthermore act as caloric energy sensor [9, 10]. HDACs are grouped in four classes, which all have in common to regulate gene expression by removing acetyl groups in histones [11]. HDAC class III includes seven members of sirtuins (SIRT1-7), identified in mammals, which are localized in different cellular compartments [9, 11]. SIRT1, 2, 6, and 7 can be found in the nucleus; SIRT1 and SIRT2 in cytoplasm; and SIRT3, 4, and 5 are localized in the mitochondria [9]. In-creasing data support their role in modulation of cellular senescence and lifespan in different animal models [5]. SIRT3 regulates several aspects of mitochondrial function and is a promising candidate to diminish oxidative stress, thus inflammation and age- related diseases [9]. Interestingly, it has been demonstrated that activation of nuclear factor erythroid 2–related factor 2 (NRF2) induces SIRT3 gene expression via antioxidant response element (ARE) in its enhancer region [12]. NRF2 is a transcription factor important as the first cellular defense against oxidative stress [6, 12]. Usually sequestered together with Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm, NRF2 dissociates during oxidative stress and translocate to the nucleus and furthermore induces transcription of genes with ARE in their regulatory region [12]. NRF2 and SIRT3 have been discussed as therapeutic targets to reduce senescence in

adipose tissue and thus decreasing chronic low-grade inflammation and prevent different disorders [5, 12–14].

In the last decade, a large group of bioactive compounds, including flavonoids, like EGCG and anthocyanidins as well as the stilbene resveratrol, have been identified to target senescence via multiple pathways [15]. Many effects are due to the modulation of epigenetic mechanisms [5, 8, 14]. A large group of bioactive compounds have been identified to activate SIRTs and NRF2 [5, 12, 13, 16]. In general, senescence of cells can be eliminated by the activation of the immune system or targeting individuals SASP factors, which might prevent the paracrine spread of senescence, thus inflammation [8]. An-other approach is to identify compounds with senolytic effects inducing apoptosis in senescent cells and prevent their accumulation [17]. Especially, secondary plant ingredients like polyphenols, and their synergistic effect when combined, are highly interesting compounds with anti-SASP activity. Furthermore, their anti-inflammatory and antioxidative capacity might be useful in the treatment and prevention of metabolic syndrome [15, 16, 18]. Some bioactive compounds are known to activate sirtuins, thus having beneficial effects for human health [11]. Also, caloric restriction and ketogenic diet have become more popular for their anti-inflammatory effect and health benefits. Caloric restriction is linked to higher levels of ketone bodies such as β-hydroxybutyrate (BHB), which is produced by ketogenesis in liver mitochondria and released into the bloodstream as energy fuel. The production of ketone bodies is mediated by SIRT3 and may prevent metabolic dysfunctions, like insulin resistance and obesity by activating antioxidative defenses [19]. Butyrate, another short-chain fatty acid produced by the gut microbiota, can inhibit HDAC class 1 and thus maintain homeostasis and oxidative status [20]. A further promising molecule in this context is spermidine, a polyamine which stabilizes DNA and RNA, has antioxidative capacities, and able to modulate various enzyme functions. In mammals, polyamine levels strongly depend on their nutritional supply as well as its synthesis by the intestinal micro-biota. However, polyamine proportion declines within age [21].

The aim of this study was to characterize the effects of different bioactive compounds on senescence status and gene expression of senescence-induced cells. As polyphenols constitute a promising substance group in this context, we investigated EGCG, anthocyanidin, resveratrol, and phloretin, but also spermidine, butyrate, and BHB with regard to their effect on *SIRT3* via *NRF2*, modulation of senescence, and SASP in senescence induced 3T3-L1

preadipocytes. Senescence was induced with a sublethal dose of BrdU, which is widely used to measure DNA synthesis in proliferating cells and has been known to alter growth and phenotype of different cells. BrdU as a 5-halogenated thymidine analogue is incorporated into the DNA which can result in DNA hypermethylation, mutations, chromatid breaks, and other lesions and induce a senescence-like phenomenon in every type of mammalian cell [22, 23]. To assess potential synergistic effects, cells were treated with these substances only, as well as with a mixture of EGCG, resveratrol, and spermidine.

Material and Methods

1.1. Cell Culture. 3T3-L1 preadipocytes were sponsored by the Department of Nutritional science Vienna. Cells were cultured as a monolayer in Dulbecco's modified Eagle medium (DMEM) high glucose (4.5 g/l) containing L-gluta- mine, 5% penicillin/streptomycin and 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. Studies were performed in the passage numbers 3 to 5. Cells were passaged before reaching confluency using 1x PBS and Accutase solution (all substances from Merck, Germany). This cell line was chosen because senescence in preadipocytes impairs their function and the cytokines released in senescent cells can be spread to non-senescent neighbored preadipocytes. Moreover, the release of cytokines is highly secreted in preadipocytes of older rats and can impair the recruitment of immune cells [15].

1.2. Substances. All substances were chosen from literature based on their impact to modulate senescence, their antioxidative properties, or their impact on health. Polyphenols and the polyamine used for this study were EGCG, anthocyanidin, resveratrol, and spermidine sponsored by System- Biologie AG (Switzerland). As secondary plant ingredients are report-ed to have potential synergistic effects, we tried a mixture of EGCG 40%, resveratrol 40%, and spermidine 20%. Used plant compounds are extracts of different plants. EGCG was extracted from the leaf of Camellia sinensis O. Kuntze; anthocyanidin was obtained from blueberries, with the major component of cyanidin. Resveratrol was extracted from the roots of Polygonum cuspidatum sieb. et Zucc and spermidine from wheat germ. Butyrate (B5887), β-hydroxy-butyrate (54965), phloretin (P7912), and roxithromycin (R4394) were purchased from Merck (Germany). After test- ing for cytotoxicity, we used the following concentrations for experiments: EGCG 50 µM and 100 µM, anthocyanidin 50 µM and 100 µM, phloretin 50 µM

and 100 µM, resveratrol 10 µM and 15 µM, EGCG-resveratrol-spermidine mix 20 µM and 30 µM, spermidine 5 µM and 10 µM, butyrate 2.5 mM
and 5 mM, and BHB 4 mM and 10 mM. Roxithromycin has antisenescence properties after BrdU treatment [23] and was used as positive control.

1.3. Cell Proliferation. The proliferative potential of cells dur ing treatment was assessed by the 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT; Merck) assay. Cells were seeded in 96-well microplates at a density of 5000 cells/well for the 24 h assay, 3000 cells/well for 48 h, and 1000 cells/wells for the 72 h assay. They were cultured for two days. Different densities and incubation times were only used for assessment for cytotoxicity to see potential cytotoxicity in confluent and nonconfluent cultures. Cells were treated with several different concentrations for each substance and each concentration was done in triplicates. After the respective treatment times, the media of the plates was discarded, and MTT was added to the wells together with DMEM high glucose without phenol red (Merck). This was followed by another incubation for 4 h at 37°C in a humidified CO2 atmosphere. The media was again discarded and 100 µl of acid isopropanol (0.1 N HCl in anhydrous isopropanol) were pipetted to each well to solubilize formazan precipitates. The absorbance of purple formazan was measured at a wavelength of 540 nm using a FLUOstar OPTIMA micro- plate reader (BMG Labtech). Each compound was assessed for cytotoxicity with several different concentrations and different timepoints. Proliferation rate and cytotoxicity were calculated relatively to the proliferating control cells, and concentrations for further experiments were determined by IC50 and listed in the chapter substances.

1.4. Senescence Induction and Treatments. Cells were plat-ed in 24-well plates at a density of 3000 cells/cm2. Around 80% confluency, cells were exposed to 100 µM BrdU for 8 days, including one medium change containing BrdU after 4 days. The concentration of BrdU and incubation length of all substances was determined based on the literature and proliferation assay results [23]. After final treatment with BrdU, cells were washed with PBS and kept for additional 96 h in fresh DMEM containing different substances, each in different concentrations. For testing anti-SASP effects all concentrations and substance combinations as mentioned above were applied for experiments. This set of experiments was done for β-galactosidase staining, ELISA and gene expression analysis, and for each analysis done in tripli-

cates and different timepoints. By day 8 of exposure to BrdU, treated cells acquire the senescence-like phenotype. The most widely used assay for senescence detection is the senescence- associated β-galactosidase activity at pH 6.0. The activity is based on the increased lysosomal content and reflects the increased autophagy as well as the enlarged lysosomal compartments in the cells. Cells were plated in 6-well culture plates at a density of 3000 cells/cm2. Cellular senescence was identified using the β-galactosidase assay (Biovision). At the end of the experiment, medium was aspirated from the cells, washed with PBS, and then fixed with fixation solution. Cells were washed again, stained, and incubated over night at 37°C in absence of light and CO2. Next day, cells were observed under microscope (Leitz LG91 Diavert wetzlar Germany) for the appearance of blue color as a marker of senescence associated β-gal activity. Several images were taken, and cells were manually counted. The experimental design is outlined in Figure 1.

1.5. ELISA. After respective treatments, the supernatants of cells were collected for detecting IL6 levels using a sandwich ELISA kit (Mouse IL-6 ELISA Kit Invitrogen by Thermo Fisher Scientific).

1.6. RT2-PCR. RNA was extracted using the MagMAX™ mir- Vana™ Total RNA Isolation Kit via King Fisher Duo Prime (ThermoFisher Sientific). Up to 1 µg template RNA was used for reverse transcriptase and cDNA amplification using Tag-Man Reverse Transcription Reagents (ThermoFisher Sientific). Real-time PCR was performed using GAPDH as housekeeping gene and SIRT3, CDKN1a and NRF2 as genes of interest using PCR condition, primer assay, and master-mix according to the manufacturer's protocol (all Thermo- Fisher Scientific). For comparison of runs, an untreated control was used on every plate. Relative expression was calculated using $^{\Delta\Delta}$ct method and expressed as $2^{-\Delta\Delta Ct}$ using the Gene Expression Software.

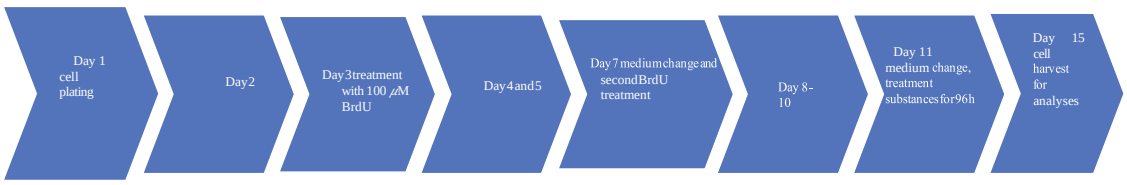


FIGURE 1: The experimental design outlined per day. Timeline for cell plating and senescence induction and incubation with different substances are addressed in this figure.

1.1. Statistical Analyses. Data was analyzed using the Graph- Pad Prism (Version 6) software and

data are presented as mean ± standard deviation (SD). Each experiment was done at least three times. Statistical differences between control and experimental groups were determined using one-way ANOVA ($p < 0:01$) with Dunnett's post hoc test.

Results

3.1. BrdU Induces Senescence-Like Morphology in 3T3-L1 Cells. Cells treated with BrdU developed a typical senescence like morphology. β-gal staining displayed large, flat, multinucleated, and enlarged nuclei compared to the control (Figure 2(b)). Furthermore, BrdU inhibited proliferation of 3T3-L1 cells.

3.2. Polyphenols Reduce Senescence Genotype Significantly. Assessment of *CDKN1a* gene expression of treated cells compared to untreated control was applied for verification of β-gal activity results ($p < 0:01$). Most substances could decrease β-gal activity, whereas not all changes were significant (Figure 3). Besides cells treated with 50 µM anthocyanidin, all other polyphenols reduced the senescence genotype significantly. BHB in a physiological concentration of 4 mM, which can be reached after a 5 days fasting period [24], reduced *CDKN1a* gene expression significantly ($p < 0:05$). Higher concentrations of BHB, representing a long-time fasting intervention, attenuated gene expression of this cell cycle inhibitor. For the other endogenous substances, no significant changes could be observed (Figure 4).

3.3. Polyphenols except Resveratrol Decrease IL6 Secretion in BrdU-Treated Cells. To assess the SASP state of treated cells, secretion of proinflammatory cytokine IL6 was assessed with ELISA. Analysis included only the highest concentration of substances. We could observe, that IL6 secretion increased strongly in BrdU-treated cells ($p < 0:002$). Except for resveratrol, all polyphenols inversed cytokine secretion significantly, with EGCG 100 µM showing the strongest effect ($p < 0:002$) (Figure 5(a)). Furthermore, IL6 secretion positively correlates with *CDKN1a* expression ($p < 0:002$) (Figure 5(b)).

3.4. NRF2 Gene Expression Was Higher in BrdU-Treated Cells. BrdU-treated cells showed a significant increase *in NRF2* gene expression compared to untreated control cells ($p < 0:05$). NRF2 gene expression of polyphenol treated cells positively correlates with senescence induction ($p < 0:01$) (Figure 6(a)). Conversely, cells treated with endogenous substances as

well as treatments with 100 µM anthocyanidin and 50 µM or 100 µM phloretin inhibited *NRF2* gene expression (Figure 6(b)). Although resveratrol and EGCG are known as *a NRF2*-activating polyphenol, only resveratrol 15 µM ($p < 0:01$) and anthocyanidin 50 µM ($p < 0:05$) could activate *NRF2* significantly.

3.5. SIRT3 Gene Expression Could Be Elevated by Anthocyanidin and EGCG. No correlation of *SIRT3* activation could be seen by increased *CDKN1a* gene expression. Our results show positive correlation of *SIRT3* expression and *NRF2* ($p<0:05$) (Figure 7(a)) and reduced IL6 levels ($p<0:05$) (Figure 8(a)). Latter could only be generated for the secondary plant ingredients. Only anthocyanidin 50 µM and EGCG 50 µM increased *SIRT3* significantly compared to roxithromycin 50 µM and 100 µM (Figure 7(b)). Although EGCG did not significantly stimulate *NRF2* expression, there is a dose-dependent correlation of *NRF2* and *SIRT3* activation ($p<0:05$), which we could not see for anthocyanidin (Figure 8(b)).

Discussion

Senescence cells can have both positive and adverse effects, depending on the disease or tissue. In cancer or liver fibrosis, senescence can be beneficial, thus restricting tumor progression and fibrosis, whereas in metabolic disorders it may be detrimental by contributing to the disease [2]. Reaching the threshold of storage capacity in adipocytes by caloric over- load triggers a stress response and macrophage recruitments [2]. Oxidative stress is of great interest to the study of obesity and its pathologies, like metabolic syndrome and diabetes, thus diminishing health span [25]. 3T3-L1 cells were chosen because the release of cytokines from preadipocytes influences the function of fat tissue, further the recruitment of immune and inflammatory cells leading to inflammatory states and pathological complications [15]. Exposure to a high fat diet leads to oxidative stress in a variety of tissues within the body. For this reason, studying senescence and oxidative stress and their effects on adipose tissue as well as the anti-SASP effects of different secondary plant ingredients and endogenous substances is of critical importance. NRF2 and SIRT3 play essential roles in the regulation of antioxidant defense [25]. Mitochondrial DNA damage is the molecular basis of cell senescence, and mitochondrial oxidative stress accumulation is a major factor to determine age related dis- eases and lifespan [13, 26]. Like Ozsvari et al., we used BrdU as a senescence inducer in 3T3-L1 preadipocytes to mimic obesity induced inflammation. Consistent with his study,

we observed that an application of sublethal BrdU concentrations could activate the senescence program in cells,

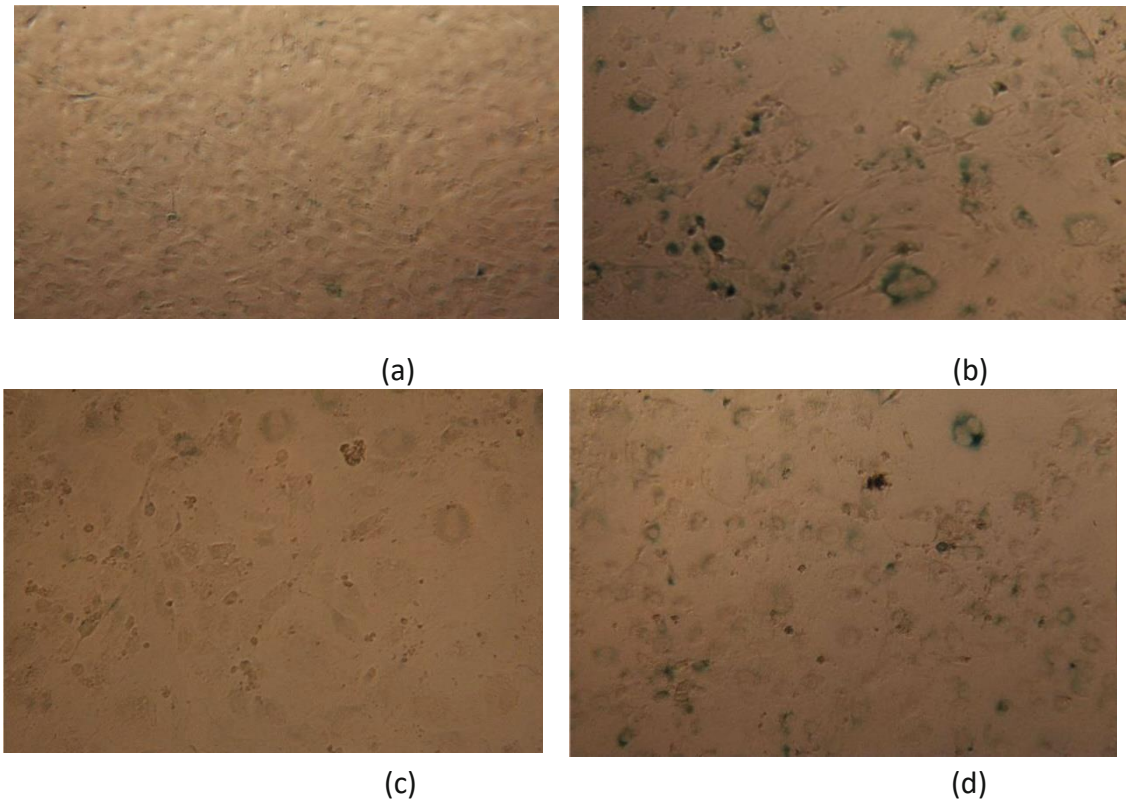


FIGURE 2: Beta galactosidase activity staining (blue) in 3T3-L1 preadipocytes. Untreated confluent 3T3-L1 preadipocytes show minor amount of senescence cells (a). After BrdU treatment for 8 days, cells show a typical senescence phenotype and increased beta galactosidase activity, which is indicated by blue staining (b). After induction of senescence with BrdU for 8 days, following a treatment with roxithromycin 100 µM for 96 h beta gallactosidase activity decreased resulting in significant less blue stained cells (c). EGCG 100 µM treatment for 96 h after 8days of BrdU treatment could decrease β-gal activity but not in the extend as roxithromycin (d).

including enlarged cell sizes, expression of SA-β-Gal, increased *CDKN1a* expression and development of SASP, by measuring IL6 levels [23]. Proinflammatory cytokines including IL6 are detrimental for the nearby cells by spreading inflammation leading to a disturbed tissue function [17]. BrdU activates cell cycle inhibitor CDKN1a and suppresses cell proliferation. In recent years, secondary plant ingredients, such as EGCG, resveratrol, and other flavonoids, have been investigated regarding their senolytic and antiaging properties [5]. In our study, all secondary plant ingredients but also BHB at a lower concentration diminished *CDKN1a*

expression and improved cell proliferation, but only polyphenols reduced the levels of IL6 secretion significantly. IL6 is a major cytokine, secreted in the SASP. Polyphenols have anti-inflammatory and antibiotic properties and may in addition activate the transcription factor NRF2. NRF2 plays a key role in cellular protection against oxidative stress and inflammation [27]. In the study of Liu et al., cells were treated with different substances to induce genotoxic stress. In combination with BHB p53 and its downstream target *CDKN1a* was deceased, induced by β-hydroxybutyrylation which is a novel histone posttranslational modification [28]. Spermidine and butyrate are known to reduce inflammation by targeting NF-κB, G-protein–coupled receptors, autophagy, or inhibiting HDAC class1 [29, 30]. In addition, the expression of many SASP components such as IL8 or IL6 are regulated by the activity of the transcription factor NF-κB, responsible for the development of inflammation [31]. In our study, we could not observe any significant anti-inflammatory effects of spermidine and butyrate. In this regard, the use of BrdU as strong genotoxic agent could alleviate potential beneficial effects of compounds. Further studies could include lower BrdU concentrations to assess anti-inflammatory effects. A chronic SASP has been associated with the spread of senescence and a high proinflammatory status, consequently contributing to a faster aging process, which can be also found in obesity and diabetes type 2 [5]. Therefore, a targeted modulation of SASP and senescence may constitute a powerful tool to increase health span. Polyphenols are auspicious compounds with multiple beneficial health effects including antioxidative and anti-inflammatory properties [5]. Their anti-SASP properties are of special interest. Their ability to activate ARE and NRF2 is determined by structural features. EGCG, phloretin, and related substances have the potential to induce *NRF2*, but only anthocyanidin and resveratrol could significantly increase *NRF2* in our study [16]. NRF2 is a stress responsive transcription factor balancing redox homeostasis by activating genes that encode cytoprotective, antioxidant, and phase II detoxifying enzymes [16, 32]. Several natural compounds have been identified as electrophilic NRF2 inducers, like resveratrol, as we could also demonstrate in our results [33]. The stimulation of NRF2

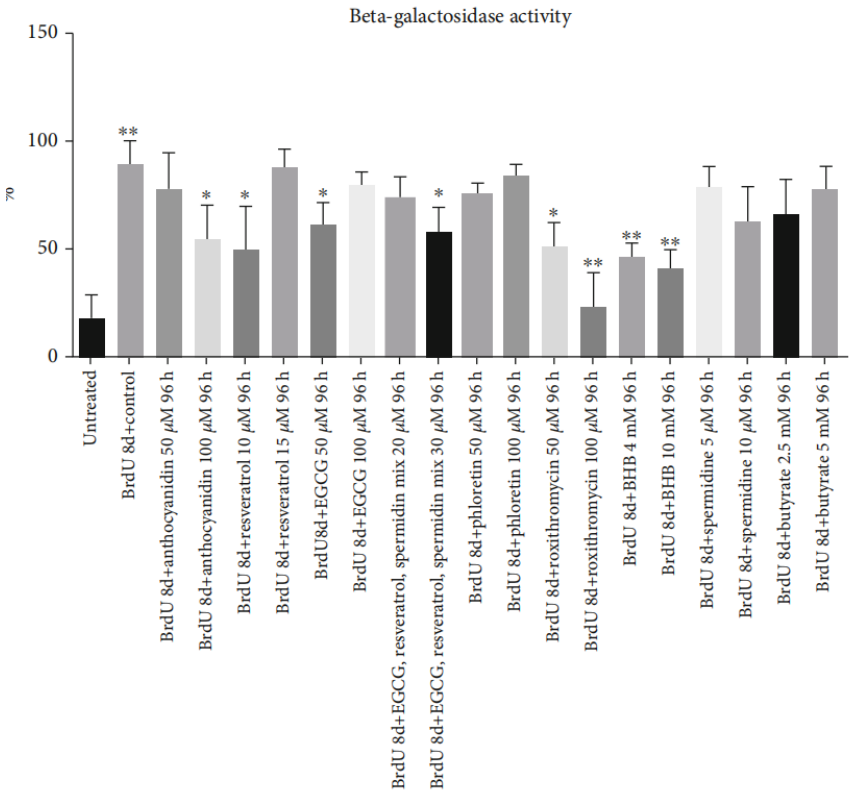


FIGURE 3: Percentage of β-galactosidase activity of all substances and concentrations in response to BrdU control. Beta-gal activity was significantly enhanced in BrdU cells compared to untreated cells ($p < 0:01$). Anthocyanidin, EGCG, resveratrol, EGCG-resveratrol- spermidine mix, BHB, and roxithromycin could change senescence phenotype and diminish beta-gal activity significantly ($^{*}p < 0:05$; $^{**}p < 0:01$). The results were expressed as mean ± SD. Statistical significance between compounds and concentrations to the control was determined by one-way ANOVA with Dunnett's post hoc test.

by exogenous substances (e.g., sulforaphane) leads to a decreased translocation of NF-κB to the nucleus, consistent with a lower DNA binding capacity and diminished proinflammatory action [34]. Nevertheless, NRF2 can also interact with CDKN1a, indicating that NRF2 is upregulated in cellular senescence [16], which goes in line with our observations, in particular after treatment with 50 µM anthocyanidin. When exposed to electrophiles or oxidative stress, cysteine residues of Keap1 are modified to prevent it from targeting NRF2 for proteasome degradation, resulting in rapid accumulation of NRF2. CDKN1a can stimulate or promote NRF2 activation while p53 or p65 decrease NRF2 transcription [34]. CDKN1a protects cells against oxidative stress through upregulation of the NRF2 signaling pathway and may be the first defense mechanism used to reduce reactive oxygen species (ROS) under low stress conditions

[16]. Mitochondrial ROS production induces different cell signals mediated by protein phosphorylation, NO synthase, and NRF2, which downregulate ROS by their feedback [35]. Apoptosis requires ROS accumulation, and consistent with that apoptosis is induced at high oxidative stress. NRF2 antioxidant response pathway must possibly be suppressed in order to induce apoptosis, which might mean that anthocyanidin (in higher concentration) and phloretin as well as some of the endogenous sub- stances used in our study act as a senolytic [16]. Kumar et al. investigated the effect of EGCG on senescent cells and observed a senolytic effect by diminished *NRF2* expression and inhibiting *Bcl2* [17]. In contrast, different studies summarize antiapoptotic effect of natural and synthetic compounds due to increased *NRF2* expression and ameliorating *Bcl2* expression [5, 16, 36]. In our study, endogenous substances, like spermidine, BHB, and butyrate, did neither increase *NRF2* nor decrease *CDNK1a* and IL6 levels. Nevertheless, most secondary plant ingredients, including the EGCG-resveratrol-spermidine mix, diminish *CDKN1a* and IL6 levels, although only anthocyanidin and resveratrol activated *NRF2* significantly. NRF2 can be activated both dependently and independently from Keap1. Latter activation is either due to oxidative stress or other NRF2 activators. Following this, NRF2 translocate from the cytoplasm into the nucleus and binds to ARE of different genes [32, 37]. Butyrate, but also BHB, has been shown to be potent NRF2 activators in different studies [38–40]. In contrast to our results, Kwak et al. demonstrate spermidine as a phase 2 enzyme inducer due to NRF2-ARE pathway activation [37]. In addition, recent studies indicate that *NRF2* binds directly to the *SIRT3* promoter, which leads to an increase in its expression [41, 42]. Although resveratrol increased *NRF2* expression in our study, *NRF2* further did neither stimulate *SIRT3* expression nor IL6 secretion was reduced. One possible explanation could be that resveratrol is not able to diminish genotoxic stress, caused by BrdU while inducing senescence.

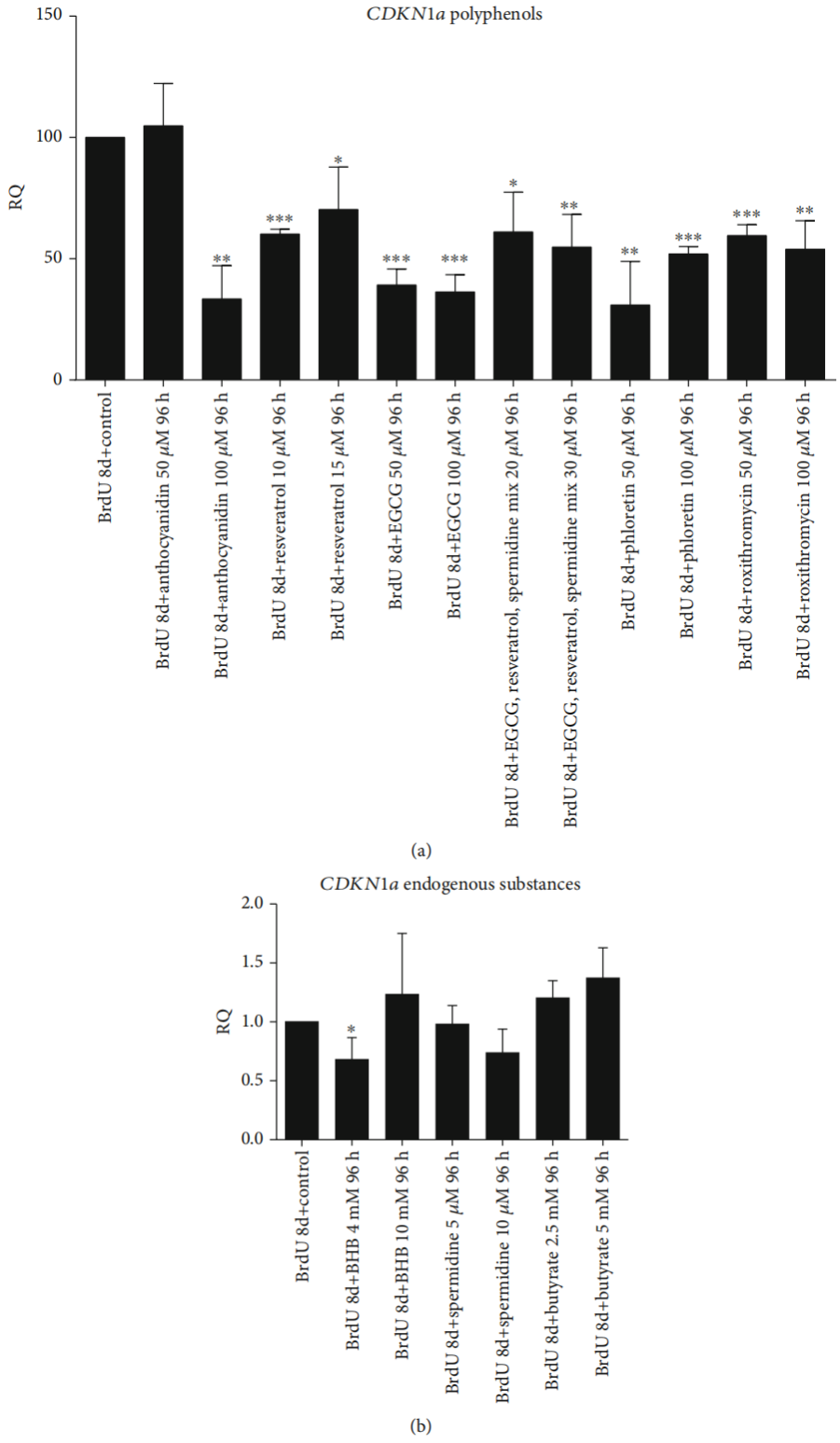
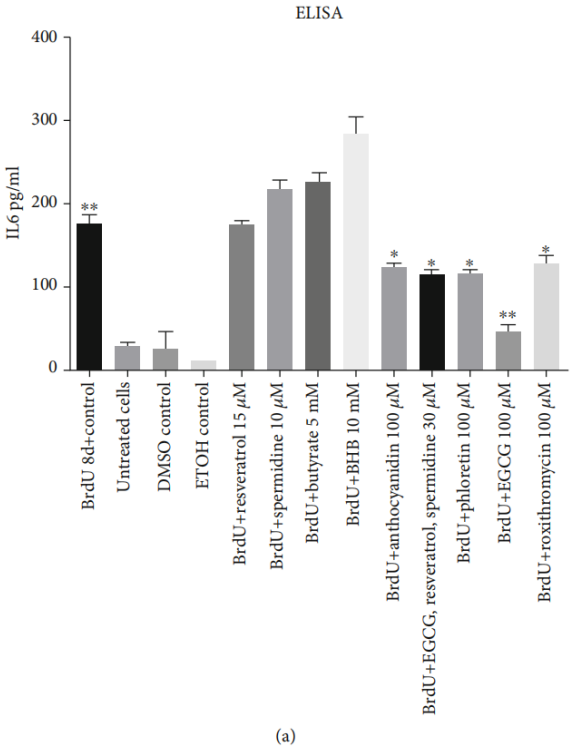
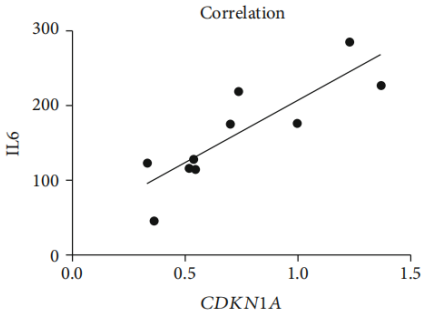


FIGURE 4: RQ value of *CDKN1a* mRNA expression. Cells incubated with BrdU showed an increase in gene expression, thus cell cycle arrest. Subsequent treatment with different substances showed a decrease of *CDKN1a* expression with secondary plant ingredients (a), but for endogenous substances this result could only be reached for 4 mM BHB (b). Statistical significance was defined $^{*}p < 0{:}05$; $^{**}p < 0{:}01$; $^{****}p < 0{:}001$. The results were expressed as mean ± SD. Statistical significance between compounds and concentrations to the control was determined by one-way ANOVA with Dunnett's post hoc test.





Pearson r	
r	0.8537
95% confidence interval	0.4845 to 0.9648
R square	0.7289
p value	
p (two-tailed)	0.0017
p value summary	**
Significant? (alpha = 0.05)	Yes
Number of XY pairs	10

(b)

FIGURE 5: IL6 levels pg/ml measured with ELISA in cells treated with BrdU and different substances, using the highest concentration. Compared to untreated cells IL6 secretion increased significantly ($p = 0:002$). Regarding BrdU control, most secondary plant ingredients besides resveratrol could decrease IL6 secretion significantly. Statistical significance was defined $^{*}p < 0:05$; $^{**}p < 0:002$. The results were expressed as mean ± SD. Statistical significance between compounds and concentrations to the control was determined by one-way ANOVA with Dunnett's post hoc test (a). Pearson's correlation analysis showed a significant correlation of *CDKN1a* expression with IL6 secretion including all substances ($^{**}p < 0:002$) (b).

The most consistent data was generated for roxithromycin, a drug described for their antisenescence properties [23]. Roxithromycin attenuated senescence status regarding *CDKN1a* and IL6 secretion and increased *SIRT3* expression via *NRF2*. Compared to the other treatment compounds, EGCG showed similar characteristics in terms of results in gene expression. Although EGCG did not increase *NRF2* significantly in our study, it activated *SIRT3* leading to reduced pro- inflammatory cytokine secretion and decreased *CDKN1a* expression. EGCG is known for its beneficial effects on human health, preventing inflammatory diseases attributed to its antioxidative, anti-inflammatory, radical scavenging, metal chelating, and anticarcinogenic properties [43]. Although IL6 levels decreased in senescent cells after most polyphenol treatments, our results suggest that this is not only by reason of *NRF2* activation

but rather, due to a direct activation of *SIRT3*. However, *NRF2* activation is depending on the chemical structure of the bioactive compounds [14, 32]. Interestingly, SIRT3 activity can reduce ROS levels by directly modulating key antioxidant enzymes, thereby acting as a shield against oxidative damage. SIRT3 deacetylates manganese superoxide dismutase (MnSOD) via FoxO3a in mitochondria and increases the ability to diminish ROS. Further, it activates isocitrate dehydrogenase 2 (IDH2) which produces NADPH needed for generating glutathione (GSH). Together with FoxO3a, SIRT3 upregulates all 13 mitochondrial encoded genes, resulting in an increase in mitochondrial respiration and biogenesis [19, 41, 44]. Thus, SIRT3 protects mitochondrial function, including ATP generation and mitochondrial membrane potential. However, studies show a *SIRT3* deficiency does not diminish increased ROS [41, 45]. Furthermore, reduced *SIRT3* expression is associated with cell aging and downregulated in metabolic syndrome, hyperlipidemia, diabetes, and smoking, thus related with human longevity [35, 46]. Dysfunction in the largest organ in humans, the fat tissue with its central role in metabolism is related to the onset of many age-related diseases [7]. Targeting compounds able to activate SIRT3 in senescent; proinflammatory cells can have profound clinical consequences.

Limitations

Like in various other in vitro studies, high concentrations of substances over physiological levels were used. However, in vivo studies would not achieve the concentrations of some sustances. Moreover, it would be interesting to investigate not only anti-SASP effects of these substances but also com- pare their senolytic effect, including markers, like Blc2 and a comparison of preadipocytes and mature adipocytes.

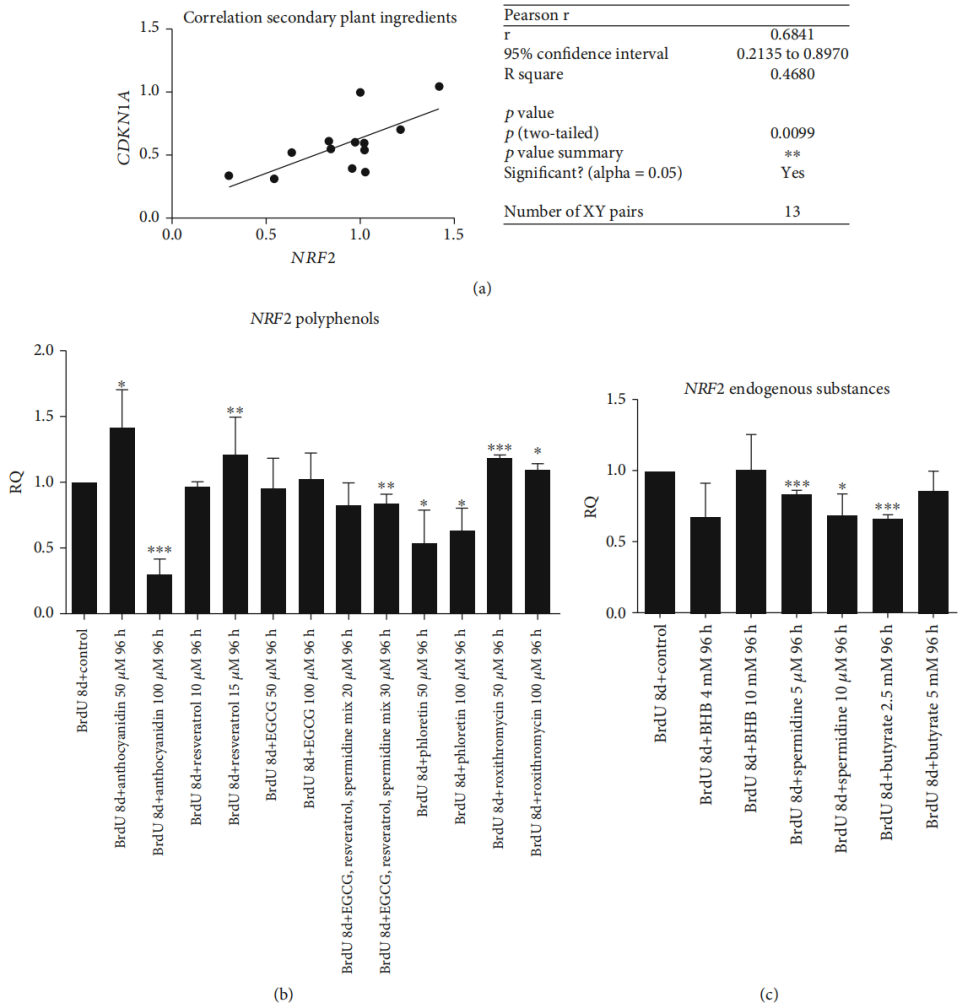
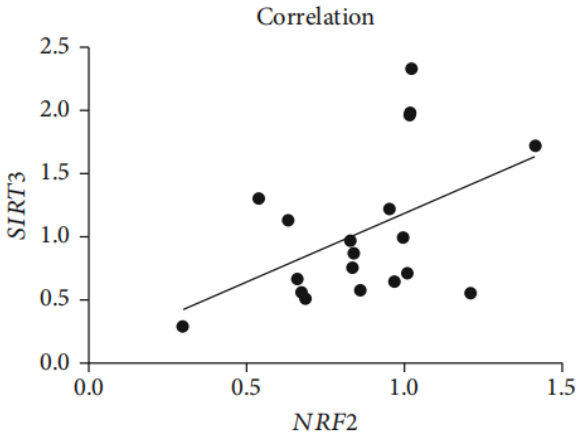


FIGURE 6: Pearson's correlation between *CDKN1a* and *NRF2*. Cell cycle arrest thus increase in *CDKN1a* positively correlates with *NRF2* expression ($p < 0:01$) (a). Not all secondary plant ingredients are *NRF2* activators. Relative quantification values of *NRF2* comparing all treatments regarding BrdU control (b+c). Anthocyanidin 50 µM, resveratrol 15 µM, and both roxithromycin concentrations increased *NRF2* antioxidative defense pathway significantly (b). EGCG, resveratrol, spermidine mix 30 µM, both phloretin concentrations, but also endogenous substances, like spermidine and butyrate diminished *NRF2* assuming lower ROS (b+c). Statistical significance was defined $^{*}p < 0:05$; $^{**}p < 0:01$; $^{***}p < 0:005$; $^{****}p < 0:001$. The results were expressed as mean ± SD. Statistical significance between compounds and concentrations to the control was determined by one-way ANOVA with Dunnett's post hoc test.

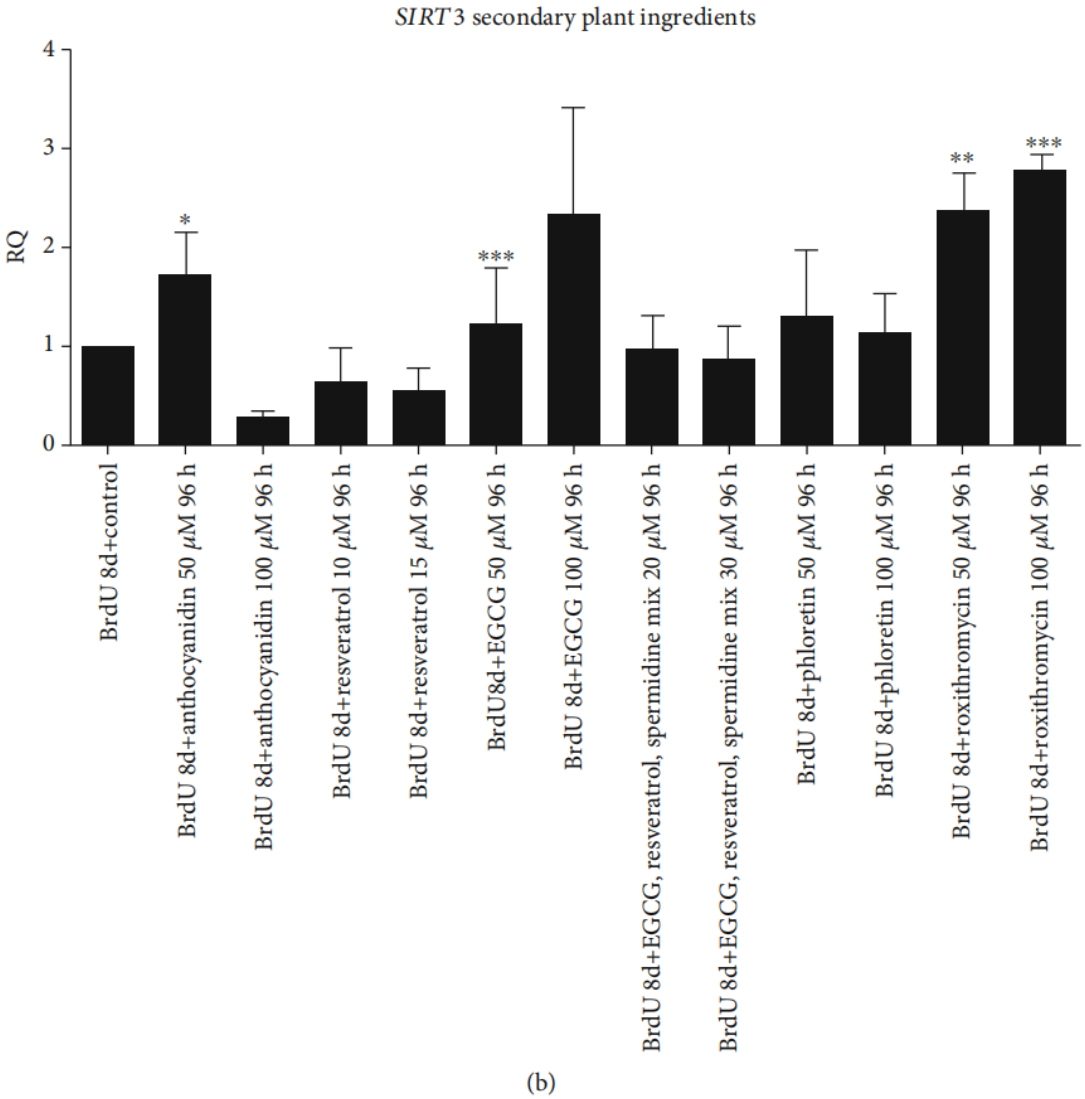
Conclusion

Accumulation of senescence cells is a hallmark of aging. Moreover, it is known that accumulation of these cells is increased in obesity. Although arrested in their cell cycle, these cells stay metabolically active and develop SASP, including secretion of cytokines thus leading to low-grade inflammation. Chronic low-grade inflammation which can be found in obesity leads to different disorders, like insulin resistance, diabetes, and hypertension. The present study demonstrates an anti-SASP effect of the selected polyphenols as well as spermidine, butyrate, and BHB on senescence induced preadipocytes. Additionally, resveratrol, anthocyanidin, and EGCG induced SIRT3/NRF2, a pathway believed to be reduced senescence.



Pearson r	
r	0.4773
95% confidence interval	0.02936 to 0.7656
R square	0.2278
p value	
p (two-tailed)	0.0388
p value summary	*
Significant? (alpha = 0.05)	Yes
Number of XY pairs	19

(a)



(b)

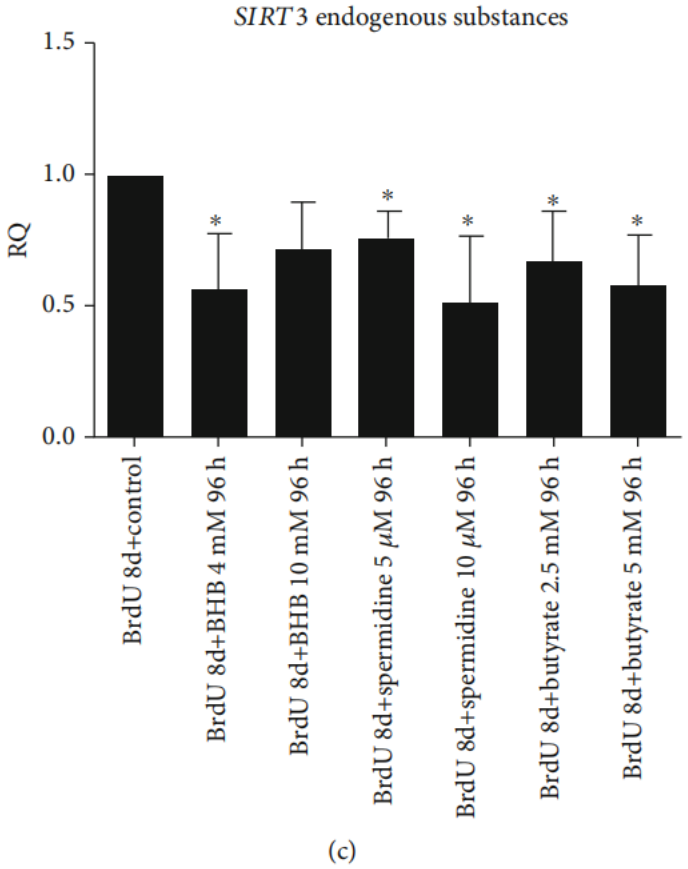


FIGURE 7: Positive Pearson's correlation between *NRF2* and *SIRT3* expression of all substances (a). Relative quantification values of *NRF2* comparing all treatments regarding BrdU control (b+c). Significant activation of *SIRT*3 can be seen with anthocyanidin and EGCG both at a concentration of 50 µM. EGCG 100 µM increased *SIRT3* expression but not significant (b). Both concentrations of roxithromycin increased *SIRT3* expression. The other secondary pant ingredients did either not influence *SIRT3* expression or like the endogenous substances ameliorate *SIRT3* (c). Statistical significance was defined $^{*}p < 0{:}05$; $^{**}p < 0{:}01$; $^{***}p < 0{:}005$; $^{****}p < 0{:}001$. The results were expressed as mean ± SD. Statistical significance between compounds and concentrations to the control was determined by one-way ANOVA with Dunnett's post hoc test.

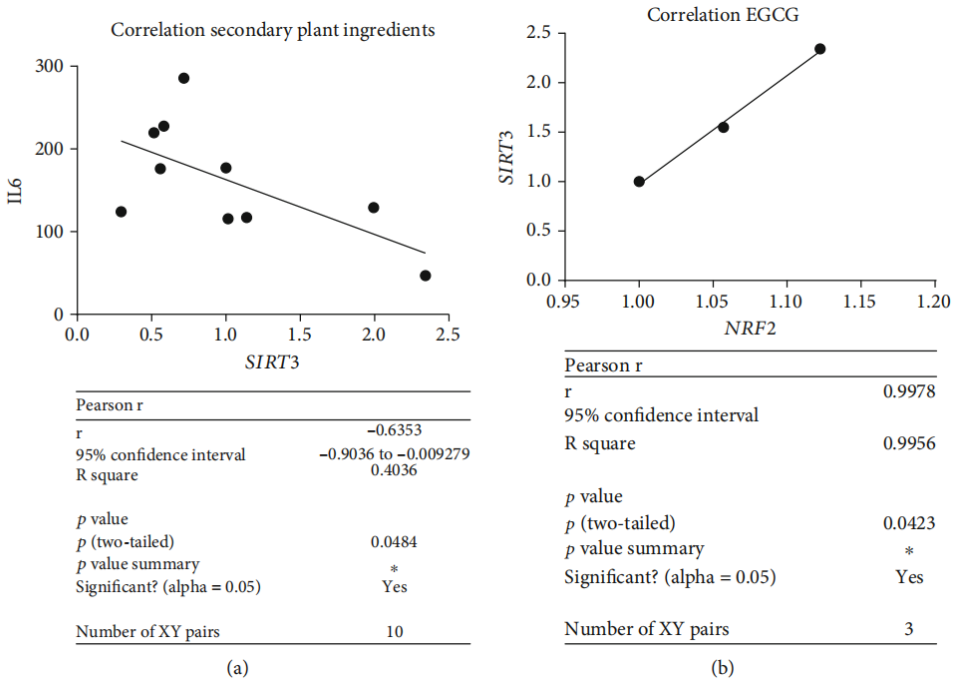


FIGURE 8: Pearson's correlation IL6 levels pg/ml and *SIRT3* gene expression RQ regarding treatment with secondary plant ingredients. *SIRT3* activation diminished IL6 secretion in senescent cells, thus ameliorating inflammation regarding secondary plant ingredients but notendogenous substances ($p < 0:05$) (a). Positive Pearson's correlation *SIRT3* and *NRF2* of EGCG in a dose dependent manner (0 µM, 50 µM, and 100 µM) ($p < 0:05$) (b).

Data Availability

Data will be made available in a special file on request.

Conflicts of Interest

The authors have no conflict of interest to declare.

Acknowledgments

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12.1.2 Fasting and fasting mimetic supplementation address sirtuin expression, miRNA and microbiota composition.

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Abstract

Background: Fasting and fasting mimetics - bioactive compounds mimicking fasting effects, are of growing interest as potential means to slow down the aging process and increase health span. Sirtuins are known as enzymes that interfere with mitochondrial energy metabolism and molecular pathways involved in longevity. Although their activation is determined as a response to stress i.e. caloric restriction. Sirtuin activating nutraceuticals are believed to mimic the effects of nutrient deprivation, thus activating signaling pathways correlated to an improved health span. In this study, we compare 5 days periodic buchinger fasting intervention with 3 months shot supplementation, a drink formula, containing secondary plant ingredients considered to activate sirtuins.

Methods We analyzed pathways in response to fasting and a sirtuins activating drink. Genetic and epigenetic biomarkers including telomere length, *LINE1* methylation, and a set of mRNAs and miRNAs were assessed using qPCR analysis. Gut composition and metabolites were compared using Illumnia sequencing and mass spectrometry.

Results Fasting, but also the fasting mimetic could increase expression *of FoxO1, SIRT1*, and *MLH1* mRNA, all genes discussed in aspects of longevity. A positive correlation between telomere length and both *SIRT1*, and *SIRT6* was observed. Furthermore, a

significant change in the gut composition was measured. Actinobacteria increased in the supplementation group, whereas after buchinger fasting a rise in the distribution of Proteobacteria could be observed. Firmicutes/Bacteroidetes ratio decreased and correlated with the body mass index (BMI).

Conclusions Our results confirm the effects of fasting on longevity associated mechanisms but also suggest that SIRTFOOD shot intervention addresses some of these effects.

INTRODUCTION

The hallmarks of aging are often summarized as genomic instability, epigenetic modification, telomere shortening, epigenetic alterations, altered intracellular communication and more[1]. Epigenetic mechanisms are agreed to be at the center of regulation of aging and the epigenetic clock is considered to be the most reliable marker for aging. As age increases, reactive oxygen species (ROS) amass and drive senescence pathways, impairing epigenetic methylation of CPGs, histones as well as miRNAs. Aging can address various mechanisms differently and recently, even certain age types were defined2,3. Calorie restriction (CR) has been discussed as a potential method of delaying the onset of aging conditions associated with increased morbidity and mortality. CR is defined as a moderate reduction of around 20-40% calorie intake compared to ad libitum without malnutrition and was found to slow down aging and extend healthspan[4,5]. Periodic fasting, like 'buchinger fasting' with a total daily intake of <250kcal is mostly unstudied.

During fasting pyruvate dehydrogenase kinase isoform 4 (PDK4) is upregulated, muscle and liver stores of glycogen are depleted to release glucose into the bloodstream6,7. Then, ß-oxidation provides energy by metabolizing the released free fatty acids to ketone bodies to serve as an alternative energy source[8,7]. The energy status in the cell is reflected by the adenosine monophosphate (AMP)/ adenosine triphosphate (ATP) ratio, which first increases during fasting or CR and triggers AMP-activated protein kinase (AMPK). AMPK promotes an intracellular increase in nicotinamide adenine dinucleotide (NAD+) levels, the rate-limiting substrate for silent information regulator 2 (SIR2), which is one of the critical mediators of CR-induced lifespan extension in yeast. In mammals, 7 sirtuins, SIRT1–7, have been identified [10,9]. Sirtuins are located in different parts of the

cells and have multiple functions including DNA repair, cell survival, interaction with metabolism, lipid and glucose homeostasis, stress resistance as well as insulin secretion[11,12]. SIRT1 is the most studied isoform among family members. It is associated with longevity and is often observed to be expressed in calorie-restricted cells[12]. A secondary molecular link between AMPK, CR, and SIRTs provides the Forkhead box subgroup O (FoxO) family[9]. The different transcription factors of this family promote fatty acid oxidation, suppress the generation of ROS and are linked to autophagic processes, thus slowing the accumulation of oxidative damage that might accelerate aging. FoxO is activated by its phosphorylation via AMPK and its activity is controlled through acetylation and deacetylation, which is altered by SIRT1. This further illustrates the networking interaction between AMPK, FoxO and SIRT1 in the regulation of many cellular processes involved in the adaptations to CR and promotion of longevity[9,13]. However, CR can affect gene expression through multiple mechanisms, like chromatin modifications, mRNA transcription and mRNA translation. In particular, control of the expression of regulatory RNAs such as microRNAs (miRNAs) is an important determinant in this regard[15]. MiRNAs have a variety of important functions including their modulating role in cell proliferation, differentiation, apoptosis and senescence[16]. mRNA can have several miRNAs regulators and vice versa17. More than 16 miRNAs regulate SIRT1 expression and activity under which miR34a has been the most studied. MiRNAs do not only have a crucial impact on a human's physiology, they are also able to enter microbial organisms. The host's miRNA may potentially affect the gut microbial ecosystem. The composition of the intestinal bacterial community can influence the digestion of various dietary compounds and thus, affect the host's health. Food components can themselves also impact the growth and metabolic activity of gut microbiota, their composition and/or potential functions[18,19]. These include secondary plant ingredients or other bioactive substances, known as functional food or nutraceuticals - a dietary supplement that in addition to its nutritional value, beneficially modulates body functions[19].

Recently, the terms CR mimetics or fasting mimetics have been used for substances that mimic the biological effects of nutrient deprivation involving the activation of different signaling pathways with beneficial effects in metabolism, DNA repair and thus improving

health span[20,21,22] . Polyphenols show increasing evidence to enhance the function of immune cells i.e natural killer cells to eliminate senescent cells. Moreover, multiple bioactive compounds, like EGCG, resveratrol, phloretin, butyrate and many more can act as epigenetic modifiers, modulate gene expression, DNA methylation and miRNAs[21,23]. Based on the observation that fasting and certain bioactive compounds have strongly overlapping physiological features regarding their impact on age-related pathways, this study has been conducted to compare the outcomes of buchinger fasting with a dietary supplementation containing different secondary plant ingredients, considered to activate sirtuins, and their relevance for salutogenesis24. 151 healthy men and women were included. We measured and compared the relative telomere length, Long Interspersed Nuclear Elements (LINE1) methylation, expression of 6 different mRNAs and 7 different miRNAs as well as the changes in the gut composition.

Material and Method

Experimental design: 180 people were enrolled for the study performed at the University of Vienna, Department for nutritional science. The study included three different intervention groups: buchinger fasting, fasting mimetics supplementation and placebo/control. After dropouts the fasting intervention group consisted of 20 people, the 131 that attended the fasting mimetic supplementary intervention, were divided into 100 participants for the active supplement and 31 participants consuming placebos, acting as the control group. The subjects were between 21 and 75 years (mean 43 years). The mean BMI was 26.255 kg/m2 ± 4.545 kg/m2 and weight 76.562 kg ± 15.420 kg. 44 participants (29.1%) were male and 107 participants (70.9%) were female. 124 participants of the total study population were nonsmokers and two thirds indicated to be physical active at least once a week. Exclusion criteria were metabolic diseases thus its medication. Moreover, participants taking probiotics, antibiotics or sirtuin activating compounds/medicine like metformin were excluded.

Dietary intervention: 20 participants joined a oneweek fasting program prescribed by Dr. Buchinger in Pernegg Monastery, defined and supervised by Mrs. Ingrid Höfinger, a fasting coach. The fasting program is described elsewhere[25] but in brief, consists of a total caloric intake of 250 calories a day for 5 consecutive days. The supplementation group was divided into a placebo control group and an active group. The supplement was

composed by the Swiss company System-Biologie AG (Wollerau) and will be addressed as SIRTFOOD shot. Both groups were advised to not change their lifestyle and nutritional habits during the intervention time. Participants had to include one flacon (25ml) of SIRTFOOD shot or placebo during the day for three consecutive months. The study was randomized, controlled and singleblinded. The supplement consists of prebiotic and secondary plant ingredients, known to act as fasting mimetics and able to active sirtuins according to literature. The following compounds can be found in one falcon (25ml) SIRTFOOD shot: 3.5 mg of gallic acid, 40 mg EGCG, 25 mg phloretin, 14 mg anthocyanin, 10 mg anthocyanidins, 6.4 mg oleuropein and 0.9 mg sulforaphane, 3g galactooligosaccharides. The compounds were extracted from mango, apple, blueberry, olive leaves, broccoli and green tea.

Sample collection: Food frequency questionnaires, dried blood spots, and stool samples were collected at two different timepoints at the beginning and after the intervention periods. After collection, stool samples were stored at -80°C. In accordance with the declaration of the Viennese Human Ethics committee all study participants gave written consent for the use of data. The beginning of the intervention is defined as timepoint 1 (T1). For fasting, the end of the intervention was defined as timepoint 2 (T2) and for the SIRTFOOD shot intervention as timepoint 3 (T3).

LINE1 methylation and relative telomere length: Total DNA and RNA were isolated from dried blood spots (DBS) using MagMAX FFPE DNA/RNA ultra-kit (Thermofisher, USA) via KingFisher Duo Prime purification system. Quantity and quality of isolated RNA were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). For analysis of LINE1 methylation, qPCR and high-resolution melt analysis were applied. EpiTect HRM PCR kit (Qiagen) and primers were used as described below, and analysis was carried out in a Rotor Gene Q (Qiagen). Unknown study samples were compared to standards of known methylation, generated with REPLI-g Mini Kit and mixed accordingly, to generate standards with 0, 25, 50, 75 and 100% methylation. Bisulfit conversion was done using the EpiTect bisulfite kit (Qiagen) following the manufacturers' protocol and using a maximum of 2 µg of genomic DNA. Relative telomere length was determined in genomic DNA isolated from the dried blood spots using a StepOne Plus real time PCR Detection System (Applied Biosysteme). For PCR, single-copy gene primers,

telomere primers (Biomers, Germany) and a LightCycler® 480 Sybr®Green I master mix (Roche) were used. Relative telomere length was calculated using the formula of 2−ΔΔCt (ΔCt= CtTelomere-CtReference; ΔΔCt= ΔCt-ΔCtmean placebo controls) as described elsewhere26. Primers for analysis of LINE1 and telomere length were selected from literature and purchased from Biomers, Germany. The primers were following: LINE1 forward TGTTAGATAGTGGGTGTAGGTT; for the reverse primer a 1:1 mixture was used from following primers LINE1 reverse 1: AATACATCCGTCACCCCTTT, LINE1 reverse 2: AAATACATCCATCACCCCTTT. Telomere forward CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT; telomere reverse GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT; telomere standard TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGG GTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGG, single copy gene forward CAGCAAGTGGGAAGGTGTAATCC, single copy gen reverse CCCATTCTATCATCAACGGGTACAA, single copy gene standard CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCA GGACTCGTTTGTACCCGTTGATGATAGAATGGG. Each sample was done in duplicates.

miRNA and mRNA expression: Changes in miRNA and mRNA expression for genes associated with antiaging and longevity (FoxO1, SIRT1, PDK4, MLH1) were determined using commercially available primers (Thermofisher, USA). cDNA of mRNA was done using LunaScript RT SuperMix Kit (Biolabs, Germany). cDNA synthesizing from miRNA was done using TagMan Advanced miRNA cDNA synthesis kit and reverse transcription was conducted using a MultiGene gradient Thermal Cycler (Labconsulting). Samples were run in 10 µL reactions in doubles, using TaqMan Fast advanced Mastermix (Thermofisher, USA) for RTqPCR amplifications performed on StepOne Plus realtime PCR Detection System (Applied Biosystem). All target mRNAs were normalized to GAPDH as an endogenous control. For target miRNAs, miR24 was used as an endogenous control. Fold changes for mRNA and miRNA were calculated using the ΔΔ cycle threshold (ΔΔCT) method, with fold changes expressed relative to the mean values for the control group, placebo using the formula as described for the telomere length[27].

Microbiota and microbial metabolites: Upon sample collection, stool samples were stored at -80°C. For sequencing microbial composition all fasting samples were analyzed by Biomes NGS GmbH (Germany) using Illumina Sequencing. Microbial metabolites were analyzed using mass spectrometry at University of Vienna.

SCFA MCFA analysis: The detection technique was established based on the published method 2-NPH or 3-NPH derivatised fatty acids analysis utilising LCMS28. Shortly dried pellets were resuspended in 150µl acetonitrile, samples were centrifuged at 14000rpm at 4°C, dried in the SpeedVac concentrator The metabolites were analysed by liquid chromatography coupled to mass spectrometry (LC-MS) using an Ultimate 3000 (Thermo Fischer Scientific, Waltham, Massachusetts, US) and a micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) with an Atlantis T3 3µm column (2.1x150mm, Waters, Milford, MA, USA) kept at 40°C.

Beta-hydroxybutyrate: BHB was measured from the fasting group only via on call GK Dual blood glucose and ketone meter (swiss point of care) using blood drops from finger before and after intervention.

Statistical analysis: All data are presented as mean ± standard deviation (SD). Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, USA) and graph pad prism (Version 6). Paired t-test was used to compare the different timepoints for parametric and Wilcoxon test for nonparametric values.

RESULTS

BHB and weight management: Fasting intervention resulted in ketogenesis with ß-hydroxybutyrate (BHB) increasing significantly after 5 days of fasting ($p < 0.01$) (Figure 1A). The participants lost on average 4.5 kg, whereas participants in the supplementation groups lost 0.5 kg on average (Figure 1B). Ketone bodies were not measured in participants of the SIRTFOOD shot intervention, because ketogenesis is not expected when continuing dietary habits with normal caloric intake.

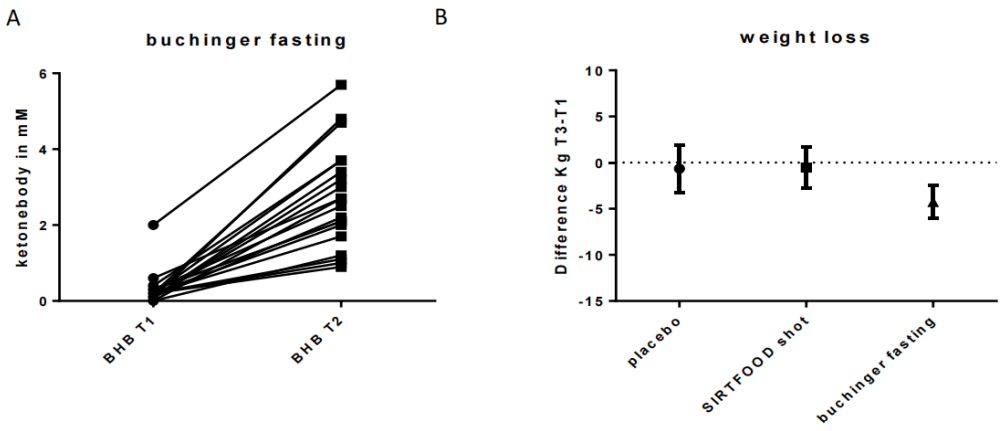
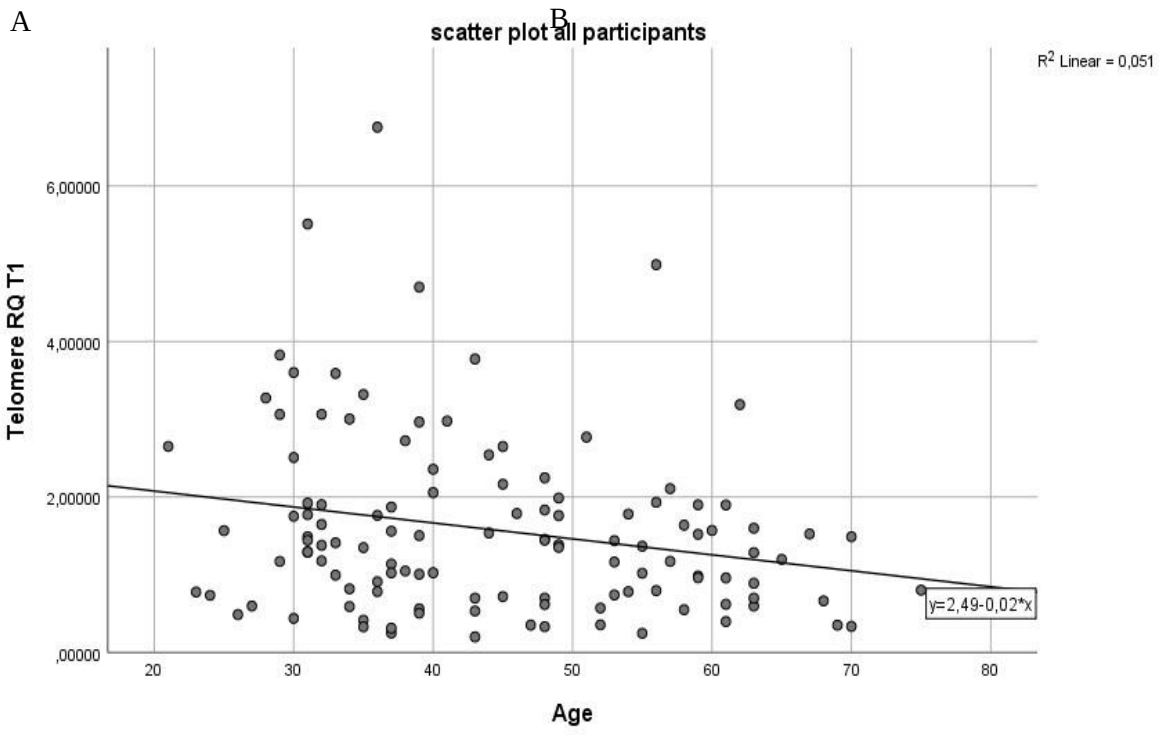


Figure 1*:* Ketogenesis measured *by* ketonebody BHB before and after buchinger fasting (A) ($p<0.01$). Weight loss with different interventions pictured in (B).



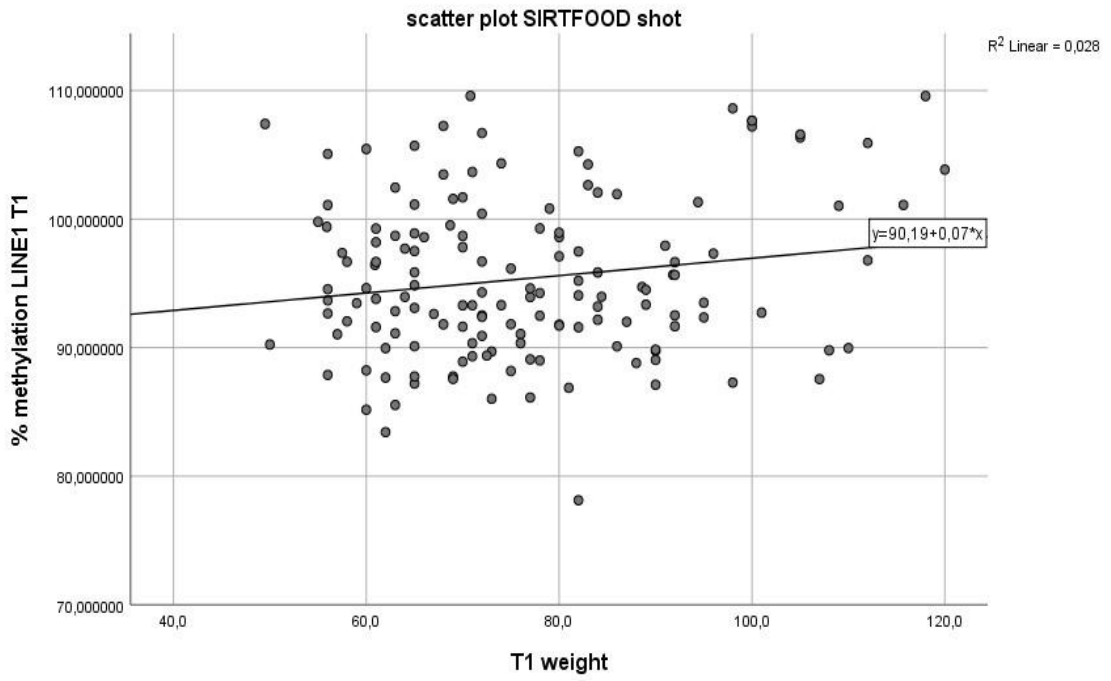


Figure 2: Output scatter plot Spss, Pearson's correlation. Negative correlation of relative telomere length and age of all participants (p< 0.05) (A). *LINE1* methylation positively correlated with weight before starting the SIRTFOOD shot intervention (p< 0.05) (B).

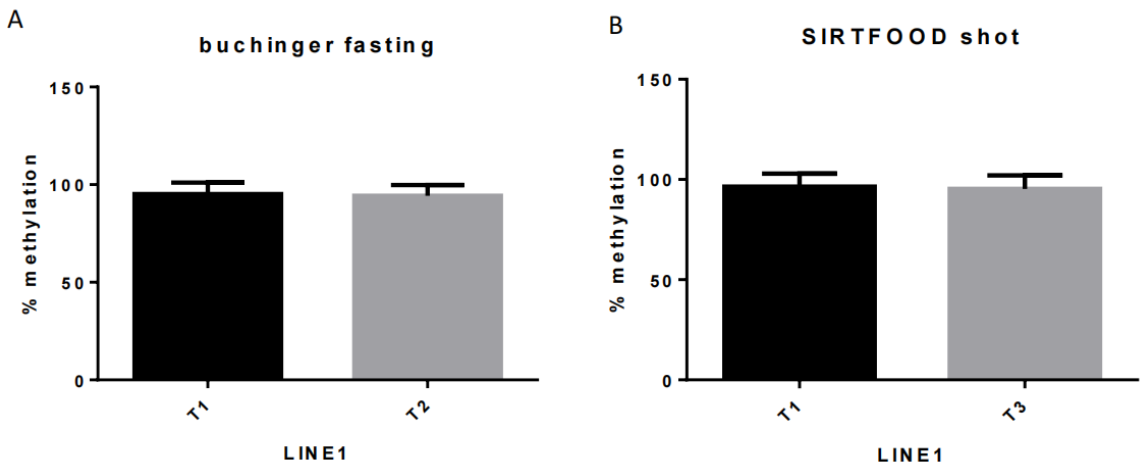


Figure 3: % LINE1 methylation for buchinger fasting and SIRTFOOD shot. No differences could be seen before and after the interventions in *LINE1* methylation (A&B).

Expression of mRNA

Both buchinger periodic fasting and SIRTFOOD shot intervention could increase mRNA gene expression relevant for mechanisms involved in longevity pathways. Both interventions significantly increased *FoxO1*, *MLH1*, *PDK4* and *SIRT1* gene expression (Figure 4A &B). *SIRT3* was elevated in the fasting group, but not significantly and *SIRT6* showed no effect (Figure 4B). SIRTFOOD shot had no effect on *SIRT3* gene expression, whereas mRNA levels of *SIRT6* decreased but not significantly (Figure 4A).

Expression of miRNAs

A set of different miRNAs was chosen depending on having an impact on the selected mRNAs or relevant for health span and longevity pathways. In the SIRTFOOD shot intervention significant changes were seen for *miR93-5p, miR16-5p, miR21-5p and miR34a5p*. *MiR125b-5p* decreased and *miRlet7b-5p* elevated but not in a significant extend (Figure 5A). buchinger fasting ameliorated *miR125b-5p, miR93-5p, miR165p, miR21-5p* and attenuated *miR34a-5p* significantly. No significant effect was seen for *miRlet7b-5p* (Figure 5B). No changes in *miR155- 5p* were seen in all samples. *MiR122-5p* and *miR33b- 5p* were under the detective rate, therefore, these two miRNAs were excluded.

Correlations mRNA and miRNA

A significant correlation was observed between the different mRNA or miRNA gene expressions. For the fasting group, *FoxO1* positively correlated with *SIRT1, SIRT3* and *MLH1* ($p < 0.05$) and *MLH1* positively correlated with all genes besides *SIRT6* ($p<0.01$). Moreover, ß-oxidation is controlled by *PDK4,* which overexpression increased *SIRT1* and *SIRT3* expression ($p<0.02$). Telomere length and *SIRT1* expression positively correlated at the beginning of the fasting intervention ($p< 0.02$) (Figure 8A), but not for SIRTFOOD shot. Nevertheless, for the SIRTFOOD shot intervention multiple correlations were observed as well, like *FoxO1* upregulation positively increased *MLH1, PDK4 and SIRT1* gene expression. Connections between miRNA and mRNA expression were also seen. *SIRT1* and *miR34a-5p* showed a negative correlation in the SIRTFOOD shot intervention, although only a strong trend. Telomere length positively correlated with *SIRT6* and *miR125b-5p* expression for the supplementation intervention ($P<0.05$) at the baseline.

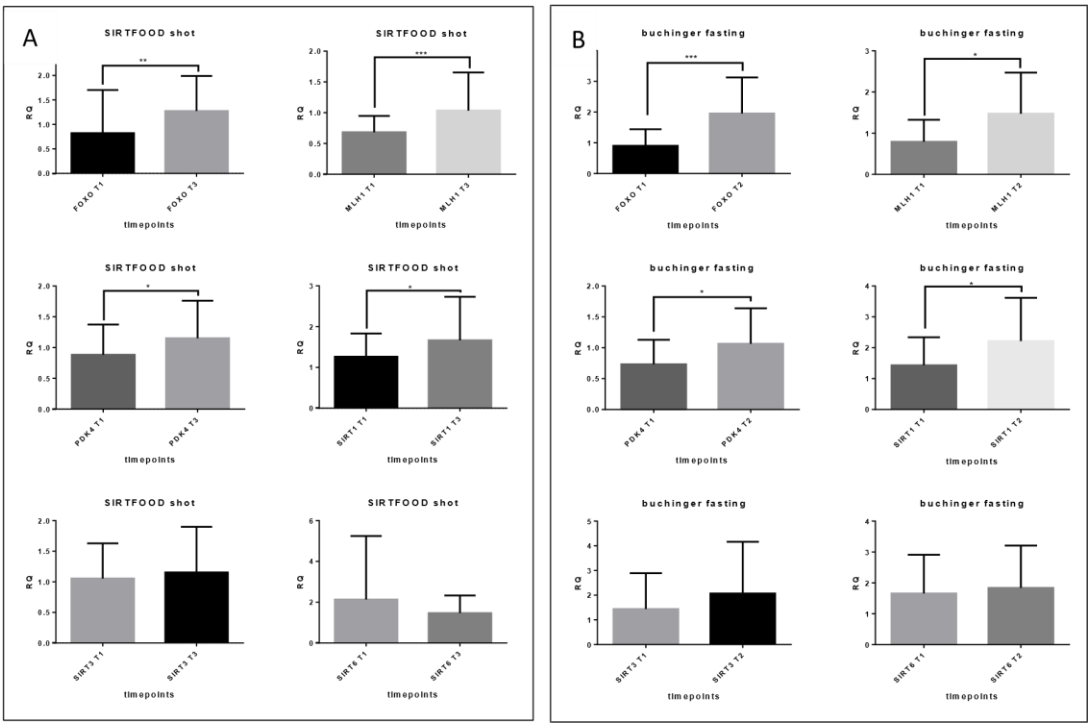


Figure 4: RQ selected mRNA gene expression (*FoxO1, MLH1, PDK4, SIRT1, SIRT3, SIRT6*) SIRTFOOD shot and buchinger fasting. The results are expressed as mean +/- SD. Statistical significance between timepoint 1 (T1) and end (T2 or T3) of the intervention was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

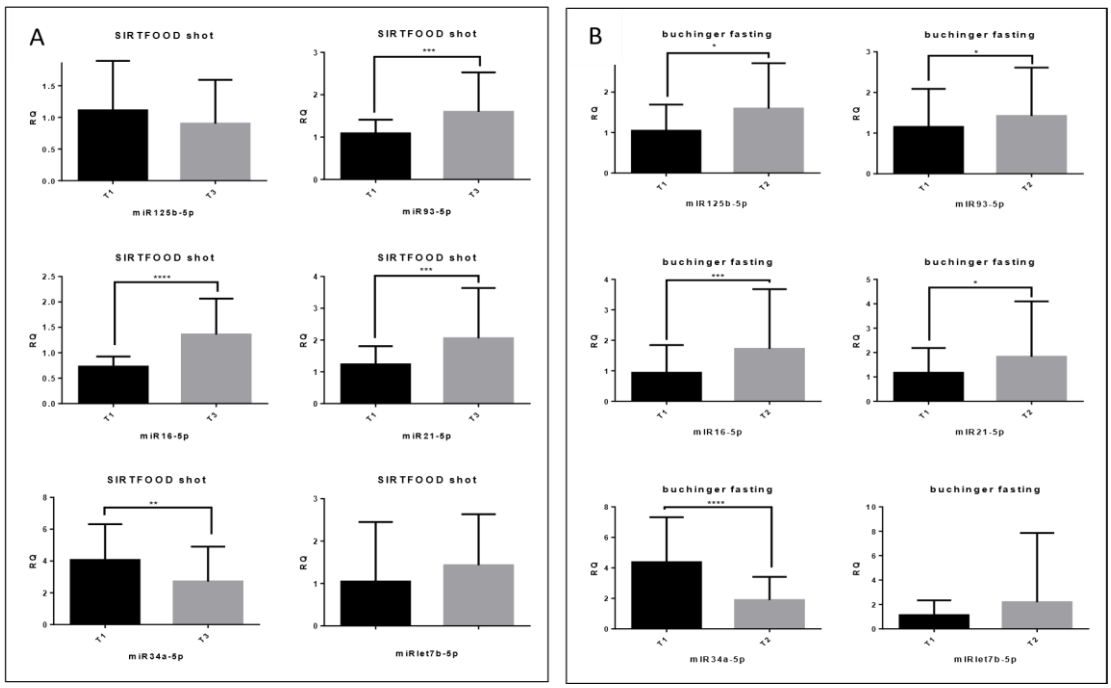


Figure 5: RQ selected miRNA gene expression (*miR125b-5p, miR93-5p, miR16-5p, miR21-5p, miR34a-5p, miRlet7b-5p)* SIRTFOOD shot and buchinger fasting. The results are expressed as mean +/- SD. Statistical significance between timepoint 1 (T1) and end (T2

or T3) of the intervention was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Microbiota and metabolites

No changes for *Actinobacteria, Bacteroidetes and Firmicutes* were seen after the fasting intervention. However, *Lentisphaerae* decreased, *Tenericutes* and *Verrucomicrobia* elevated, but not significantly. A strong trend was seen in the switch of the abundance of *Euryarchaeota* and *Cyanobacteria.* Latter was increased after the intervention, whereas *Euryarchaeota* attenuated after periodic fasting. Significant elevated changes in microbiota were observed for *Proteobacteria, TM7* and *Fusobacteria* (all $p < 0.05$) (Figure 6A). For the SIRTFOOD shot intervention, a different pattern in the switch of microbiota can be seen. *Tenericutes* had the same result comparing buchinger fasting and SIRTFOOD shot intervention. Three months of supplementation lead to an increase in *Euryarchaeta*, *Bacteroidetes*, *Lentisphaerae* and decreased *Cyanobacteria* and *Verrcoumicrobia* but not in a significant manner. In comparison to periodic fasting where a significant increase can be seen for *Proteobacteria, TM7 and Fusobacteria*, SIRTFOOD shot decreased the amount of these phyla but not significantly. Nevertheless, a strong trend in *Firmicutes* reduction and a significant elevation in *Actinobacteria* ($p < 0.05$) was observed with the supplement (Figure 6B). For the placebo group, no changes were seen in any of the phyla (Figure 6B). On the level of short-chain fatty acids (SCFAs) produced by bacteria, no changes can be seen after the SIRTFOOD shot intervention, whereas periodic fasting significantly increased butyrate level (Figure 7A). At the end of the intervention, a positive correlation can be observed for abundance in *Tenericutes* and the amount of butyrate produced ($p = 0.05$). Although only a strong trend, medium-chain fatty acid (MSCFa) caprylate decreased after periodic fasting, but not for the other intervention. Statistical interactions with miRNAs and phlya were seen for *Cyanobacteria* and *miR16-5p*, with a positive correlation after the fasting intervention. The same phylum and additionally *Lentisphaerae* positively correlated with *miR34a-p5* at the beginning of fasting ($p < 0.02$). Interestingly, *SIRT1* expression positively correlated with the abundance of *Actinobacteria* and negatively with *Cyanobacteria* at the end of fasting, although not significantly. Nevertheless, *SIRT1* expression positively correlated with *Fusobacteria* after an intervention ($p < 0.05$). In the supplementation group, Bacteroidetes positively affected *miR125b-5p* (Figure 8B), *miR16-5p, miRlet7b-5p, MLH1* ($p < 0.05$) and *SIRT1* expression, although only a trend. Moreover,

strong trends were seen for *Actinobacteria* and *Cyanobacteria* with different miRNAs, such as *Verrucomicrobia* and *miR34a-5p*. Latter bacterial phylum also significantly correlated with SIRT6 expression in a positive extend but decreases with age, observed for the fasting group ($p < 0.05$) Significant interactions are seen for *Firmicutes/Bacteroidetes* ratio with BMI and weight for all the participants and the SIRTFOOD shot intervention ($p<0.05$) (Figure 8C&D).

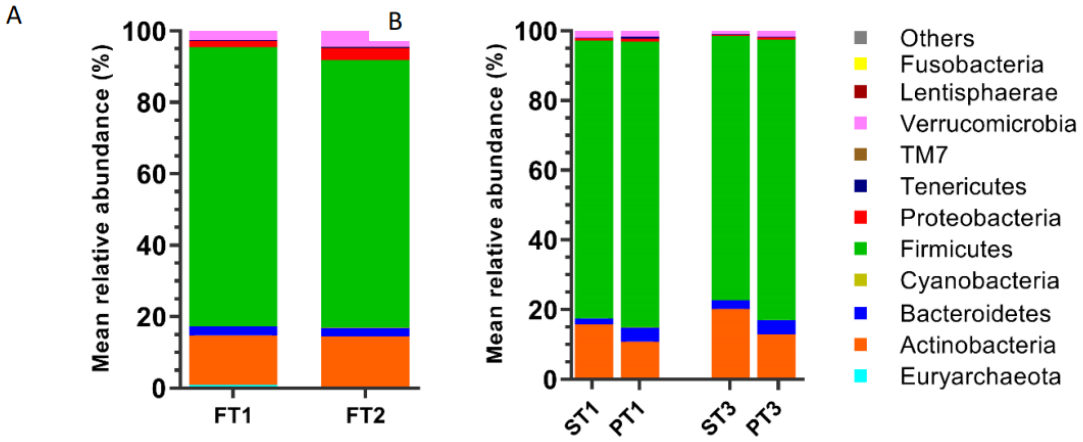


Figure 6: Abundance microbiota by phyla for fasting group (A), SIRTFOOD shot (ST1 vs ST3) (B) and placebo group (PT1 vs PT3) (B). Results are expressed in percentage of the mean of relative abundance for the different phyla. Statistical significance between timepoint 1 (T1) and end (T2 or T3) of the intervention was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

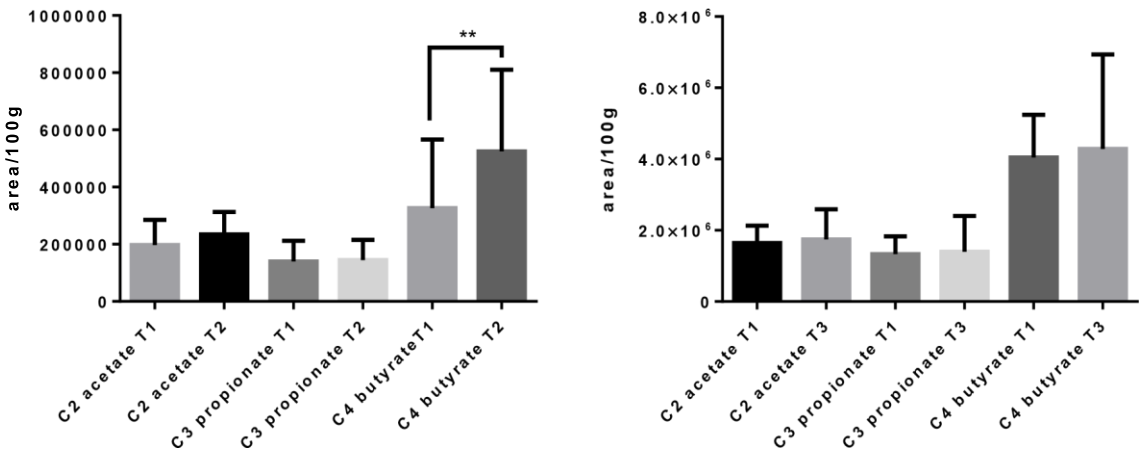


Figure 7: Amount of SCFAs produced given as area/100g stool for buchinger fasting (A) and SIRTFOOD shot (B) interventions. Statistical significance between timepoint 1 (T1) and end (T2 or T3) of the intervention was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

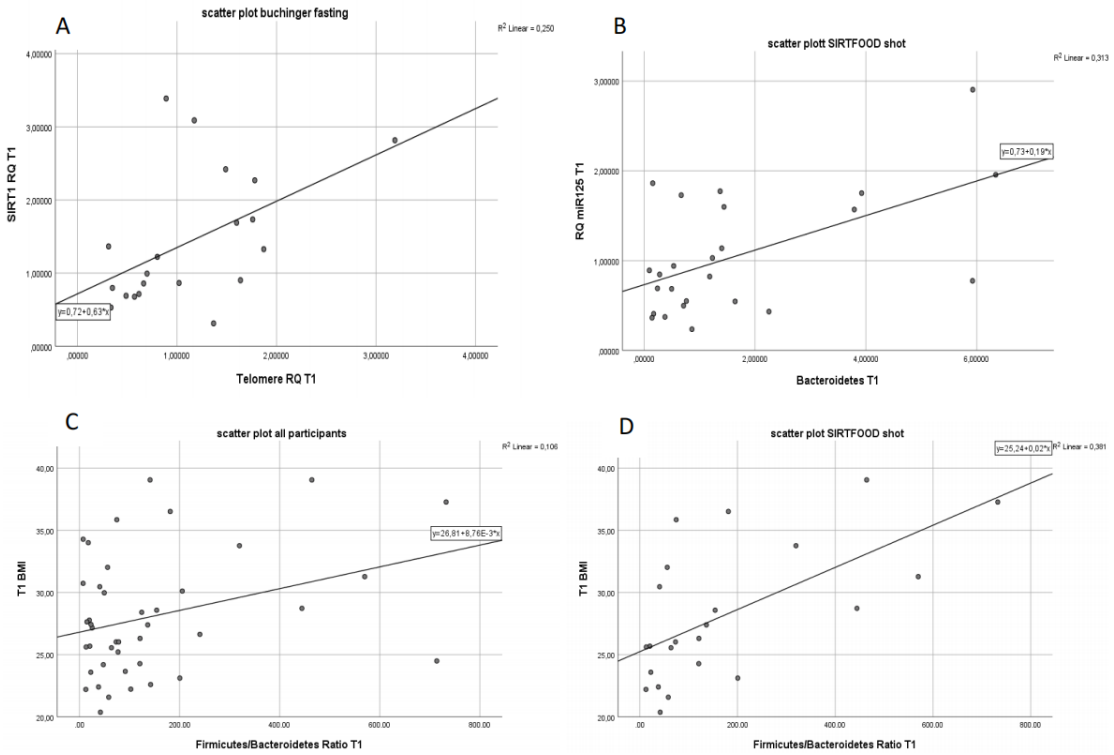


Figure 8: Spss output scatter plots. (A) shows a positive correlation between telomere length and *SIRT1* expression for buchinger fasting at baseline. *Bacteroidete*s and *miR125b-5p* positively correlated in the SIRTFOOD shot intervention at baseline(B). For all participants the ratio of *Firmicutes/Bacteroidetes* increased with higher BMI (C), which was also seen for the SIRTFOOD shot intervention Discussion (D). Statistical significance was defined as $p < 0.05$.

DISCUSSION

CR has been widely discussed for its beneficial effect on human health. In animal studies, CR was reported to elevate sirtuin expression but to our knowledge, the effect of sirtuin expression after consecutive fasting days has not yet been studied in humans. The term fasting mimicking diet has been increasingly used in literature recently and its positive impact on human health has already been revealed [5]. Anyway, many fasting mimicking diets, like the SIRTFOOD diet developed by Goggins and Matten, result in a reduction of caloric intake, when changing their dietary habits [29,5]. Our study was conducted upon our vitro study with different secondary plant ingredients regarding senescence and sirtuin expression. Now we wanted to observe the potential of periodic fasting and/or supplementing of a combination of secondary pant ingredients without changing dietary habits on sirtuin expression. Certain polyphenol combinations exhibit synergistic effects with a higher biological activity than the sum of the individual ones [30,31]. Various aging

biomarkers have been identified to monitor the personal response on lifestyle, nutrition and environment. Among them, *LINE1* methylation as a marker for global methylation level has been positively associated with BMI and obesity[32]. Our results showed no differences in *LINE1* methylation before and after the intervention, like in the study of Duggen et al. [33]. However, as Marques-Rocha et al. could observe, the methylation level of participants in our study also correlated with body weight [34,33]. Yet, they found a higher methylation level in individuals with lower body fat mass, which we could not see in our data [34]. Regarding telomere length, we observed a positive correlation with weight loss in contrast to Mason et al., who did not see any changes resulting from different diet and sport interventions. One possible explanation might be the differences in study populations [35]. Our study constitutes a very heterogenous group of individuals, to assess potential wide-range effects. Furthermore, we observed a correlation between *LINE1* hypomethylation and increased telomere length, assuming *LINE1* methylation decreases with lower body fat mass thus resulting in higher telomere length. Baseline telomere length attenuated with age in the SIRTFOOD shot group, but not for the buchinger fasting. This result may be explained by the population size, intervention time and age distribution of the different interventions. Telomere shortening is a hallmark of aging which implicates driving pathologies and aging [36,1]. Both interventions increased SIRT1 significantly. Glucose deprivation, like in fasting leads to a switch in AMP/ATP and increased NAD^+ concentrations, subsequently activating AMPK and sirtuins. Bioactive substances target sirtuins, by targeting nicotinamide phosphoribosyltransferase (NAMPT), an enzyme in the NAD salvage pathway, which leads to an increased NAD+/NADH ratio. Polyphenols can target AMPK, by blocking the F0F1ATPase/ATP synthase, the complex IV in the respiratory chain reaction in mitochondria, leading to a switch in the AMP/ATP ratio and elevation on NAD+ in the cell [37,38]. At baseline, telomere length positively correlated with *SIRT1* expression in the fasting intervention and with *SIRT6* for SIRTFOOD shot. A study conducted by Palacious et al. demonstrates *SIRT1* having a positive effect on telomere length, but only in mouse models [39]. SIRT6 was found to protect DNA by maintaining telomeres and modulating 5-hydroxymethylcytosine, the first oxidative product in the demethylation of 5 methycytosine[12]. Moreover, *SIRT6* depletion results in abnormal telomeres, leading to genomic abnormalities and premature senescence [40]. Although, we observed *SIRT1*

expression increased significantly in both groups, no changes in telomere length were seen before and after the interventions. Another longevity-related protein of the sirtuin family is SIRT3, which attenuates ROS through superoxide dismutase 2 (SOD2) delays cell senescence and decreases oxidative [12,41]. SIRT3*,* a mitochondrial sirtuin that regulates the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase, impacts ketone body production [42]. Although *SIRT3* activation was not significantly, periodic fasting elevated BHB to an extend of 5.7 mM. However, ketosis and ketone body production were very unequal because participants were allowed to consume a spoon of honey in case of dizziness, which could have an impact on ketone body generation. Ketogenesis is a metabolic mechanism in mitochondria responding to fasting[43]. Increased *PDK4* gene expression is usually observed during fasting, which inhibits pyruvate dehydrogenase complex, conversion of pyruvate to acetyl CoA decreases and at the same time ß-oxidation increases to provide energy [8]. Remarkably, we could see this for the SIRTFOOD shot group, assuming increased fatty acid oxidation also in the supplementation group [44,45,46]. It is well known, that an increased ß-oxidation and decreased glucose concentrations lead to *FoxO1* activation [46]. The axis between *SIRT1/ FoxO1* and *PDK4* is not yet fully understood, but *SIRT1* modulates upstream signaling of *FoxO1* and its activity improves glucose utilization [44]. Many miRNAs have been studied and new connections with our lifestyle and pathways can be generated, thus pattern can be seen of miRNAs and age-related diseases, like lower *miRlet7b-5p* levels in cardiovascular diseases and diabetes type 2[47]. Although our results in this regard were not significant, Cannataro et al. showed that *miRlet7b-5p* increased during ketogenic diet [17]. Overexpression of *miR21-5p*, which our results show for both interventions, is often associated with inflammation and cancerogenesis, but can also dampen cytokine secretion mediated by toll-like receptor 4 (TLR4)[48,49]. Several papers implicated the interaction of SIRT1 downregulation via *miR34a-5p* overexpression [50,51]. *miR34a* is not only associated with different cancer types, but moreover it also interacts with the cardiovascular system and metabolism by modulating SIRT1 expression. Overexpression of *miR34a* inhibits SIRT1 expression and endothelial senescence. *miR34a* is elevated in diet-induced obesity with a concomitant decrease in insulin sensitivity [16]. At the baseline for the SIRTFOOD shot group, we could also observe that an overexpression of *miR34a-5p* correlates with decreased *SIRT1* expression. However, *SIRT1* can also be expressed in the gut which plays

an important role in health and disease [52]. Gene expression is modulated by lifestyle, furthermore, miRNAs interact with our microbiome and their metabolites. Nonetheless, the microbiome can be also modulated with nutrition. Similarly like recently observed in another survey, we assessed a positive correlation of *Firmicutes /Bacteroidetes* ratio and BMI, whereas the ratio decreased after both intervention [53]. Furthermore, in the SIRTFOOD shot group a significant increase of *Actinobacteria* could be seen, which agrees with the literature, where secondary plant ingredients, like gallic acid and resveratrol were observed to modulate the abundance of

Bifidobacteria, which constitute a major part of the phylum *Actinobacteria* in the human gut [19,52]. However, the prebiotic agent, which the supplement consists of, could be a more reasonable explanation for this change [54]. Nevertheless, *Bifidobacteria* exhibit beneficial effects on the host as it promotes gut maturation and integrity, protects against pathogens, and modulates immunity. Moreover, agerelated changes in physiology and function of the gastrointestinal tract could result in a decline in *Bifidobacteria*, which could be reversed with the supplement [55]. We found only two other studies assessing changes in gut diversity after buchinger fasting. Like Mesnage et al., we observed a major decrease in *Firmicutes* and a significant increase in *Proteobacteria* after periodic fasting. *Proteobacteria* are usually associated with inflammatory bowel disease, but it is important to mention, that these are strain dependent [18]. Differences in miRNAs expression patterns can be seen in stool and plasma samples regarding nutritional habits [56]. Hewel et al., could identify that *miR125b* and *Bacteroidetes* have multiple potential identical targets, for which we saw a significant positive interaction with the supplement [57,58]. Moreover, Martinez et al. showed that *miR125b-5p* and *miR16-5p* are downregulated in participants with irritated bowel syndrome, assuming that the increased amount of *Bacteroidetes* and overexpression of *miR125b-5p* after SIRTFOOD shot supplementation could have a positive impact on intestinal barrier function [59]. This interaction of course could not be seen for the fasting intervention because without solid food for 5 days this bacterial phylum cannot grow and therefore, no changes in the amount of *Bacteroidetes* were observed for the fasting intervention. Nevertheless, for this group we observed a positive correlation of *Tenericutes* producing butyrate, which was already identified as a butyrate producer by Vital et al. [60]. The anti-inflammatory properties of butyrate have many beneficial effects on health [61]. At last, we identified a positive correlation of the

abundance of *Bacteroidetes,* another butyrate producer, with telomere length and a decrease of *Verrucomicrobia* with age, which further affects *SIRT6* expression for this group ($p<0.05$). Fransen et al. observed the same result for mice and the amount of this phylum decreases with age, but to our knowledge, little is published about sirtuins stimulating humans microbiota [62].

Limitations:

Like in various other in vivo studies, the bioavailability of the bioactive substances is a major concern. Testing the compound individually would be interesting to investigate. The effects of the different microbial compositions are very strain-specific, subsequently, changes in bacterial strains and its interaction are of high interest for our future studies. The effects of polyphenols and the galactooligosaccharides in the drink should be elucidated specifically.

Conclusion and perspectives: Our study provides results from 5 days of periodic fasting and a fasting mimetic supplement within gene expression involved in health span and longevity. Periodic fasting activated SIRT1 and modulated gut microbiota. Supplementing a combination of bioactive compounds without reducing caloric intake shows similar expression patterns of our selected mRNA and miRNAs. Changes in the microbiota, especially *Actinobacteria, a butyrate producing bacteria phylum* were observed with the supplement. Both interventions revealed results with beneficial outcomes for human health and confirm the effects of fasting on longevity associated mechanisms but also suggest that an intervention with a combination of certain sirtuin activating bioactive compounds addresses some of these effects.

List of Abbreviations: Body mass index (BMI), reactive oxygen species (ROS), calorie restriction (CR), pyruvate dehydrogenase kinase isoform 4 (PDK4), adenosine monophosphate (AMP), AMP-activated protein kinase (AMPK), adenosine triphosphate (ATP), silent information regulator 2 (SIR2), nicotinamide adenine dinucleotide (NAD+), superoxide dismutase 2 (SOD2), the Forkhead box subgroup O (FoxO), MutL homolog 1 (*MLH1*), β-hydroxybutyrate (BHB), Long Interspersed Nuclear Elements (LINE), microRNAs

(miRNAs), timepoint 1 (T1), timepoint 2 (T2), timepoint 3 (T3), short chain fatty acids (SCFAs), , medium chain fatty acid (MSCFa), tricarboxylic acid
(TCA), toll-like receptor 4 (TRL4)

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12.1.3 Five Days Periodic Fasting Elevates Levels of Longevity Related Christensenella and Sirtuin Expression in Humans

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Abstract: Periodic fasting (PF) is an increasingly popular approach that assists in the management of metabolic and inflammatory diseases as well as in preventing mechanisms involved in aging. However, little is known about the effects of fasting on gut microbiota and its impact on the epigenetic regulation of metabolically relevant enzymes, especially sirtuins (SIRTs). We analyzed the effect of periodic fasting on the human gut microbiota, SIRTs expression, and mitochondrial content in 51 males and females. The participants fasted under supervision for five consecutive days following the Buchinger fasting guidelines. Ketogenesis, selected mRNAs, miRNAs, mitochondrial (mt) DNA, and gut composition were analyzed before and after PF. PF triggered a significant switch in metabolism, as indicated by the increase in ß-hydroxybutyrate (BHB) and pyruvate dehydrogenase kinase isoform 4 (*PDK4)* expression in the capillary blood. MtDNA, *SIRT1*, *SIRT3*, and *miRlet7b-5p* expression in blood cells were elevated, whereas *SIRT6* and *miR125b-5p* were not affected. Following fasting, gut microbiota diversity increased, and a statistically significant correlation between *SIRT1* gene expression and the abundance of *Prevotella* and *Lactobacillus* was detected. The abundance of longevity related *Christensenella* species increased after fasting and inversely correlated with age as well as body mass index (BMI). Thus, this represents the first study that showing that fasting not only changes the composition of the gut microbiota, making it more diverse, but also affects SIRT expression in humans.

Keywords: Buchinger fasting; gut microbiota; sirtuins

Introduction

The occurrence of metabolic disorders has dramatically increased during recent decades in developed and developing countries. Obesity, type 2 diabetes, hypertension, cardiovascular disease, gastrointestinal disorders, and cancer often correspond to aging and contribute to increased mortality [1,2]. Restrictive diets (RD), including caloric restriction (CR), time-restricted feeding, and intermittent fasting, are known to retard age-related diseases, thus, increasing life span, at least in animal models [3]. Whether RDs extend lifespan in humans is unknown. In general, a reduction in caloric intake reduces metabolic rate and oxidative stress, improves insulin sensitivity, and alters neuroendocrine and sympathetic nervous system functioning; all known to be altered in obesity and during the progression of aging [4,5]. In contrast to RD which usually lasts long-term, periodic fasting (PF)—such as Buchinger fasting—involves a daily energy intake of a maximum of 250 kcal for approximately one week. Although, under medical supervision, it can last up to three weeks [6,7].

The beneficial effects of fasting occur by modulating the activity of multiple pathways, including nutrient responsive pathways, such as the insulin/insulin-like growth factor (IGF-1) pathway, adenosine monophosphate-activated protein kinase (AMPK), as well as different classes of histone deacetylases (HDACs), and suppression of the nucleotide binding oligomerization domain NLRP3inflammasome [9]. Decreased insulin signaling activates AMPK and mammalian Forkhead-Box-O (FoxO) proteins, such as FoxO1 and FoxO3, which stimulate the expression of many genes involved in autophagy, including sirtuins (SIRTs), the mammalian homolog of the silent mating type information regulation (SIR) genes present in lower eukaryotes [11]. AMPK promotes an intracellular increase in nicotinamide adenine dinucleotide (NAD+) levels, the rate-limiting substrate for SIR2, which is one of the critical mediators of CR-induced lifespan extension in yeast. In mammals, seven types of SIRTs have been identified (SIRT1–7) [11,12], which can be found in different parts of the cells, and have multiple functions including DNA repair, cell survival, metabolism, lipid and glucose homeostasis, stress resistance, as well as insulin secretion, mostly via their HDACs activities. SIRT1 is the most intensively studied member of the SIRT family and it is associated with longevity in animal models. AMPK and SIRT1

both regulate each other's activities and share many common targets and functions [13,14]. Both AMPK and SIRT promote mitochondrial biogenesis and functioning. Consequently, they increase cells ability to generate ATP, diminish oxidative stress, and other potentially adverse cellular events [11–14].

SIRTs are also expressed in the gastrointestinal tract. Mice lacking SIRT3 expression have altered gut microbiomes, exhibited a higher inflammatory level, and in-creased intestinal epithelial damage [15]. A decline in SIRT expression and de-creased intestinal microbial diversity is associated with aging [16]. Dysbiosis or a less diverse gut microbiome is associated with aberrations of gut barrier integrity and inflammation, which contribute to pathogenesis like type 2 diabetes, fatty liver, and hepatic steatosis, atherosclerosis, cardiovascular disease, which are comorbidities for obesity and aging [17]. The intestinal microbiome impacts gene expression inducing epigenetic changes and regulates activity of G-protein coupled receptors via short-chain fatty acids (SCFAs). SCFAs are gut microbiota metabolites generated by the fermentation of dietary fiber. One of these SCFAs—butyrate— inhibits class I HDACs, epigenetically induces the proliferation and differentiation of immune cells and upregulates the activity of the adiponectin-mediated AMPK pathway that stimulates mitochondria biogenesis and fatty acid oxidation [1].

However, RD and PF can affect gene expression through multiple mechanisms, such as chromatin modification, mRNA transcription, and mRNA translation. Control of the expression of regulatory RNAs, such as microRNAs (miRNAs), is an important determinant in this regard [18].

Health-span results from the interaction of multiple factors including genetic and epigenetic, as well as microbiota [19]. Based on previous reports showing that RDs have a beneficial impact on human health, which was exemplified by clinical parameter results and an enhanced health-span [20], we conducted a study to investigate the potential impact of Buchinger fasting on age-related pathways and the microbiome. To our knowledge, so far, most scientific studies have focused on RD or intermittent fasting and only a few have investigated the effects of PF in con-sideration of the human intestinal microbiota; however, none of these have done so by focusing on longevity related genes.

2. Results

2.1. Fasting Results in Ketogenesis, Weight loss, and Increased Levels of mtDNA

Male (n = 5) and female (n = 15) study participants underwent five days of PF following the Buchinger fasting protocol, while other groups of male (n = 11) and female (n = 20) subjects served as non-fasting controls. Approximately half of the total study population (52,9%) had a body mass index (BMI) lower than 25 kg/m2, 35.3% were defined as overweight and 11.8% were obese (Table 1). After PF, blood BHB significantly increased from 0.2 to 5.7 mM ($p < 0.01$) (Figure 1a). Due to the intervention, a mean weight loss of 4.26 kg was recorded (Table 1). Relative mitochondrial (mt) DNA content in the blood was significantly higher in the fasting group com-pared to the non-fasting control group ($p < 0.05$) (Figure 1b)

Table 1. Characterization of study population by intervention groups. Values for characterization are given in total numbers and as a percentage. Anthropometric measurements were given in mean ± SD.

Characteristics	Total Study Population *n* (%)	Fasting Population *n* (%)	Non-Fasting Control *n* (%)
Population size	51 (100)	20 (39.2)	31 (60.8)
Male	16 (31.4)	5 (25)	11 (35.5)
Female	35 (68.6)	15 (75)	20 (64.5)
BMI < 25 kg/m^2 T1	27 (52.9)	6 (30)	21 (67.7)
BMI > 25 kg/m^2 T1	18 (35.3)	9 (45)	9 (29)
BMI > 30 kg/m^2 T1	6 (11.8)	5 (25)	1 (3.2)
Characteristics	**Total study population mean ± SD**	**Fasting population mean ± SD**	**Non-fasting control mean ± SD**
Age	45.24 ± 14.625	56.55 ± 12.576	37.94 ± 10.767
Weight kg at T1	75.882 ± 12.8509	80.550 ± −13.7093	72.871 ± 11.5029
BMI kg/m^2 at T1	25.9298 ± −3.932	27.7610 ± 4.32706	24.7484 ± 3.20067
Weight loss kg T2–T1	−2.0067 ± 2.66021	−4.265 ± 1.77535	−0.2 ± 1.5

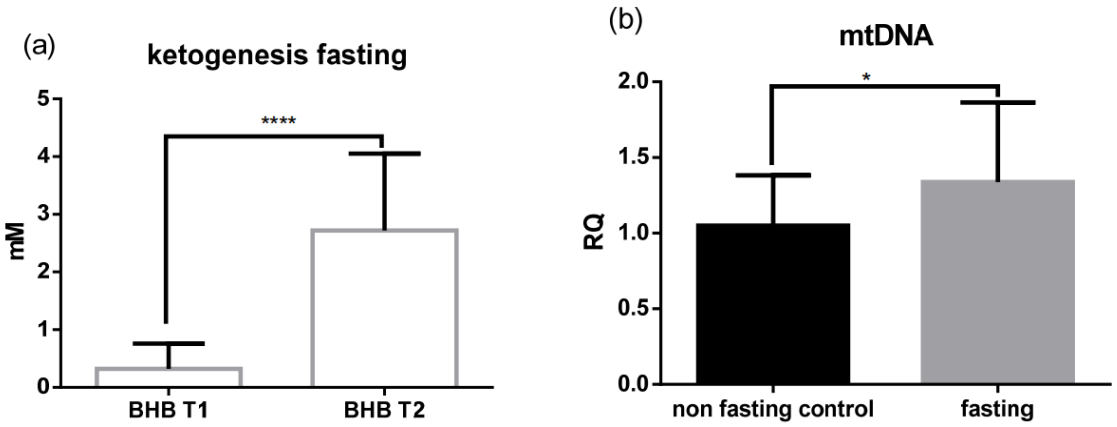

Figure1: Periodic fasting (PF) increases ketogenesis and mitochondrial (mt)DNA. (**a**) ß-hydroxybutyrate (BHB) levels were measured in the blood of fasting subjects before (T1) and after (T2) the intervention ($p < 0.01$). (**b**) Relative quantification (RQ) of mtDNA was measured in blood. The results are expressed as mean +/− SD. Statistical significance was determined using paired *t*-test for parametric values and Wilcoxon test for nonparametric values.

2.2. PF Affects mRNA and miRNA Expression

Selected mRNA and miRNA levels were assessed in the capillary blood of the study subjects. After PF, changes in gene expression were detected for all of the selected genes, apart from *SIRT6*, and *miR125b-5p*. The levels *of FoxO1, SIRT1, SIRT3*, and *miRlet7b-5*p were significantly increased compared to the non-fasting controls, whereas *miR34a-5p* levels were reduced ($p < 0.01$) (Figure 2a–h).

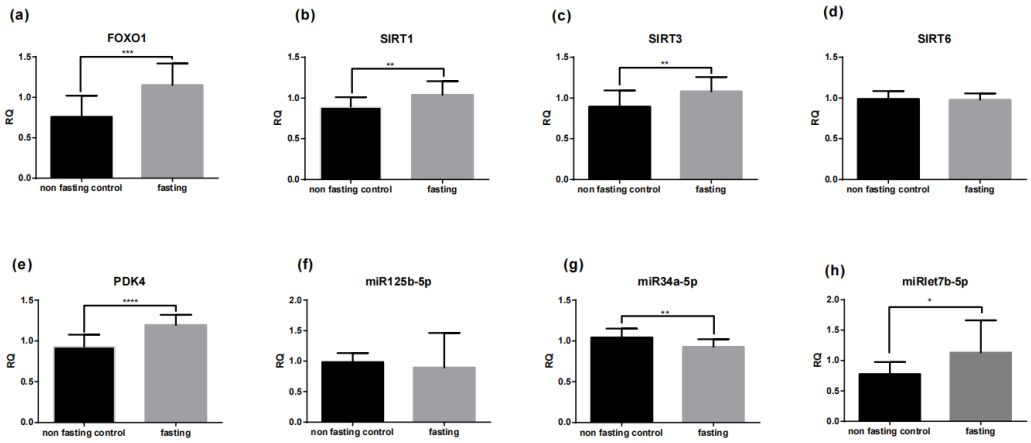

Figure 2. PF affects gene expression in blood cells. Relative quantification (RQ) after the intervention compared to the non-fasting control group of selected mRNA (*FoxO1, SIRT1, SIRT3, SIRT6 and PDK4* (a–e)) and miRNA (*miR125b-5p, miR34a-5p and miRlet7b-5p* (f–h)). The results are expressed as mean +/− SD. Statistical significance was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

PF resulted in significant correlations between the expression of *FoxO1 with SIRT1, SIRT3,* and *PDK*4 (Figure 3a–c). Similarly, S*IRT3* expression positively correlated with mtDNA levels, but only at the baseline ($p < 0.05$) (Figure 3d). Moreover, *PDK4* positively correlated with BHB blood concentrations and *SI*RT3 expression after PF ($p < 0.02$) (Figure 3e,f). Although attenuating *miR34a-5p* expression would indicate higher *SIRT1* levels, no association was obtained, yet increasing age leads to overexpression of *miR34a-5p* ($p < 0.05$) (Figure 3g).

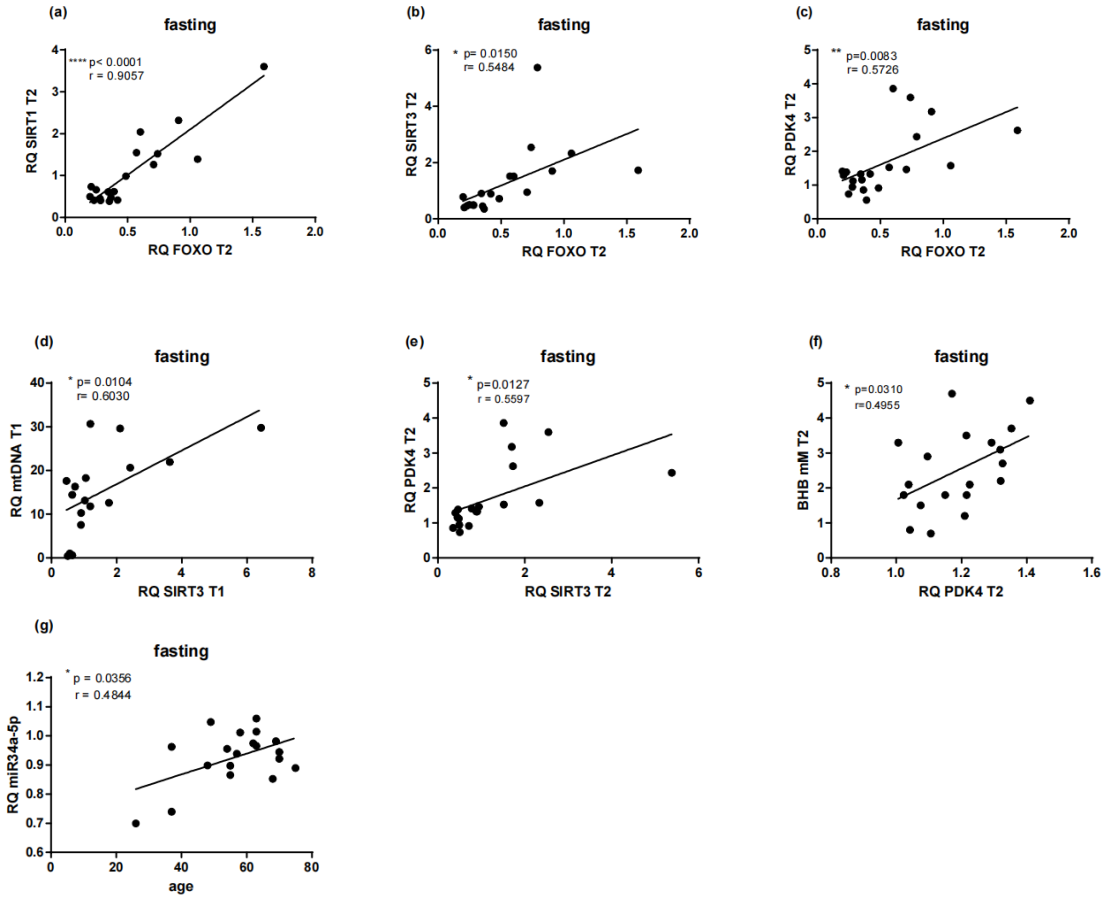

Figure 3. PF results in correlations between gene expression, age, ß-hydroxybutyrate (BHB), and mitochondrial (mt)DNA. Scatterplots illustrate Pearson correlation of different gene expressions with other markers for the fasting population and different timepoints (**a**–**g**). Statistical significance was defined as a *p*-value below 0.05.

2.3. Gut Microbiota Composition Changes and Diversity

Gut composition and metabolomics were assessed in stool samples. PF resulted in microbiota composition changes. At the phylum level *Tenericutes, Verrucomicrobia*, *Cyanobacteria, Proteobacteria, TM7*, and *Fusobacteria* were affected; however, only the last three were statistically significant ($p < 0.05$) (Figure 4a). A strong statistical trend was observed for the change in abundance of *Euryarchaeota* and *Cyanobacteria* before and after PF. The level of the latter was increased after the intervention, whereas the level of *Euryarchaeota* was reduced after PF (Figure 4a). No significant changes were seen for *Actinobacteria, Bacteroidetes*, and *Firmicutes* (Figure 4a). The non-fasting group showed no changes in microbiota composition of any of the phyla (Figure 4a). Comparing the

fasting and nonfasting group, *Verrcoumicrobia*, *Firmicute*s, *Actinobacteria*, and *Proteobacteria* are the phyla with the strongest differences at T2 (Figure 4b).

The Shannon diversity index was used to calculate α-diversity, but sequencing results showed no significant differences between non-fasting and fasting groups and within the different timepoints. The dataset was further subjected to principal coordinates analysis (PCoA). Although overlapping, PCoA showed a significant grouping of band patterns according to the two groups of study participants at T2 (Figure 4c).

All statistically significant changes at the species level for the fasting versus control groups are illustrated in Figure 5, which demonstrates strong differences in the microbiota composition at the species level before and after the fasting intervention. Significant decreases were observed for: s__*unspecific_02d06; s__unspecific_Dialister; s__prausnitzii; s__unspecific_Clostridiaceae; s__unspecific_Ruminococcaceae. Elevations were observed for: s__unspecific_Actinomyces; s__unspecific_Christensenella; s__unspecific_Coprobacillus; s__lenta; s__unspecific_Granulicatella; s__mucilaginosa; s__unspecific_Staphylococcus; s__unspecific_Erysipelotrichaceae;s__unspecific_Gemellaceae;s__unspecific_Peptostreptococcus; s__dentocariosa; s__unspecific_Rothia; s__unspecific_TM7-3; s__unspecific_Burkholderiales; s__unspecific_Succinivibrio; s__unspecific_Fusobacterium; s__unspecific_Leuconostocaceae* (Figure 5).

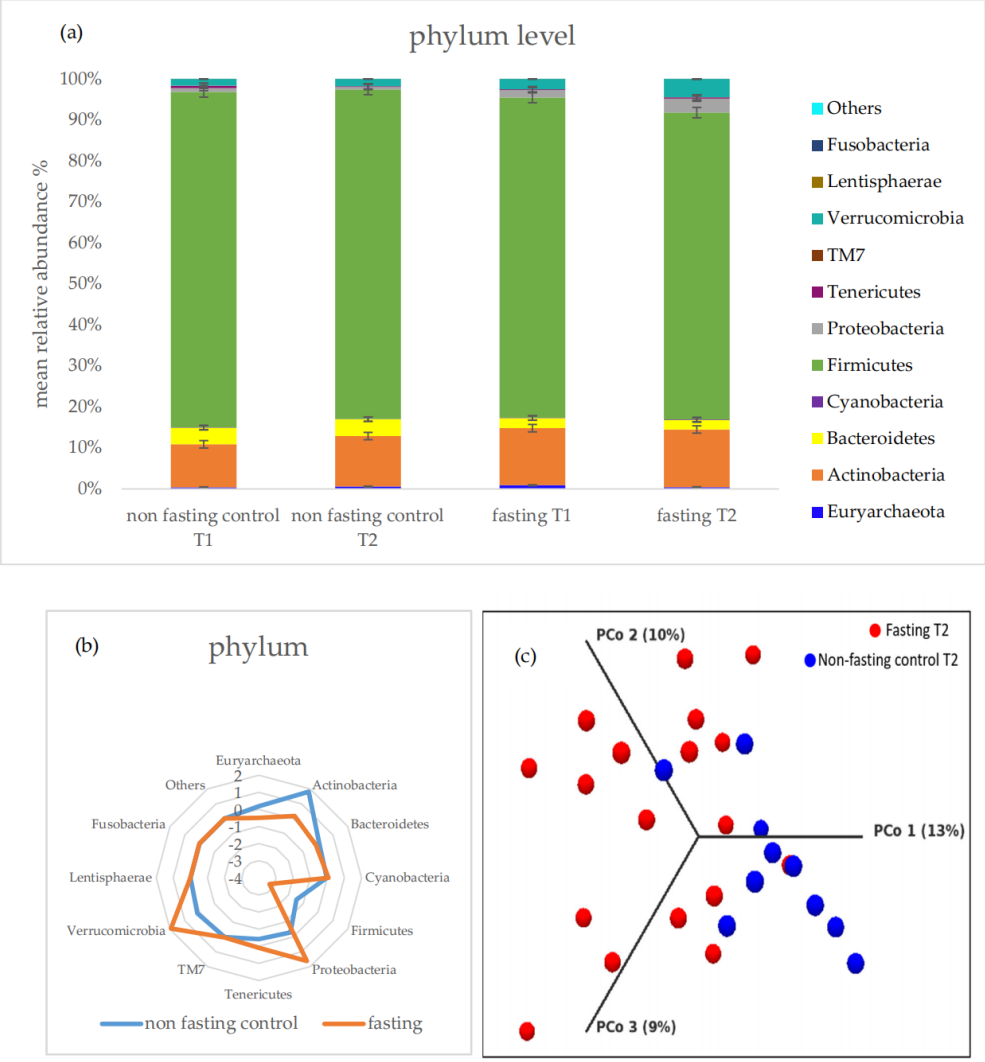

Figure 4. The dissimilarity of the microbiota composition of the non-fasting control and fasting group. (**a**) Bar charts of sequencing data given in mean +/− SD relative bacteria abundance in % at phylum level for non-fasting and fasting group. (**b**) Major differences between non-fasting and fasting groups at the phylum level. Values are given as the mean abundance of T2–T1. (**c**) PCoA based on Bray–Curtis dissimilarity index showing cluster for fasting and non-fasting group at T2. Permutational multivariate analysis of variance (PERMANOVA; p = 0.00004) was applied for the analysis.

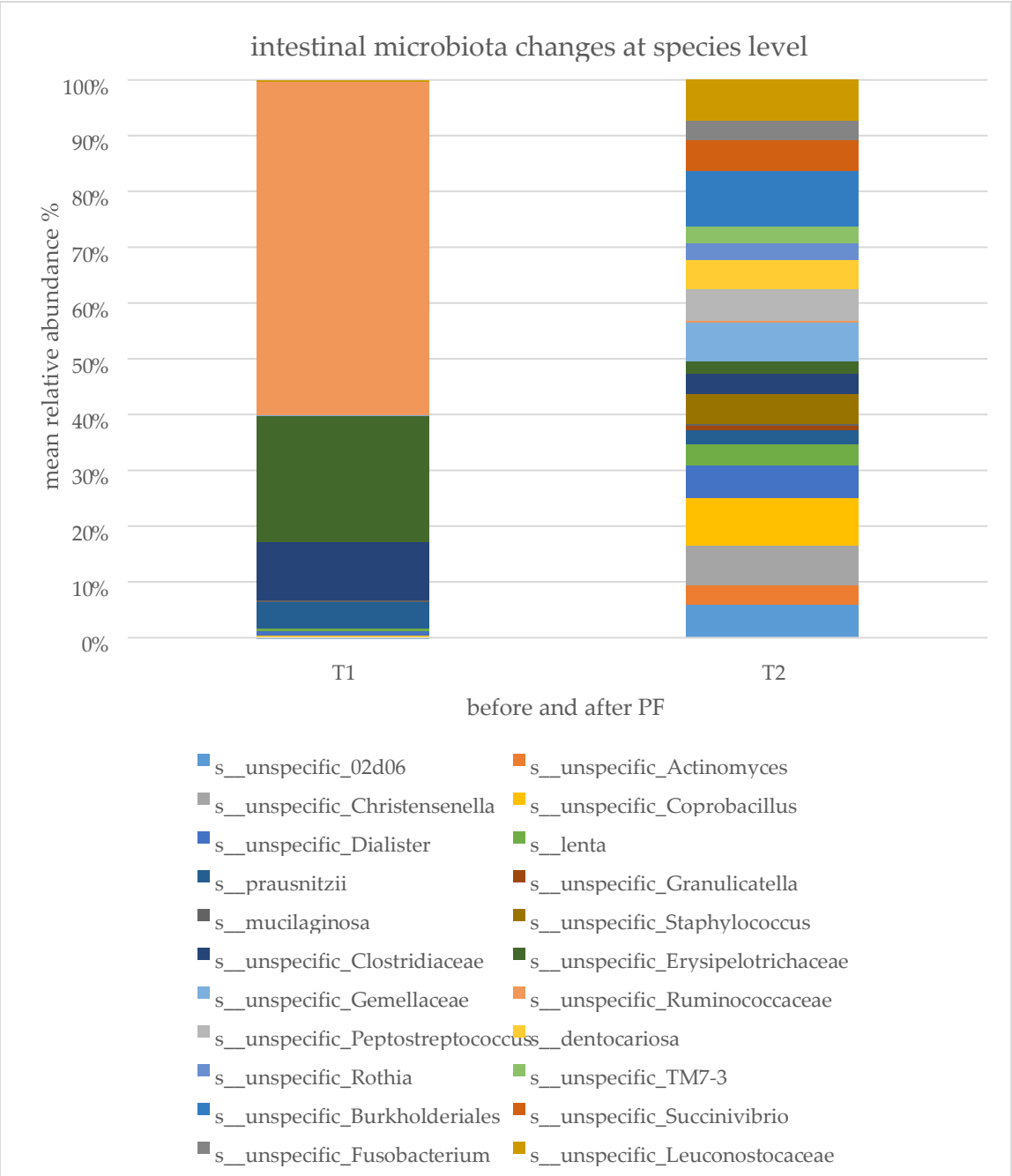

Figure 5. Microbial changes at species level before and after PF. Bar charts of all statistically significant changes of the sequencing data at species level given in mean relative bacteria abundance in % for the fasting group. Statistical significance was determined using paired *t*-test for parametric values and Wilcoxon test for nonparametric values and defined as $p < 0.05$.

2.4. PF Decreases *Firmicutes/Bacteroidetes* Ratio and Addresses Microbial Metabolites

At the end of the intervention, a positive correlation was observed for abundance in *Tenericutes* and the amount of butyrate produced, which significantly increased after the fasting period ($p < 0.05$) (Figure 6a). The *Firmicutes/Bacteroidetes* ratio decreased in the fasting and elevated in the nonfasting group (Figure 6b) but no correlations were seen with BMI or weight.

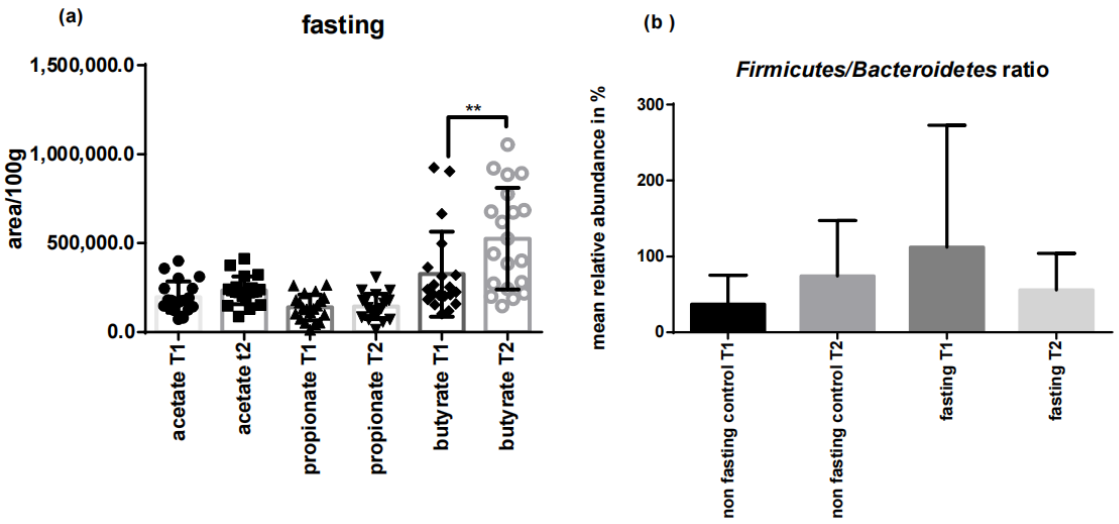

Figure 6. PF increased levels of butyrate and changed *Firmicutes/Bacteroidetes* ratio. Short-chain fatty acids (SCFAs) amount comparison of fasting group T1 versus T2 (**a**) *Firmicutes/Bacteroidetes* ratio increased for the non-fasting group and decreased after PF, although not statistically significant (**b**). The results are expressed as mean +/− SD. Statistical significance was determined using paired *t*-test for parametric values and Wilcoxon test for nonparametric values.

2.5. Microbial Changes at Genus Level and Correlations

At the genus level, PF triggered changes in the abundance of *Actinomyces, Granulicatella, Roseburia, Rothia, Rominococcus, Eggerthella*, and *Christensenella* ($p < 0.05$). The levels of longevity related *Christensenell*a increased after PF (Figure 7a). Age negatively correlated with the abundance for *Christensenella* (Figure 7b), *Eggerthella* showed a similar trend; however, this was not statistically significant ($p = 0.068$) (Figure 7c). The abundance of *Christensenellaceae* positively correlated with *SIRT3* expression ($p < 0.05$) (Figure 7d). Participants with a higher abundance of *Prevotella* or *Lactobacillus* had higher

levels of *SIRT1* expression with a statistically significant correlation ($p = 0.020$) for *Lactobacillus* and a strong trend ($p = 0.058$) for *Prevotella* (Figure 7e,f). At the genus level, unspecific *Firmicutes* and *Bifidobacteriaceae* correlated with the PF-triggered increase in the levels of butyrate ($p < 0.05$) (Figure 7g). The higher amount of butyrate showed a trend for correlation with higher mtDNA ($p = 0.0698$) (Figure 7h). *Bifidobacteriaceae* correlated with higher levels of weight loss for the study population with increased abundance of this genera ($p = 0.0189$) (Figure 7i). *Rothia* showed a similar result but only as a trend ($p = 0.07$). At the baseline, *Bilophila* was more abundant in participants with higher weight for the fasting group ($p = 0.0331$) (Figure 7j). Interestingly, at the species level, the amount of *Faecalibacterium prausnitzii* correlated with a lower level of weight loss (Figure 7k). Nevertheless, unspecific *Christensenella*, the amount of which was significantly ameliorated after periodic fasting, showed a strong correlation with a lower BMI ($p = 0.0558$) (Figure 7l).

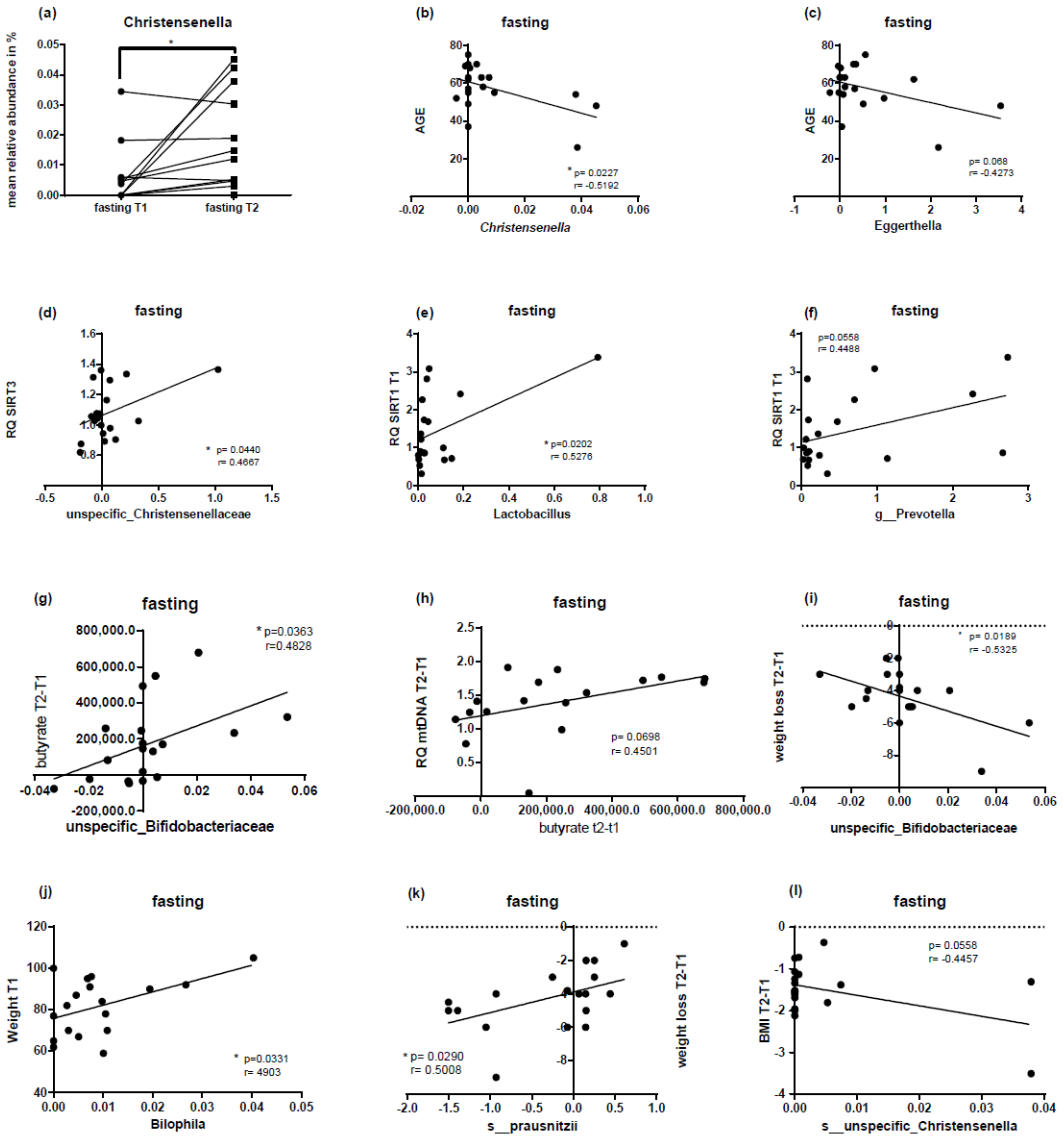

Figure 7. Correlation of gut microbiota at genus level with other biomarkers. *Christensenella* amount increased after PF described as fasting T1 and fasting T2. Statistical significance was determined using paired *t*-test for parametric values (**a**) Several Pearson correlations of gut microbiota with age, weight, weight loss, gene expression, butyrate, and BMI at different timepoints are illustrated from (**b**–**l**). Statistical significance was defined as a *p*-value below 0.05.

3. Discussion

Restrictive dietary protocols have proven to elicit beneficial effects on health. PF reduces blood pressure, insulin resistance and improves wellbeing [7,20,21]. Only a few studies have investigated PF in humans and focused on clinical blood parameters, microbiota composition changes, or longevity-related parameters.

As expected, our results show that nutrition depletion leads to weight loss and a switch in metabolism, as documented by the increased *PDK4* expression. Significant differences between the fasting and control group were seen for *SIRT1, SIRT3, FoxO1, PDK4, miR34a-5p* and *miR-let7b-5p*, BHB and mtDNA. However, no significant differences were seen between gender or BMI groups, in the fasting intervention, possibly caused by too few a number of participants or different characteristics of the control group.

In addition, ketone body production was increased during PF which is characteristic of pro-longed fasting. BHBs act on hydroxy-carboxyl acid receptors (HCA), in particular, on HCA2/GPR109a, which is a G-protein-coupled receptor. GPR109a has the capacity to prevent metabolic and inflammatory diseases, including type 2 diabetes, by suppressing the inflammasomes [22–24]. Therefore, GPR109a is a factor that represents increased health and lifespan, with it showing potential to be used as a therapeutic target for the prevention of type 2 diabetes, obesity, and inflammation [25].

Similar to the study conducted by Cannataro et al. [24] we observed an increase in *miRlet7b-5p* levels after PF. An overexpression of *miRlet7b-5p* leads to lower levels of triglycerides and insulin secretion by targeting the retinoid x receptor (RXR) and insulin receptor substrate, respectively. Further, *miRlet7b-5p* is involved in multiple metabolic regulatory processes, such as adipogenesis and fat metabolism [24], whereas an increase *in miR34a-5p* can lead to mitochondrial dysfunction [26]. Moreover, *miR34a-5p* acts as a pro-senescence factor by leading to in-creased inflammation and reduced mitochondrial content [26–28]. Overexpression of *miR34a* is known to correlate with aging and reduced levels of *SIRT1* [29]. The former was confirmed by our study. After five days of PF, we observed decreased expression of *miR34a-5p*; however, a significant correlation with its main target—SIRT1—was not detected, possibly because the study population was too small.

Sirtuins, especially *SIRT3*—located in the mitochondria—regulate many pathways involved in fasting metabolism, e.g., ketone body production, via deacetylating the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase; the rate-limiting step in β-

hydroxybutyrate synthesis [30,31]. This is reflected in our results, where non-fasting controls had significantly lower *SIRT3* expression compared to fasting subjects. In addition, positive correlations between levels of BHB and P*DK4* and the latter with *SIRT3* were observed in our study. Increased *PDK4* gene expression is usually observed during fasting, which leads to inhibition of the pyruvate dehydrogenase complex, conversion of pyruvate to acetyl CoA decreases, and at the same time ß-oxidation in-creases to provide energy [32]. Shimazu et al. [31] reported that mice lacking *SIRT3* showed decreased BHB levels during fasting. Additionally, *SIRT3* contributes to mitochondrial quality and biogenesis, by activating antioxidative factors, such as *FoxO* and superoxide dismutase [33]. Correspondingly, we found positive correlations between *FoxO*1 and *SIRT1, SIRT3* and *PDK4* in the intervention group. Likely due to its involvement in mitochondrial biogenesis, *SIRT3* expression correlated with mtDNA content in the blood.

The levels of SIRT6, also involved in aging and metabolism, did not alter during the fasting intervention, and no differences were seen between the PF and control groups. Nevertheless, *SIRT1* gene expression increased after PF and significant changes were seen between the subject groups. Overexpression of this gene is associated with attenuated adipogenesis and increased lipolysis. *SIRT1* can also be expressed in the gut, where it exerts anti-inflammatory effects in acute intestinal inflammation [1]. Harada et al. [34] identified the strain Lactobacillus brevis T2102 as an *SIRT*1 activator in the gut. Consistent with this finding, we found a positive correlation of participants with a higher amount of *Lactobacillus* and *Prevotella* and *SIRT1* expression at baseline. This correlation was not observed after the intervention, thus, a major switch in the gut microbiome occurs after nutrition depletion. Furthermore, abundance changes were seen for *Proteobacteria, TM7*, and *Fusobacteria*. Although overlapping, two different clusters were seen for the intervention groups at the phylum level. The *Firmicutes* to *Bacteroidetes* ratio decreased after PF, but contrary to the study by Mesnage et al. [7], in our outcomes, *Firmicutes* remained as the dominant phylum. At the species level, consistent with the study by Wilhelmi de Toledo et al. [21] but not with that by Remely et al. [20], we saw a significant decrease in the level of *Faecalibaterium prausnitzii* after PF. This

important butyrate producer is usually associated with a lower BMI and body weight, whereas we found the oppo-site effect for the fasting group [35]. The family *Bifidobacteriaceae*, another important butyrate producer, which declines with aging, is associated with lower BMI and higher weight loss [36], which is consistent with our results. Another highly interesting finding in our study considers *Christensenella,* which has recently been referred to as a longevity-relevant gut microbiota as it is more frequently found in centenarians [37]. The abundance of *Christensenella* is usually influenced by age, diet, and genetics [37]. In our study population, the abundance of this species decreased with age and higher abundances were inversely correlated with BMI, similar to previous reports [37,38]. Nevertheless, after PF, a significant increase was observed for *Christensenella*. As a butyrate producer, *Christensenella* might have contributed to the significant generation of butyrate following PF. Correspondingly to the beneficial impact of butyrate on mitochondrial function [39,40], we observed a positive trend between stool butyrate and mtDNA content in the blood of the intervention group.

Aging and obesity are associated with lower bacterial diversities and altered metabolic pathways, which are involved in nutrient harvesting and energy production [20]. After PF, we observed a more diverse composition of gut microbiota at the species level, which was distinctive from the composition of the control group. Together, these findings indicate that PF increases the expression of genes and the diversity of gut microbiota relevant for longevity.

Results from larger fasting and control groups are desirable, however, such type of study is difficult to coordinate at the same time and in a single given location. Sustainable effects after fasting are of high interest and promise to be useful for further therapeutic approaches, thus, we are already investigating this topic.

4. Material and method

4.1. Experimental Design and Dietary Intervention

A total of 55 participants were enrolled for a five-day fasting study performed at the University of Vienna, Department of Nutritional Sciences, in cooperation with the

Monastery of Pernegg (Austria). All study participants gave written consent for the use of data. The study population comprised participants, who decided to participate in the fasting group (N = 24) and a non-fasting control group (N = 31). Dropouts were only present in the fasting intervention, due to a lower tolerability of nutrition and this consequently reduced the group to a total number of 20 participants. During fasting, the subjects were supervised by a fasting coach in the Monastery in Pernegg. Following Buchinger fasting, all subjects were asked to drink 2–3 L of water or non-energy herbal teas daily. Furthermore, an organic freshly squeezed fruit juice (250 mL) was served at noon and a vegetable soup (liquid only) in the evening. To remove intestinal remnants of the last meals, the intestinal tract was emptied through the intake of a laxative super-vised by the fasting coach. The study population was between 23 and 75 years (mean 45.24). The mean BMI was 25.93 ± 3.93 kg/m2 and the mean weight was 75.9 ± 12.85 kg. Only four participants indicated to be smokers. Physical activity and stress load including age and gender were determined not to be confounding factors. In total, 16 males and 35 females participated in the study population. Participants supplementing probiotics, antibiotics, or sirtuin activating compounds/medicine such as metformin were excluded from the study.

4.2. Sample Collection

A food frequency questionnaire, general health questionnaire, dried blood spots, and stool samples were collected from the total population. Blood ketone body levels were measured before the beginning of fasting and before the fast break—for the fasting group only. The stool samples were collected before starting the fasting and from the first stool after the fasting break. After collection, stool samples were immediately stored at −80◦C. In accordance with the declaration of the Viennese Human Ethics Committee, all study participants gave written consent for the use of data generated during the study. The beginning of the intervention is defined as time point 1 (T1). For fasting, the end of the intervention was defined as time point 2 (T2).

4.3. BHB Measurement

GK Dual Blood glucose and ketone meters (Swiss Point of Care, Zurich, Switzerland) were used to measure BHB, from the fasting group only, using blood drops from the finger for both timepoints.

4.4. DNA and RNA Extraction

Capillary blood drops were collected on Whatman® protein saver cards (Sigma Aldrich, St. Lou-is, MO, USA), and are described as dried blood spots (DBS). Total DNA and RNA were isolated from DBS using MagMAX FFPE DNA/RNA ultra-kit via a KingFisher Duo Prime purification system (both Thermo Fisher Scientific, Waltham, MA, USA). A nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) was used for quantity and quality checks of DNA and RNA samples.

4.5. Mitochondrial DNA

Relative mitochondrial DNA content was determined in genomic DNA isolated from the DBS. For qPCR, a StepOne Plus real-time PCR Detection System (Applied Biosystems, Waltham, MA, USA) and single-copy gene primers, mtDNA primers (Biomers, Ulm, Germany), and a Light-Cycler® 480 Sybr®Green I master mix (Roche, Penzberg, Germany) were used.

Relative mtDNA content was calculated using the formula $2^{-\Delta\Delta Ct}$ ($\Delta Ct = Ct^{mtDNA} Ct^{singlecopygene}$; $\Delta\Delta Ct = \Delta CtT2 - \Delta CtT1$) as described elsewhere [41,42]. The sequences of the forward and reverse mitochondrial primers were: CAT CTG GTT CCT ACT TCA GGG and TGA GTG GTT AAT AGG GTG ATA GA. Following primers sequence were used for forward and reverse single-copy gene: CAG CAA GTG GGA AGG TGT AAT CC and CCC ATT CTA TCA TCA ACG GGT ACAA. An initial heating step of 95 ◦C for 10 min was followed by 40 cycles of 95 ◦C for 15 s and 60 ◦C for 1 min.

4.6. mRNA and miRNA Expression

Genes of relevance to RD and longevity were selected. Changes in mRNA expression were determined using available primers (Thermofisher, Waltham, MA, USA). LunaScript RT SuperMix Kit (New England BioLabs, Frankfurt, Germany) and TagMan Advanced miRNA cDNA synthesis kit (Thermofisher, Waltham, MA, USA) was used

for cDNA synthesis of mRNA and miRNA. cDNA synthesis was performed using MultiGene gradient Thermal Cycler (Labconsulting, Vienna, Austria). A total of 10 µL reactions were run in duplicates using TaqMan Fast advanced Mastermix (Thermofisher, Waltham, MA, USA) and StepOne Plus real-time PCR Detection System (Applied Biosystem, Waltham, MA, USA).

All target mRNAs levels were normalized to GAPDH, and miRNAs to miR24 as housekeeping genes. Relative quantification (RQ) for mRNA and miRNA were calculated using the ΔΔ cycle threshold (ΔΔCT) method, with fold changes using the formula, as described in the section be-fore. RQ for T1 or T2 were calculated using the formula 2−ΔΔCt expressed relative to the mean values for the control group [43].

4.7. 16S rRNA Gene Amplification and Sequencing and Microbial Metabolites

For sequencing microbial composition, all fasting samples were analyzed by Biomes

NGS GmbH (Wildau, Germany) via 16S rRNA gene amplification and sequencing. Microbial genomic DNA from fecal material was extracted by bead-beating technique.

As the most promising for bacterial and archaeal primer pairs [44], the V3–V4 region of the 16S rRNA gene was amplified and sequencing was performed on the Illumina MiSeq platform using a 2 × 300 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Microbial metabolites were analyzed using mass spectrometry at the Department of Nutritional Sciences, University of Vienna. The detection technique was established based on the published method 2-NPH or 3-NPH derivatized fatty acids analysis utilizing LC-MS [45]. The metabolites were detected by liquid chromatography coupled to mass spectrometry (LC-MS). Therefore, Ultimate 3000 (Thermo Fischer Scientific, Waltham, MA, USA) and a micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) with an Atlantis T3 3 µm column (2.1 × 150mm, Waters, Milford, MA, USA) were used, and kept at 40 ◦C.

4.8. Bioinformatics and Statistical Analysis

Data are presented as mean ± standard deviation (SD). Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, USA) and graph pad prism (Version 6). The paired t-test was used to compare the different time points for parametric values and the Wilcoxon test was used for nonparametric values. Statistical significance was defined by a p-value < 0.05.

Raw microbial sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [46]. High-quality reads were binned into operational taxonomic units (OTUs) at a 97% similarity threshold using UCLUST [47] and a "de novo" approach. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier against Greengenes database. All singleton OTUs were removed in an attempt to discard the majority of chimera sequences. Microbial alpha diversity was analyzed by using the Chao1 index, Shannon entropy, Simpson's index, and phylogenetic diversity whole tree metrics and beta diversity was estimated based on Bray–Curtis dissimilarity index and plotted as a multidimensional scaling or Principal Coordinates Analysis (PCoA) by the CLC Genomics Workbench version 20.0.4 (QIAGEN). The Mann–Whitney U test was used to analyze the mean difference of the alpha diversity index using GraphPad Prism version 9.0.0 (San Diego, CA, USA) and plotted as mean ± SD. p-value < 0.05 was considered statistically significant.

The difference in the microbial community composition (beta diversity) of the groups was test-ed using the permutational multivariate analysis of variance (PERMANOVA).

5. Conclusions

RD or fasting, in any version, beneficially changes clinical blood parameters, which has already been studied and documented. In addition, in animal models, RDs have been documented to increase lifespan by activation of SIRTs. We show that in humans, five days of consecutive nutrition depletion increases *SIRT1* and *SIRT3* expression in comparison with non-fasting controls. Additionally, PF leads to a switch in the gut microbial composition. Interestingly, the abundance of *Christensenella*, which is associated with longevity, increased after PF. To our knowledge, this is the

first study assessing SIRT expression, including its interaction with the gut composition in PF subjects.

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Informed Consent Statement: All study participants gave written consent for the use of data.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

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12.1.4 Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin- activating drink

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ABSTRACT

Background: Sirtuins attract high attention considering their properties to reverse molecular hallmarks of aging and age-related disorders. Many secondary plant ingredients (SPI) are known for their sirtuin-activating activities as well as epigenetic regulation of telomers, autophagy, senolysis, DNA repair but also improvement of gut microbiota. Furthermore, prebiotics enhanced butyrate and was shown to interact with SIRT pathways. This study investigated the effects of a drink containing a mix of different SPIs in combination with galactooligosaccharides (GOS) and their effect on SIRT activation, markers of aging relevant mechanisms, and gut microbiota composition in correlation with subjective wellbeing and skin structure appearance.

Methods: We analyzed gene expression, mtDNA amount, and microbial composition in response to a sirtuin- activating drink in humans compared to a control group consuming

a placebo. Food frequency, beauty, and general health questionnaires were asked, and a set of mRNAs and miRNAs were assessed using qPCR analysis. The gut composition was analyzed using Illumnia sequencing.

Results: SPI increased *SIRT1*, *SIRT3,* and modulated cell cycle relevant *miR16* and senescence regulating *miR34* expression. Additionally, mtDNA amount was higher in the group consuming the active supplement indicating an improved mitochondrial activity. The combined effect of SPI and GOS lead to an increase of *Actinobacteria*, especially *Bifidobacterium,* but also *Veillonellaceae* which was not observed in the control group. Significant correlations between *SIRT3* expression and the gut microbiota *Bifidobacterium* and *Veillonellaceae* were observed. Additionally, statistical analysis of subjects self-reporting indicated beneficial effects regarding beauty and wellbeing.

Conclusion: Our results show that the combination of sirtuins inducing SPI and prebiotic GOS influences molecular pathways counteracting aging, senescence, inflammation, and enhanced groups of gut microbiota which are known to improve the innate and adaptive immune system.

Keywords*:* secondary plant ingredients, prebiotic, Sirtuins, subjective wellbeing, Bifidobacterium

INTRODUCTION

Due to their regulatory roles in many pathophysiological conditions sirtuins (SIRTs) attract high attention. In mammals, seven SIRTs (SIRT1-7) have been identified and belong to the nicotinamide adenine dinucleotide (NAD+) dependent histone deacetylases (HDAC) [1-3]. HDAC removes the acetyl group from histones, which causes a condensed chroma-tin structure, thus results in gene silencing [1-2]. SIRT deacetylate histones and non-histone proteins and have been identified in distinct subcellular localization, thus being involved in multiple physio-logical processes, namely DNA repair, cell cycle regulation, energy metabolism, neuronal protection, gene expression regulation, and epigenetic activity

[1][2][4]. SIRTs, located in the mitochondria are in-volved in amino- and fatty acid metabolism, oxidative phosphorylation, mtDNA replication, and regulation of anti-inflammatory agents [2-5].

Restrictive diets and their activation of SIRT1 have been intensively studied in vitro and in vivo, whereas the latter mostly in animal models than in humans [6]. Nevertheless, SIRT expression increases also in humans following Buchinger fasting guidelines [7]. During nutrition depletion, adenosine monophosphate (AMP) increases and adenosine triphosphate (ATP) decreases within the cell thus activating AMP-activated protein kinase (AMPK) and NAD+, further activates SIRTs expression [8-9].

Secondary plant ingredients (SPI) are a broad range of bioactive botanicals and have been described to mimic this effect, thus defined as SIRT activating compounds (STACS). SPI can activate AMPK or SIRT directly or activating SIRT by targeting nicotinamide phosphoribosyltransferase (NAMPT), the enzyme in the NAD salvage pathway, which leads to an increased NAD+/NADH ratio [1][3][10]. Other studies show that polyphenols can target AMPK, by increasing the AMP/ATP ratio by interfering with the respiratory chain reaction in mitochondria, thus leading to activation of SIRTs [11]. SPI can also modulate micro RNAs (miRNA) leading to SIRT activation [12-13].

SPI which are defined by their low abundance have been used for centuries in traditional medicine due to their free radical scavenging action, antioxidative, antimicrobial and antiviral capacities. Polyphenolic compounds can therefore reduce cardiovascular risk due to low density lipoprotein oxidation inhibition [1][14][15]. Other SPIs exhibit anticancer, anti-obesity, cardio and neuroprotective effects and prevent type 2 diabetes by increasing insulin sensitivity [1].

Additionally, SPIs modulate gut microbiota (GM) [16]. Besides protecting against pathogens colonization, the GM protects integrity and permeability of the gut barrier and is part of the innate and adaptive immunoregulation. Moreover, the GM has several metabolic functions, such as syn-thesis of vitamins and short-chain fatty acids, absorption of dietary compounds, regulation of lipid, protein and carbohydrate metabolism and enzymatically modifies SPI bioavailability and healthy effect [16-17]. Age, diet, physical inactivity, stress and drugs are the main factors for GM dysbiosis and are linked to diseases

regarding the gastrointestinal tract but also metabolic disorders, like obesity or diabetes [17].

SPIs and prebiotics favor the generation of short-chain fatty acids (SCFAs) and the SIRT expression in the gut mucosa. Both exert anti- inflammatory effects, maintains gut barrier functions and beneficially effects clinical blood parameters 18-20] but are also linked to be neuroprotective and regulate hormones, such as leptin, ghrelin, melatonin and serotonin, thus influencing hunger, satiety and mood [21-22]. Nevertheless, the range of short chain fatty acid (SCFAs) activities are limited comparing SPIs metabolites produced, within the gut[23]. Additionally, SPIs are usually found in combination in nature. Due to their heterogeneity, they exhibit different biologic activities and show synergistic properties [23].

Based on their multiple biological benefits and as potential STACs they are nowadays used in nutraceuticals or functional foods and in cosmetics to treat multiple (skin) aging signs [14][24][25]. We conducted a randomized, placebo-controlled, one blinded study to investigate if the SIRTFOOD®SHOT supplement, consisting of several potential natural SIRT activating compounds and SCFA producing prebiotic galactooligosaccharides (GOS) can address SIRTs expression in humans. Additionally, we observed the changes in the microbial gut composition, Food frequency questionnaire outcomes, regarding general health and beauty aspects and possible connections.

MATERIALS AND METHODS

Experimental design: 150 people were enrolled for the study performed at the University of Vienna, Department for nutritional science. The study was randomized, controlled, single-blinded and consists of two intervention groups: SIRTFOOD®SHOT supplementation (active) and placebo/control. The supplement was composed by the Swiss company System-Biologie AG (Wollerau). After dropouts, 100 participants attended the active group and 31 participants were consuming placebos, considering them as the control group. Dropouts were only seen in the intervention group, due to the lower tolerability of the bitter taste. The subjects were in average 41.12 years. The mean BMI was 26.0219 ± 4.54906 kg/ m2 and weight 75.993 ± 15.6306kg. 39 participants (29.8%) were males and 92 participants (70.2%) were female. Exclusion criteria were participants

taking pre- and probiotics, antibiotics, or sirtuin activating compounds/medicine like metformin were excluded. Both groups were advised to keep their lifestyle and nutritional habits during the intervention. Only one flacon (25ml) of SIRTFOOD®SHOT or placebo had to be included during the day for three consecutive months. Following compounds can be found in one falcon (25ml) SIRTFOOD®SHOT: 3.5 mg of gallic acid, 40 mg EGCG, 25 mg phloretin, 14 mg anthocyanin, 10 mg anthocyanidins, 6.4 mg oleuropein and 0.9 mg sulforaphane, 3g galactooligosaccharides. The compounds were extracted from mango, apple, blue-berry, olive leaves, broccoli, and green tea.

Sample collection and preparation: Food frequency questionnaires, general health and beauty questionnaires, dried blood spots, and stool samples were collected at two different time points at the beginning and after the intervention. In accordance with the declaration of the Viennese Human Ethics committee, all study participants gave written con-sent for the use of data. The beginning of the intervention is defined as time point 1 (T1) and T2 defines the end of the study.

Nutritional behavior and subjective well-being were validated via several standardized questionnaires. Total DNA and RNA were isolated from dried blood spots (DBS) using MagMAX FFPE DNA/RNA ultra-kit (Thermofisher, USA) via King-Fisher Duo Prime purification system. Quantity and quality were assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). Relative mitochondrial DNA content was determined in genomic DNA isolated from the dried blood spots using a StepOne Plus real-time PCR Detection System (Applied Biosystem). For PCR, single-copy gene primers, mitochondrial primers (Biomers, Germany) and a LightCycler® 480 Sybr®Green I master mix (Roche) were used. cDNA was done eighter using LunaS-cript RT SuperMix Kit (Biolabs, Germany) for mRNA or TagMan Advanced miRNA cDNA synthesis kit for miRNAs. miRNA and mRNA of interest were determined using commercial primers (Thermofisher, USA) and TaqMan Fast advanced Mastermix (Thermofisher, USA) for RT-qPCR amplifications performed on StepOne Plus real- time PCR Detection System (Applied Biosystem). Fold change was calculated using the formula of $2^{-\Delta\Delta Ct}$ ($\Delta Ct = {}^{Ctgene\ of\ interest}\_Ct^{housekeeper}$; $\Delta\Delta Ct = \Delta CtT2 - \Delta CtT1$ or $\Delta\Delta Ct = \Delta Ct - \Delta Ct^{targt\ mean\ placebo\ control}$) [26] [7]. Samples were generally run in 10 µL reactions in doubles. Upon sample collection, stool samples were stored at -80°C. For sequencing microbial composition all fasting samples were

analyzed by Biomes NGS GmbH (Germany) using Illumina Sequencing, written elsewhere [7] [27]

Statistical analysis: Raw microbial sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline [28]. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier against Greengenes database. All data are presented as mean ± standard deviation (SD). Data were analyzed using IBM SPSS Statistics for Windows Version 22.0 (IBM Corp., Armonk, NY, USA) and graph pad prism (Version 6). Paired t-test was used to compare the different time points for parametric and Wilcoxon test for nonparametric values.

RESULTS

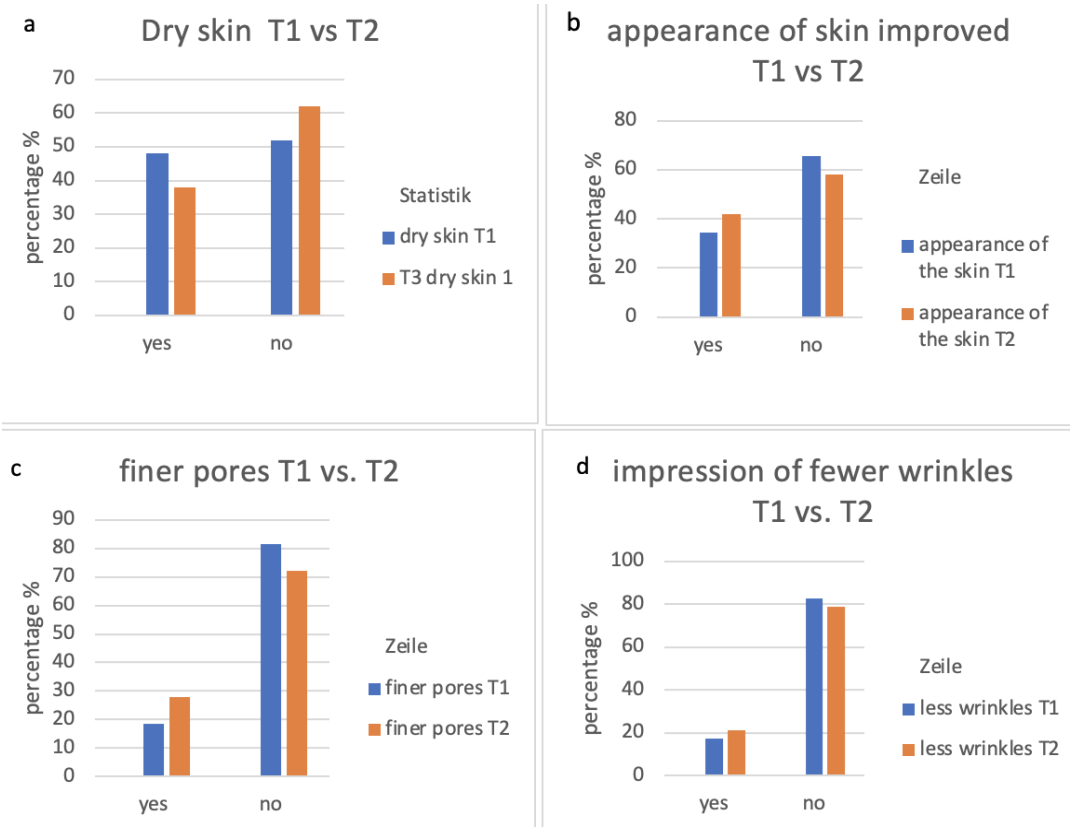
Population characteristics: The total study population size was 131 participants, divided into two different groups. The SIRTFOOD®SHOT group consists of 100 participants, consuming an active supplement, whereas 31 subjects were addressed as a control group, consuming the placebo shot. Males and females were almost normally contributed between the groups, although more females were interested to attend the study. Subjects were between 21 and 67 years, as well in the SIRTFOOD®SHOT as the placebo group. Although most characteristics were equal between the groups, participants in the control group had lower BMI (Table 1).

Table 1: Characteristics of the study population. Values were eighter given in total number (N) and percentage or mean ± standard deviation (SD)

	Total study population	SIRTFOOD®SHOT	PLACEBO
N	131 (100%)	100 (76.3%)	31 (23.7%)
Male	39 (29.8 %)	28 (28%)	11 (35.5%)
Female	92 (70.2%)	72 (72%)	20 (64.5%)
Mean ± SD			
Age	41.12 ± 11.252	42.11 ± 11.267	37.94 ± 10.767
Weight kg T1	75.993 ± 15.6306	76.915 ± 16.6584	72.871 ± 11.5029
BMI kg/ m^2 T1	26.0219 ± 4.54906	26.4248 ± 4.84322	24.7484 ± 3.20067
Weight kg T2	75.643 ± 15.5512	76.477 ± 16.4663	72.910 ± 11.9174
BMI kg/m^2 T2	25.9309 ± 4.53196	26.3211 ± 4.73664	24.6524 ± 3.56280

Subjective well-being and beauty aspects: All participants were asked to interpret their skin appearance before and after the intervention. The placebo study population did not see any beneficial effects regarding beauty aspects. Study participants who consumed the active product had the impression of improved skin texture and its appearance. After the intervention period, 13% of the SIRTFOOD®SHOT population complained less about dry skin (Figure 1a). Moreover, 7.66% stated improved appearance of their skin (Figure 1b), including finer pores (9.63%) (Figure 1c) and fewer wrinkles (3.65%) (Figure 1d). At T1 30% of the SIRTFOOD®SHOT population have stated to have brittle nails (Figure 1e). Thus 14.72% mentioned faster nail growth (Figure 1f), brittle nails deceased of 13% (Figure 1e). Moreover, 34% of the active group population stated a faster hair growth due to the intervention (Figure 1g), consequently, 21.9% reported less hair loss (figure 1h). General

health aspects were collected of the total study population. After the intervention period, 38% of the SIRTFOOD®SHOT group reported to feel healthier (Figure 2a) and 43% felt more energy (Figure 2b), due to supplementation. Moreover, almost a quarter of the inter-vention group stated improved sleep (Figure 2c) and digestion (Figure 2d).



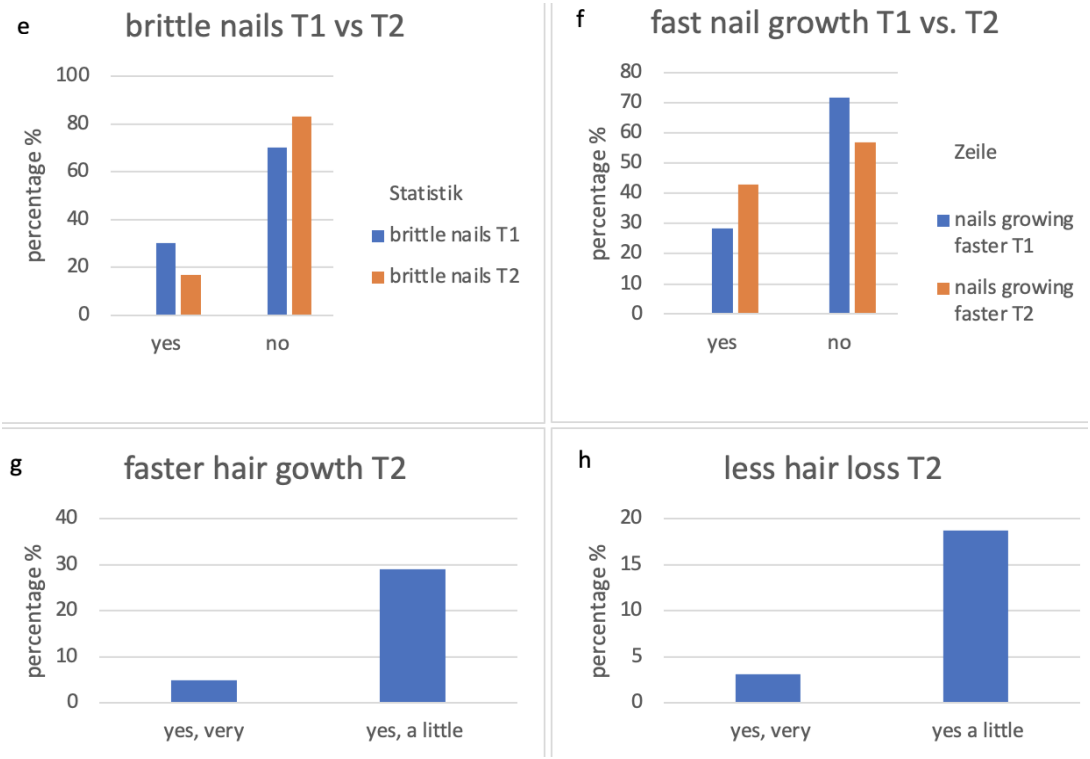


Figure 1. Analysis of questionnaire regarding beauty aspects. All results were given in percentage (%) of total answers of the SIRTFOOS®SHOT group. Figure 1a- f compared the answers of this study population between T1 and T2, whereas figure 1g and h illustrates the results at the end of the timepoint. The impression of dry skin decreased between T1 and T2 (Figure 1a). After the intervention, 7.66% more subjects consuming the active shot stated a better appearance of the skin (Figure 1b), including finer pores and fewer wrinkles (Figure 1c and 1d). Thus 14.72% mentioned faster nail growth (Figure 1f), brittle nails deceased of 13% (Figure 1e). 34% of the active group population stated a faster hair growth due to the intervention (Figure 1g), consequently, 21.9% reported less hair loss (figure 1h).

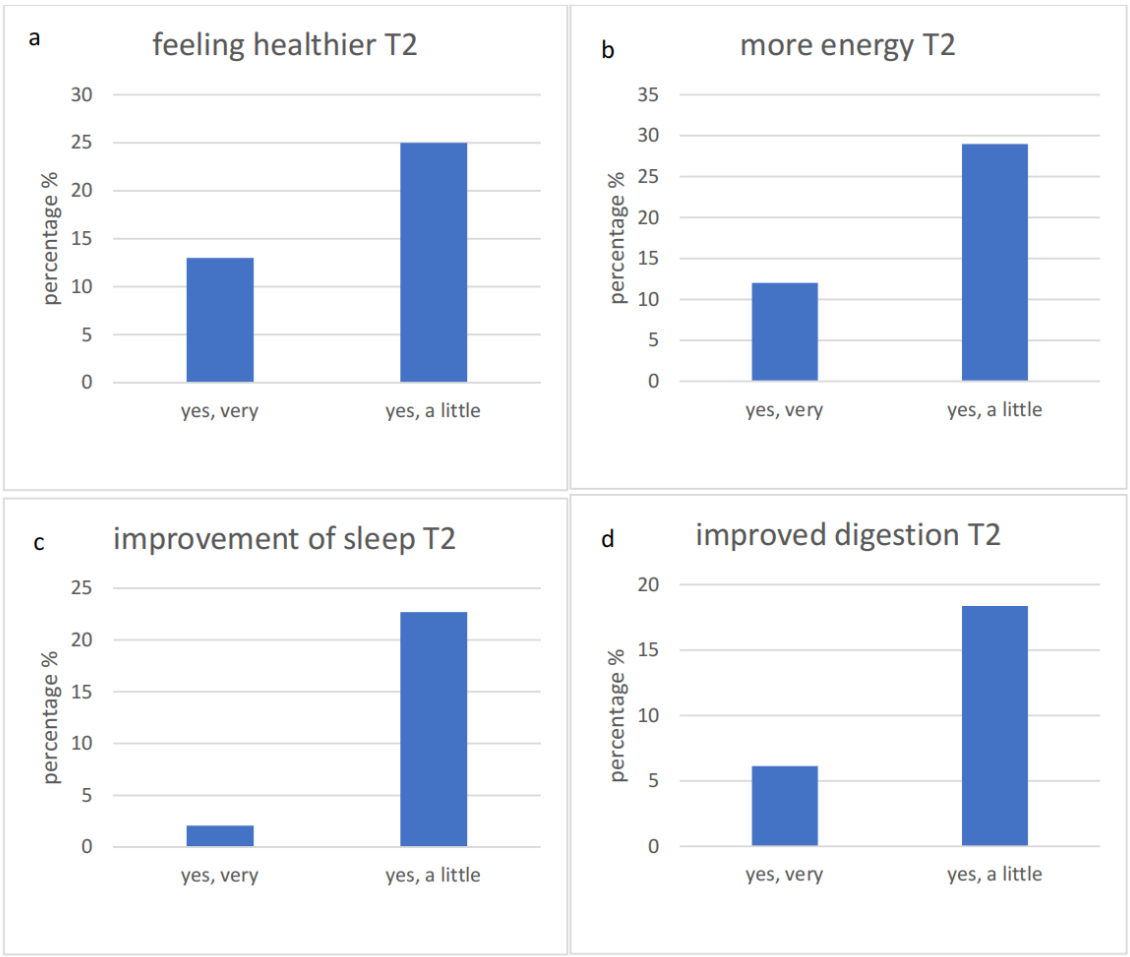


Figure 2. Analysis general health questionnaire. All results were given in percentage (%) of total answers of the SIRTFOOS®SHOT group. 38% reported to feel healthier (Figure 2a) and 43% felt more energy after the intervention (Figure 2b). Moreover, almost a quarter of the intervention group stated improved sleep (Figure 2c) and digestion (Figure 2d), due to the supplementation.

Mitochondrial DNA content and gene expression: Higher *mtDNA* content could be detected in the SIRTFOOD®SHOT intervention compared to the control group, although only a statistical trend (Figure 3a). Moreover, changes in gene expression were seen after SIRTFOOD®SHOT supplementation. Lower *miR34a- 5p* (Figure 3b) and increased miR16-5p (Figure 3c) expression were seen in the SIRTFOOD®SHOT group. Moreover, this study population showed different expression levels of *MLH1, SIRT3* and *SIRT1* compared to the control (figure 3d-f), Nevertheless, *MLH1* and *SIRT1* (Figure 3g) gene expression significantly increased and *miR34a- 5p* significantly decreased after supplementation (data not shown)

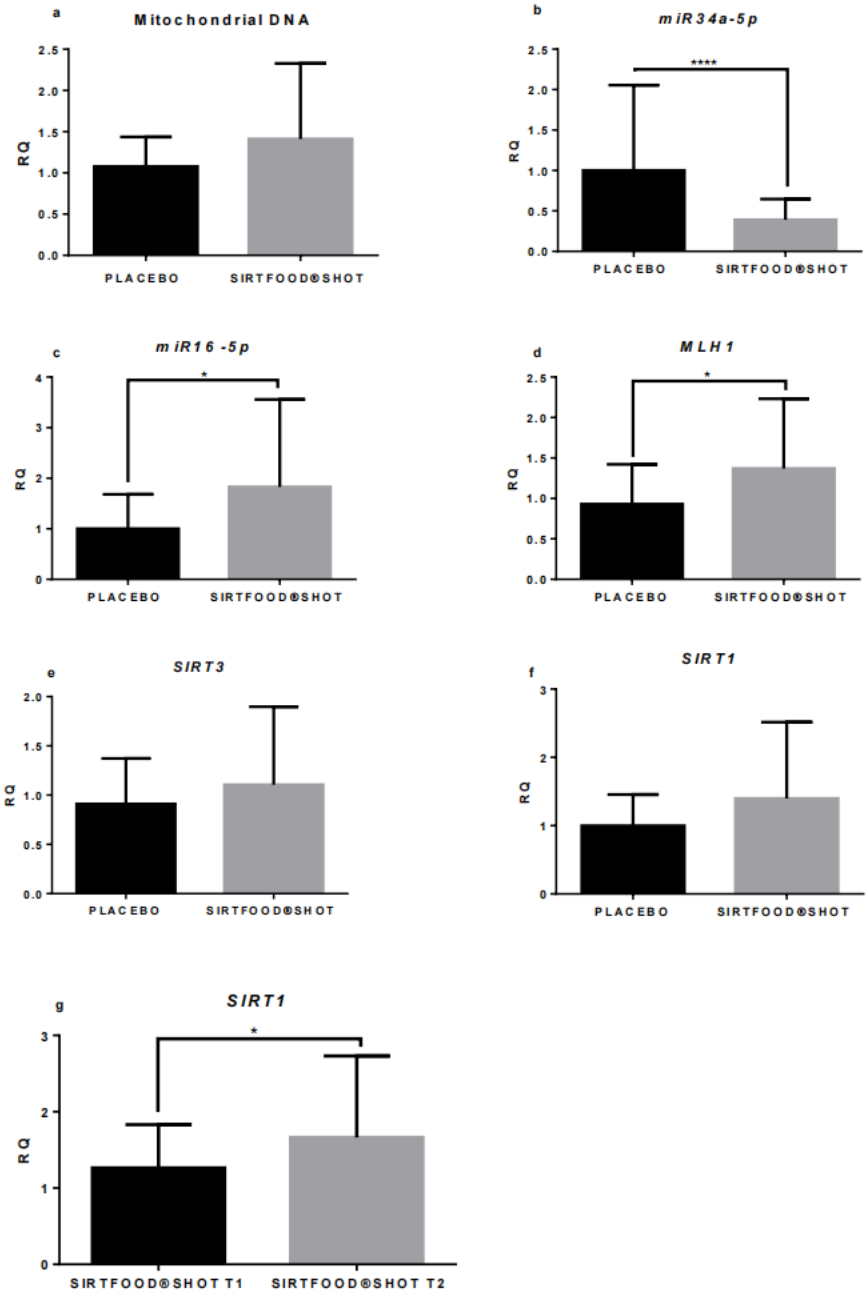
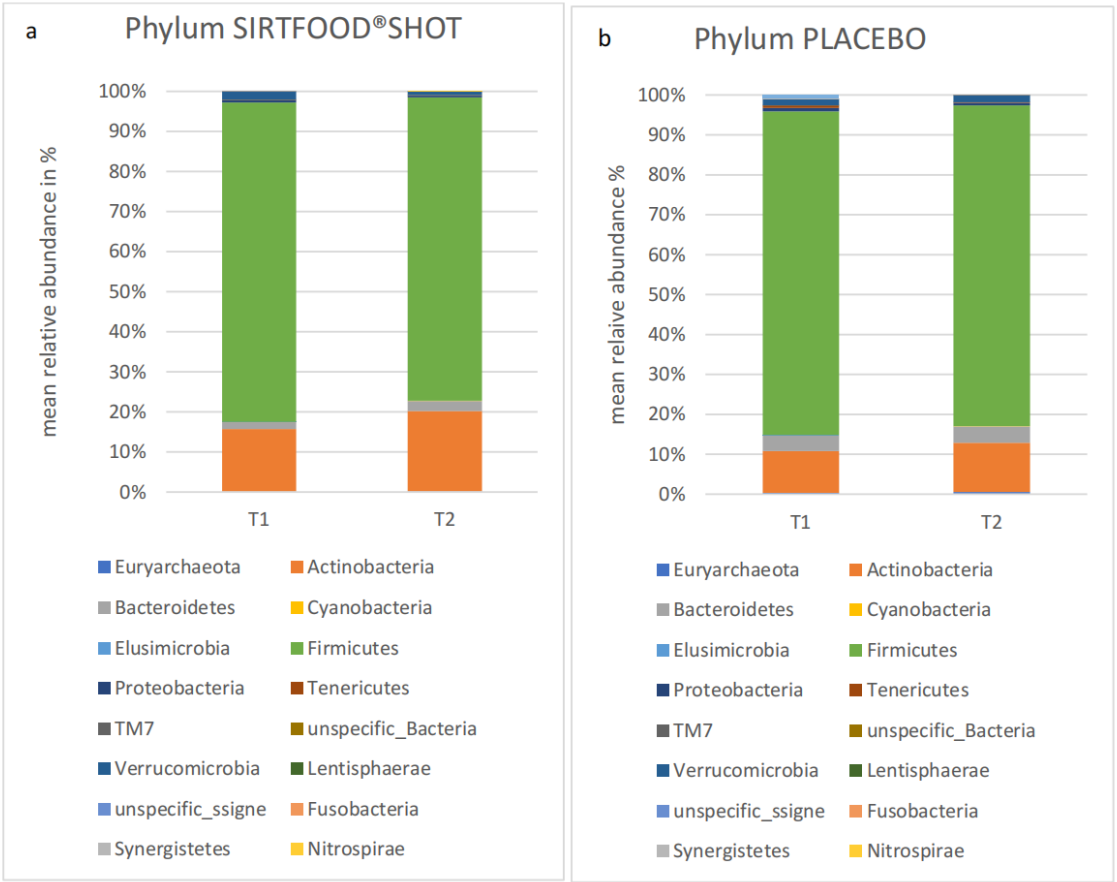


Figure 3: Results gene expression analysis. The results are expressed as mean +/- SD. Statistical significance between placebo and SIRTFOOD®SHOT (Figure 3a-f) and T1 and T2 of the SIRTFOOD®SHOT intervention (Figure 3g) was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Gut microbiota composition: After SIRTFOOD®SHOT supplementation changes in the gut microbiota were observed. At the phylum level of *Tenericutes, Lentisphaerae*, *Bacteroidetes, Euryarchaeota, Lentisphaerae* and *Actinobacteria* increased, whereas only latter showed a strong statistical trend (Figure 4a). Decreased abundance of

Cyanobacteria, Elusimicrobia, Proteobacteria, Verrucomicrobia, TM7 and *Fusobacteria* were observed after supplementation (Figure 4a). No changes were detected in Firmicutes abundance (Figure 4a). The same effects as observed in the Supplementation group were observed for *Euryarchaeota, Lentisphaeae, Cyanobacteria, Elusimicrobia*, *Proteobacteria, Verrucomicrobia* and *Firmicutes* in the control group (Figure 4b). Contrary to the SIRTFOOD®SHOT group, no significant changes were seen in the abundance of *Actinobacteria, Bacteroidetes, TM7*. The amount of *Tenericutes* decreased (Figure 4b), whereas not statistically tested. In addition, at species level, no significant changes were detected for the placebo group (figure 4d), whereas B*ifidobacterium* increased in the intervention group, although only as a strong trend (figure 4c). *Veillonellaceae* increased for the SIRTFOOD®SHOT (Figure 4e) group but not for the placebo (Figure 4f). All SCFAs propionate, acetate and butyrate only increased slightly (data not shown).



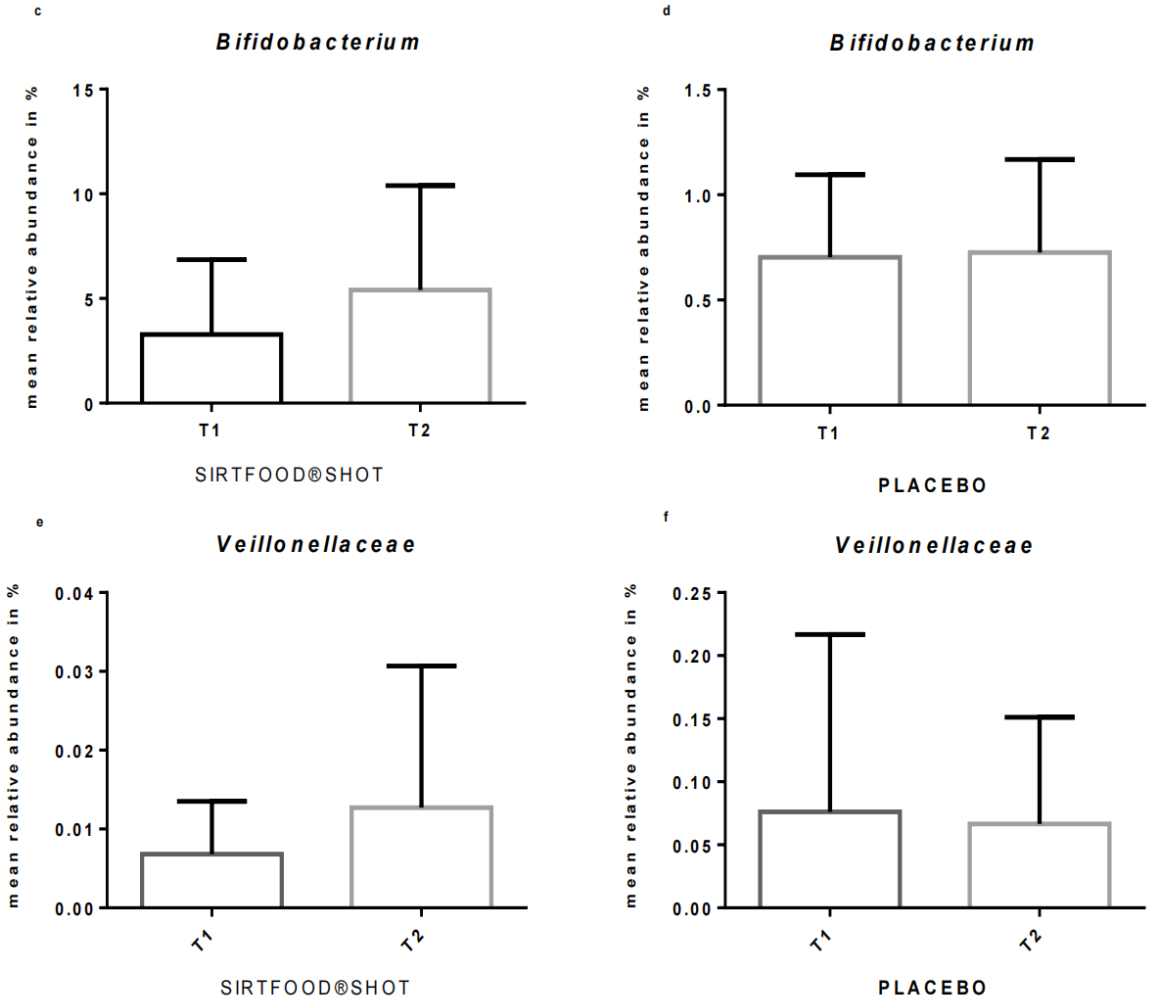


Figure 4. Microbial changes before and after treatment. Gut microbial changes before and after the treatment at phylum level showing *Bifidobacterium* and *Veillonellaceae* with the greatest change. Boxplot graphics demonstrating the elevation of *Bifidobacterium* before and after the intervention comparing to the placebo group, although only a trend p= 0.06 for the SIRTFOOD®SHOT (c&d). *Veillonellaceae* changes are demonstrated in figure e and f. The results are expressed as mean +/- SD. Statistical significance between T1 and T2 was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Correlations gut microbiota and gene expression: Several correlations were obtained regarding gene expression and different gut microbiota members. *MLH1* and *Veillonellacea*e, in addition *SIRT3* and *Actinobacteri*a especially *Bifidobacterium* significant positive correlations were seen ($p<0.05$) (Figure 5a-c). Increasing *SIRT3* negatively correlated with *Bilophilia* amount, although not statistically significant (Figure 5d)

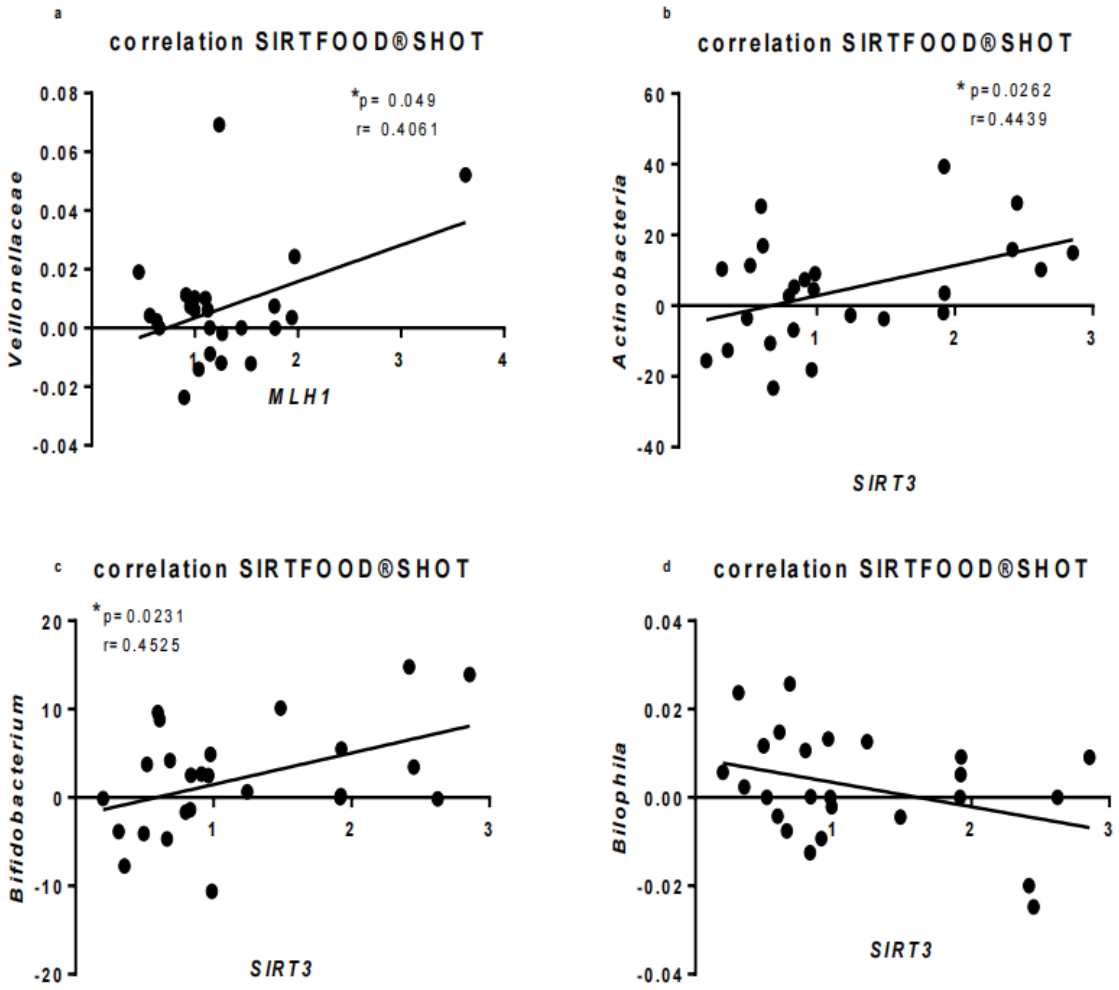


Figure 5: Pearson's correlations between microbiota and gene expression. a) positive correlation between *MLH1* and *Veillonellaceae* (p=0.049). Positive correlation between *SIRT3* and *Actinobacteria* (b), especially *Bifidobacterium* (c) (p<0.05). *SIRT3* and *Bilophilia* negatively correlated, although only a trend (d) (p= 0.07).

DISCUSSION

The human gut microbiota is a dynamic system that is influenced by its host lifestyle, genetics and epigenetics. The human genome and gut microbiome networking within metabolic pathways directly influence both normal physiology and disease processes [29]. SPIs and galactooligosaccharides exhibit prebiotic effects, which have beneficial immune, metabolic and cognitive functions [23]. The short chain fatty acids (SCFAs) produced by the gut microbiota are known to inhibit class I Histone deacetylases (HDACs) and also activate the UCP2- AMPK-acetyl-CoA carboxylase (ACC) pathway with decreased PPARγ gene expression, thus resulting in decreased lipogenesis and increased AMP: ATP ratio.

Latter induces AMP-activated protein kinase (AMPK) and sirtuins (SIRT) activation, having multiple beneficial effects on human health [23][30].

Improved digestion was reported by the participants after SIRTFOOD®SHOT supplementation. Kwon et al. investigated the effect of GM on defecation and concluded the increasing amount of *Bifidobacterium* is associated with increased stool frequency and better defecation [31]. This means increasing the amount of *Bifidobacterium,* which we observed after the active supplementation, might be helpful for people with obstipation.

Almost half of the participants from our study mentioned to feel healthier, having more energy, and better sleep after SIRTFOOD®SHOT treatment. Although, these results reflect subjective well-being, SPI and GM metabolites are known to exhibit anti- inflammatory, cardio- and neuroprotective properties and influence metabolism [23]. Via methoxyindole pathway, the gastrointestinal tract is responsible for 95% of bodies serotonin synthesis from dietary tryptophan and is transported via the blood-brain barrier [23]. Because microbial composition is individual, metabolism and health effect can differ within individuals. Due to their prebiotic effect and modulation of GM, SPI supports tryptophan synthesis, the precursor of serotonin [23]. SPI favor the growth of beneficial GM, like *Bifidobacterium*, which we observed after SIRTFOOD®SHOT treatment but not in the control [17]. *Bifidobacterium* acts probiotic and Tian et al. [32] reported serotonin enhancement via modulating tryptophan biosynthesis pathways after *Bifidobacterium* supplementation [33][32]. Additionally, GM and its metabolites influence the sleep duration and quality of its host by influencing clock gene expression. *Bifidobacterium* has been found to improve the subject's sleep, both factors increased in our study [34]. Short sleep and bad quality are associated with GM dysbalance and counteract age-related diseases and increased mortality [34].

GM and its modulation by SPI play an important role in mood and gut health. Nevertheless, the host body is a fine-tuning network of many factors and epigenetic mechanisms play an essential role in gene expression thus human health. Thus, participants self reported better well-being and sleep, this might also be explained by the increased *miR16- 5p* expression in the SIRTFOOD®SHOT group compared to the placebo group. *MiR16-5p* can be modulated by SPIs [13][35] and regulates not only gut health by targeting claudin 2, which latter is predominantly expressed in leaky epithelia and

upregulated in participants with bowel dysfunction [36]. Additionally, host miRNA can enter bacterial cells and regulate growth behavior and certain bacterial gene transcripts [37]. *MiR16-5p*, which decreases within age [38] is also an important regulator of the cell cycle, but also of serotonin thus regulating mood, satiety and sleep, factors protective against depression, eating disorders, and aging [39]. SPIs also impact *miR34a- 5p*, involved in the regulation of senescence. Additionally, overexpression is known to silence *SIRT1* mRNA expression [40][41]. Obesity is associated with elevated *miR34a -5p* levels thus resulting in decreased SIRT1 expression. *SIRT*1 and *SIRT3* enhance mitochondrial function, oxidative metabolism and counteract obesity [42]. Interestingly, the SIRTFOOD®SHOT group showed increased mitochondrial DNA amount compared to the control group. Weight loss (data not shown) was the same in both groups with an average -0.5 kg after three months, thus more studies are needed to investigate sirtuins counteract obesity via weight loss in humans. SIRT can also be expressed in human gut epithelial cells. Khalili et al. [19] reported a significant increase of *SIRT1* expression after probiotic supplementation with *Lactobacillus casei* in participants with type 2 diabetes [19]. The same connections were seen in our study. A higher amount of *SIRT3* expressed were seen with increased amount of *Actinobacteria*, especially *Bifidobacterium.* All three biomarkers were elevated after SIRTFOOD®SHOT treatment but not with the placebo. Interestingly, Chen et al [43] demonstrate, mice lacking *SIRT3* leads to gut dysbiosis, intestinal permeability and inflammation following a high fat diet [43]. Additionally, Natividad et al. [44] reported increased amount of *Bilophilia* after high fat diet in mice, contributing to intestinal barrier dysfunction, inflammation, and metabolic syndrome. After SIRTFOOD®SHOT supplementation, we saw lower *Bilophilia* amount correlated with *SIRT3* expression, implicating protective properties against metabolic syndrome.

Veillonellaceae increased in the active study population, but not in the control group. This specific group of microbiotas is known to metabolite lactate for energy production, a substrate contributing to fatigue during a physical performance and produced by *Bifidobacterium* [45][46]. Thus, an increase in the amount of *Veillonellaceae* may contribute to better physical performance, thus more energy, which the subjects reported. In addition, we observed a correlation between V*eillonellaceae* and MutL homolog 1 (*MLH1*). Latter belongs to DNA mismatch repairs and persons hyper-

methylated of one allele in MLH1, which contributes to lower *MLH1* expression, having a predisposition to developing colorectal cancer [47] [48].

Veillonellaceae contribute to hosts adaptive immune system. Gut microbial dysbiosis can release toxins that induce human DNA damage, concurring its mutability, tumor induction and progression in gastrointestinal cancer [49]. EGCG, the polyphenol of green tea can reverse DNA damage [48]. Moreover, SPI can modulate the abundance of *Veillonellaceae* [50]

SIRTs are mostly known for their longevity related properties, which are all protecting against the hallmarks of aging [51][52] [4]. Based on this knowledge SIRTS are getting more attention to slowing down skin aging processes and develop cosmetics including natural STATs, although bioavailability and skin permeability are restricting factors for use of many potential active substances [25].

Premature skin aging is manifested by accelerated induction of wrinkling, scaling, roughness, dryness, laxity, and hyperpigmentation. After SIRTFOOD®SHOT supplementation up to 10% of the participants reported improved skin appearance, less dryness, finer pores and fewer wrinkles [53]. Even here the tight network between SPI, GM and Skin aging needs to be highlighted, thus an imbalance in GM leads to dermatological manifestations [54]. This communication is not only via the skin gut axis but also more directly via metastasis of GM and their metabolites in cases of disrupted intestinal barriers, which can be detected in patients' bloodstream [55] [29]. Besides being antioxidative and prebiotic, SPIs protect against skin aging via several mechanisms, including via SIRT activating, thus reducing inflammation and limit oxidative damage in tissues via SIRT3/SOD pathway in the mitochondria [56]. Additionally, SPI, like EGCG and resveratrol inhibit the collagen degradation enzyme MMP1, leading to wrinkles and tyrosinase, which leads to Hyperpigmentation [53][25].

More than one-third of the active gup reported faster hair and nail growth. Kubo et al. investigated the effect of several polyphenols regarding hair growth in mice. The results show resveratrol and fisetin as the strongest compound promoting hair growth mainly keeping hair follicles in the anagen phase via telomerase reverse transcription[57]. Several

other studies investigated the hair growth potential of SPI, like cyanidins or EGCG [58][59] [60].

Many polyphenol rich botanicals are considered to be apoptogenic: stress-modifying phytochemicals that increase organisms' nonspecific resistance to stress by increasing their ability to adapt and survive to external stressors and stimuli.

Thus, being one of the first studies investigating the possibility of SIRT activation by a combination of SPI and GOS in human and some outcomes reflect subjective wellbeing via FFQ this study needs to be considered as a pilot study and further interventions would be interesting to prove causality regarding specific mechanisms for a specific aspect.

CONCLUSION

Microbial dysbiosis or SIRT depletion leading to different pathologies, namely e.g. obesity, Alzheimer disease, Cancer, Diabetes type 2, Liver stenosis, depression, skin diseases. The results of the study demonstrating a broad range of beneficial effects in humans by supplementing a synergistic combination of prebiotics. Secondary plant ingredients not only elevate SIRT expression, it modulates gut microbiota and im-proved subjective wellbeing.

List of Abbreviation: SIRTs: sirtuins, STACs: sirtuin- activating compounds, SPI: second-ary plant ingredients, mtDNA: mitochon-drial DNA, NAD+: nicotinamide adenine dinucleotide, HDAC: histone deacetylases, AMP: adenosine monophosphate, ATP: adenosine triphosphate, NAMPT: nicotin-amide phosphoribosyltransferase, miRNA: micro RNAs, GM: gut microbiota, SCFAs: short chain fatty acids, SD: standard devia-tion, MLH1: MutL homolog 1Author Con-tributions: S.L., A.H designed the research. S.L., H.B., C.S., conducted the research. S.L., H.B, T.D., A.M and B.H. performed clinical analysis. S.L., H.B., C.S. performed statisti-cal analyses. S.L., A.P and A.H. wrote the manuscript. A.H. had primary responsibility for the final content. All authors read and approved the final version of the manu-script.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence their work in this paper.

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12.2 Additional papers

12.2.1 The green tea polyphenol EGCG is differentially associated with telomeric regulation in normal human fibroblasts versus cancer cells

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ABSTRACT

Introduction: Topical investigations have demonstrated that oxidative stress and inflammation play key roles in biological aging and determine incidence and course of age-related diseases. Lifestyle and environmental factors hugely impact epigenetic regulation and DNA stability with telomere attrition and epigenetic instability providing a potential

record of the cumulative burden of endogenous and exogenous oxidative noxae. Certain physiologically active plant components exhibit antioxidative activities affecting epigenetic regulation of inflammation response and DNA repair.

Methods: Against this background, the present study investigated green tea polyphenol epigallocatechin gallate (EGCG) in the context of telomere regulation in Caco-2 colorectal adenocarcinoma cells vs. ES-1 primary skin fibroblasts. Cell lines were treated with 20 and 200 µM EGCG for 36, 72 and 144 hours, respectively. Telomerase activity, relative telomere length as well as methylation status of hTERT and c-Myc from different culture conditions were assessed. Malondialdehyde (MDA) served as a surrogate marker of potential prooxidative effects of EGCG in a physiologically relevant tissue model.

Results: EGCG incubation was associated with telomere shortening and decreased telomerase activity in Caco-2 cells, and relatively longer telomeres along with increased methylation of six 5'—C—phosphate—G—3' (CpG) sites in the promoter region of human Telomerase Reverse Transcriptase (hTERT) in fibroblasts. At low concentrations, EGCG significantly decreased oxidative damage to lipids in Caco-2 cells and attenuated H2O2 induced oxidation at higher concentrations.

Conclusion: These results suggest differential EGCGmediated telomeric modulation in cancer vs. primary cells and a specific antioxidant activity of EGCG against
oxidative damage to lipids in abnormal cells.

Keywords: Caco-2, epigallocatechin gallate, telomeres, hTERT, DNA methylation, telomerase, oxidative stress, malondialdehyde

INTRODUCTION

Over the past decades, redox research has demonstrated key roles for oxidative stress and inflammation in biological aging and associated diseases. Today it is commonly accepted that lifestyle and environmental factors hugely impact epigenetic regulation and DNA stability through telomere attrition. Further, epigenetic instability provides a potential record of the cumulative burden of those endogenous and exogenous oxidative stressors encountered over time [1,2,3]. Redox homeostasis is essential for regulating cell

growth, senescence and aging, and antioxidants are key players in maintaining an appropriate threshold cellular redox state [4]. Hence, unsurprisingly, an overt increase in reactive oxygen species (ROS) is associated with oxidative stress in the intracellular redox system, damage to macromolecules such as lipids, proteins and DNA, and has been linked to several diseases including neurodegenerative disorders [5,6]. Due to increased metabolic demand, ROS producing processes are typically upregulated in cancer. Concurrently, quantitative and qualitative changes in metabolic pathways and cell cycle control result in hyperproliferation, aggravating DNA damage, inflammation and genomic instability [7]. Cell culture studies have repeatedly shown oxidative stress levels dose-dependently accelerate telomeric attrition [8,9]. Human telomeres are comprised of tandem repeats of a hexameric nucleotide sequence (TTAGGG) that is associated with the shelterin group of proteins. The telomere protein complex is crucial for genomic stability and chromosomal integrity hence why telomere length has been suggested as a biomarker of biological aging[10]. Conversely, telomeric dysfunction and accelerated attrition have been linked to age-related conditions like cancer, cardiovascular disease, type 2 diabetes and neurodegeneration [11,12]. With its specialized ribonucleoprotein structure, the enzyme telomerase is a critical determinant of telomere length as it synthesizes telomeric repeat DNA, consequently slowing down telomere attrition. Human telomerase contains two core components, a catalytic unit called the human telomerase reverse transcriptase (hTERT) and an RNA template (hTERC), along with associated proteins. In adults, most healthy somatic cells express very low telomerase activity in contrast to cells with high replicative demands including fetal epithelial cells, and cells of the immune system [13]. Telomerase enzyme activity is regulated by an intricate multi-stage control machinery including transcriptional, posttranscriptional and post-translational mechanisms with the transcriptional regulation of human hTERT representing a major rate limiting factor. The hTERT gene is located in chromosome 5p15.33 [14]. The hTERT core promoter forms three parallel G- quadruplexes that play key roles in telomere homeostasis and gene regulation [15,16]. In a pathophysiological context, telomerase is essential for tumor progression and indeed, a high telomerase activity is observed in 80-90 % of invasive metastatic tumors. This makes telomerase an important therapeutic target in hyperproliferative and other age-related conditions [17]. hTERT is

switched off in differentiated cells, whereas hTERC is ubiquitously expressed in most tissues [18,19]. A vast number of transcription factors have been assumed to be involved in hTERT expression, most notably cMyc (together with estrogen), stimulating the expression of hTERT while in contrast Rb, p21 and CCCTC-binding factor (CTCF) have been implicated in hTERT suppression [20].In addition to given sequence-based genetic pre-dispositions for age-related diseases [21,22], epigenetic regulation of gene expression and DNA stability and reversible changes therein are known to impact disease pathogenesis and progression, and should be increasingly trialed. In recent decades, research has shown chemopreventive and antioxidative properties of a wide range of physiologically active plant-derived compounds. Anti-cancer effects have been reported including signaling pathways that address epigenetic mechanisms of inflammation, DNA repair and telomeric regulation [23]. In that respect, several phytochemicals such as curcumin, genistein or the polyphenol epigallocatechin-3-gallate (EGCG) studied here, have been shown to positively influence telomere length [24,25,26]. These natural bioactive compounds have the potential to act at multiple molecular target sites either directly through their antioxidative capacities or indirectly by affecting signaling pathways including DNA damage repair, epigenetic mechanisms or the mitogen activated protein (MAP) kinase pathway [27,28]. The present work focused on EGCG as the most abundant polyphenol in green tea. With its eight phenolic groups EGCG not only has it been described for its marked antioxidative potential, but also for its ability to specifically impair cancer cell progression by blocking signal transduction pathways, and thereby suppressing telomerase activity [18,19,29]. These effects have been strongly connected to the inhibition of NF-κB activity, affecting a wide array of processes including MAP kinase-dependent- as well as growth factor-mediated pathways [30]. Next to these findings, EGCG has repeatedly demonstrated anti-proliferative effects by inducing apoptosis and cell cycle arrest in cancer cell studies [31,32,33]. In this context, our objective was to advance EGCG-targeted research by exploring the compound's effects on telomere regulation in both cancer and non-cancerous primary cells. We wanted to further evaluate positive effects in contrast to possible adverse impacts of high doses of EGCG. As green tea polyphenols are ingested and therefore have high relevance to the digestive tract as their first environment of interaction, this study's experiments were performed in colorectal adenocarcinoma Caco- 2 cells alongside ES-1 primary skin

fibroblasts to unravel aspects of the cell type specific telomeric and redox activity of EGCG *in vitro*. Cell lines were treated with 20 and 200 µM EGCG for 36, 72 and 144 hours, respectively and telomerase activity, relative telomere length as well as methylation status of *hTERT* and *c-Myc* were assessed to identify EGCGdependent alterations in the epigenetic regulation of telomeres. Malondialdehyde (MDA) served as a surrogate marker of lipid peroxidation.

METHODS

Cell culture: In this study, Caco-2 colorectal adenocarcinoma cells (DSMZ Leibniz Institute, Braunschweig, Germany) and primary human skin fibroblasts ES-1 (provided by the Institute of Cancer Research, Medical University of Vienna, Austria) were cultured to form monolayers in 25 cm^2 filter cap tissue culture flasks (SPL Life Sciences Inc., Austria) in high glucose (4.5 g/l) Dulbecco's modified Eagle medium (DMEM, PAA Austria) at 37 °C in a humidified atmosphere of 95 % ambient air and 5 % CO2. Media were supplemented with 5 % (w/v) penicillin/streptomycin, 20 % (v/v) fetal bovine serum (FBS, PAA, Austria) and 1 ml of 100 mM Na-pyruvate. In all experimental setups, cells were passaged upon reaching 70 % confluence, using Accutase® solution (2 ml/flask; Sigma Aldrich Austria). Experiments were conducted from passage 18 for Caco-2 and passage 17 for ES-1 fibroblasts.

Cell treatments: For treatments, Caco-2 and ES-1 cells were seeded in 24-well plates. After 24 hours (h) initial incubation, cells were treated for a further 36, 72 and 144 h, respectively, with different concentrations of EGCG (20 and 200 µM). For lipid peroxidation studies, 100 µM EGCG was used. EGCG of the same grade and purity (> 95 %) was acquired as water soluble powder isolated from green tea leaves from Sigma Aldrich Austria and Biosysteme AG Zürich, respectively, and dissolved in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich Austria). DMEM served as vehicle control. For assessment of telomere length dynamics over time in Caco-2 cells, untreated cells were incubated for 36, 72, 96 and 192 h using the above- mentioned media conditions. Phenol red free media were used to avoid interference with MDA detection. For H2O2 induced oxidation (following 24 h of standard culture), cells were treated for 48 h with 250 and 500 µM H2O2, together with 20, 100 or 200 µM EGCG. If not indicated elsewhere, cell treatment was carried out in quadruplicate in at least 3 independent experiments.

Cell viability (≥ 85 % live cells) was monitored for each time point using the trypan blue assay.

DNA Isolation: After incubations, cells were washed with cold PBS (4 °C) and detached using 150 µl Accutase® per well. After an additional washing step with PBS, DNA extraction was carried out using the DNA Mini Kit (Qiagen, Germany) following the manufacturer's protocol. DNA concentration was measured using a Pico100 UV/VIS spectrophotometer (Picodrop Ltd, Hinxton, UK).

Malondialdehyde (MDA) as marker of lipid peroxidation in Caco-2 cells: After harvesting, cell numbers were determined and MDA levels assessed using HPLC and fluorescence detection at 533 nm as previously described by Zappe et al. [34]. Resulting MDA levels were expressed as MDA concentration relative to cell number and calculated as ratio to untreated control.

Assessment of relative telomere length and telomerase activity: To determine relative telomere length (rTL), quantitative real-time polymerase chain reaction (qPCR) was conducted with primer sets targeting telomeres (T; ForwardTEL: 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGG GTT-3' and ReverseTEL: 5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT -3') and the single copy control gene (S), acidic ribosomal phosphoprotein 36B4 (Forward36B4: 5'-ACTGGTCTAGGACCCGAGAAG-3' and Reverse36B4: 5'-TCAATGGTGCCTCTGGAGATT-3') as previously described by O'Callaghan and Fenech [35]. PCR results and CT mean values were analyzed using the StepOne Software v2.3TM (Thermo Fisher). Relative telomere length was calculated based on the 2−ΔΔCT algorithm using the normalized (to positive control) difference in cycle threshold between telomere-and single copy gene samples. For calculations to be deemed valid, amplification efficiency between telomere and single copy gene samples must be approximately equal. [36] Telomerase activity was determined by the Real-Time Quantitative Telomeric Repeat Amplification Protocol Assay combining a real-time PCR technique with the conventional TRAP method as previously described [37]. The assays were performed using individual protein extracts of both treated and untreated cells (DMEM ± ECGC) after 36, 72 and 144 h. Telomerase activity was expressed as percentage of the activity of control cell extracts (0 h, 100 %).

Bisulfite sequencing analysis: To assess the methylation status of nine auspicious CPG sites in the Telomerase Reverse Transcriptase (*TERT*) region, sodium bisulfite

pyrosequencing was performed. Approximately 1 µg of genomic DNA was used for bisulfite modification using the EpiTect-Bisulfite modification kit following the manufacturer's protocol (Qiagen, Austria). Modified DNA was then amplified by PCR using the GoTaq mix (Promega, Austria). DNA concentrations and purity were determined using a Pico100 UV/Vis spectrophotometer (Picodrop Ltd, Hinxton, UK). For analysis of nine CpGs in the promoter region of *hTERT*, PCR was performed with the following primers: Forward: 5'-GAGGGGTTGGGAGGGTT-3', Reverse: 5'-TCCTACCCCTTCACCTTCCAA-3'.Analyzed CpGs were located at -184, -175, -173, -171, -164, - 159, -154, -144, -136 bp to the translational start site. The reverse primer was biotinylated. 30 µl total volume for each reaction contained 15 µl PyroMark 2×PCR Master Mix (Qiagen), 3 µl CoralLoad (Qiagen), 5 pmol of each primer and 25 ng of template DNA. The cycling program was performed with an initial denaturation step for 15 min at 95 °C, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 10 s each and a final elongation of 10 s at 72 °C. Subsequently, sequencing was performed on a PyroMark Q24 MDx work station (Qiagen) using a specific sequencing primer: 5'-CCTTCACCTTCCAACT-3'.

High Resolution Melting analysis: CpG methylation of the *c-Myc* promoter region was assessed by using the method of Methylation-Sensitive High-Resolution Melting (MS-HRM), differentiating sequences based on their melting behavior that is dependent on GC content. The reaction mix contained 5 µl MeltDoctor™ HRM Master Mix 5-10 pmol/µl of each primer, 10 ng bisulfite converted DNA, 2 mM MgCl2 and RNase-free water. PCR was conducted with the following primers, previously described by Rahat et al. [38]: Forward 5'-TGAGGATTTTCGAGTTGTGTTGT-3' and Reverse 5'-CTTCTCGAAACAAAAAAAAAACCAAAA-3'. For MSHRM a Rotor-Gene® Q (Qiagen) including a 72-well rotor was used, PCR amplification was performed with one step of 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 58 °C for 1 min each, followed by 95 °C for 10 min and 60 °C for 1 min. HRM was conducted with a continuous acquisition to 90 °C with increments of 0.1 °C. Fully methylated and unmethylated methylation standards were acquired commercially (Qiagen) and bisulfite conversion was conducted as described above. Standards were prepared to obtain following ratios of methylation: 5.5, 8, 12.5, 25, 50 and 100 %. HRM runs were analyzed with Rotor Gene Software Q (Qiagen). Normalized relative fluorescence units were imported to Prism 6 (Graphpad) and area under the curve (AUC) was calculated as described previously by

Switzeny et al. [39]. Linear regression of standard AUC was then used to interpolate methylation of unknown samples.

Statistical Analysis: Treatment effects were tested by a two-way ANOVA followed by Sidak's multiple comparisons test, or a two-samples t-Test using the GraphPad Prism6 software. Statistical significance (*) was based on a 95 % level of confidence (p-value ≤ 0.05).

RESULTS

Associations of EGCG-treatment and telomeric modulation are inverted between cancerous and normal cells: Relative telomere length was assessed in ES-1 and Caco-2 cells at 36, 72 and 144 h, respectively, following treatment with 20 or 200 µM EGCG or DMEM only for controls. After 72 and 144 h incubation with EGCG a higher rTL was found in ES-1 cells compared to the untreated control, with an significant increase after the 144 h incubation time (p=0.01) (Table 1, Fig. 1). In Caco-2 cells, significant differences were found after 36 (p=0.03) and 72 h (p=0.02) incubation. High-dose EGCG (200 µM) was associated with a significant decrease in rTL compared to untreated controls. For incubation with 20 µM EGCG no significant changes in rTL were seen in both cell lines

Table 1. Relative telomere length in ES-1 human fibroblasts and Caco-2 adenocarcinoma cells after 36, 72 and 144 h treatment with 200 µM EGCG over untreated control (DMEM).

		Control			EGCG 200 µM		
	Incubation hours (h)	**rTL**	**SD**	**N**	**rTL**	**SD**	**N**
ES-1	36h	0.91	0.14	6	0.79	0.20	6
	72h	1.20	0.13	6	1.29	0.27	6
	144h	0.92	0.13	6	2.29	0.88	6
Caco-2	36h	1.11	0.18	4	0.78	0.05	4
	72h	0.74	0.08	4	0.58	0.06	4
	144h	0.80	0.09	4	0.91	0.05	4

Values of rTL are expressed as means.

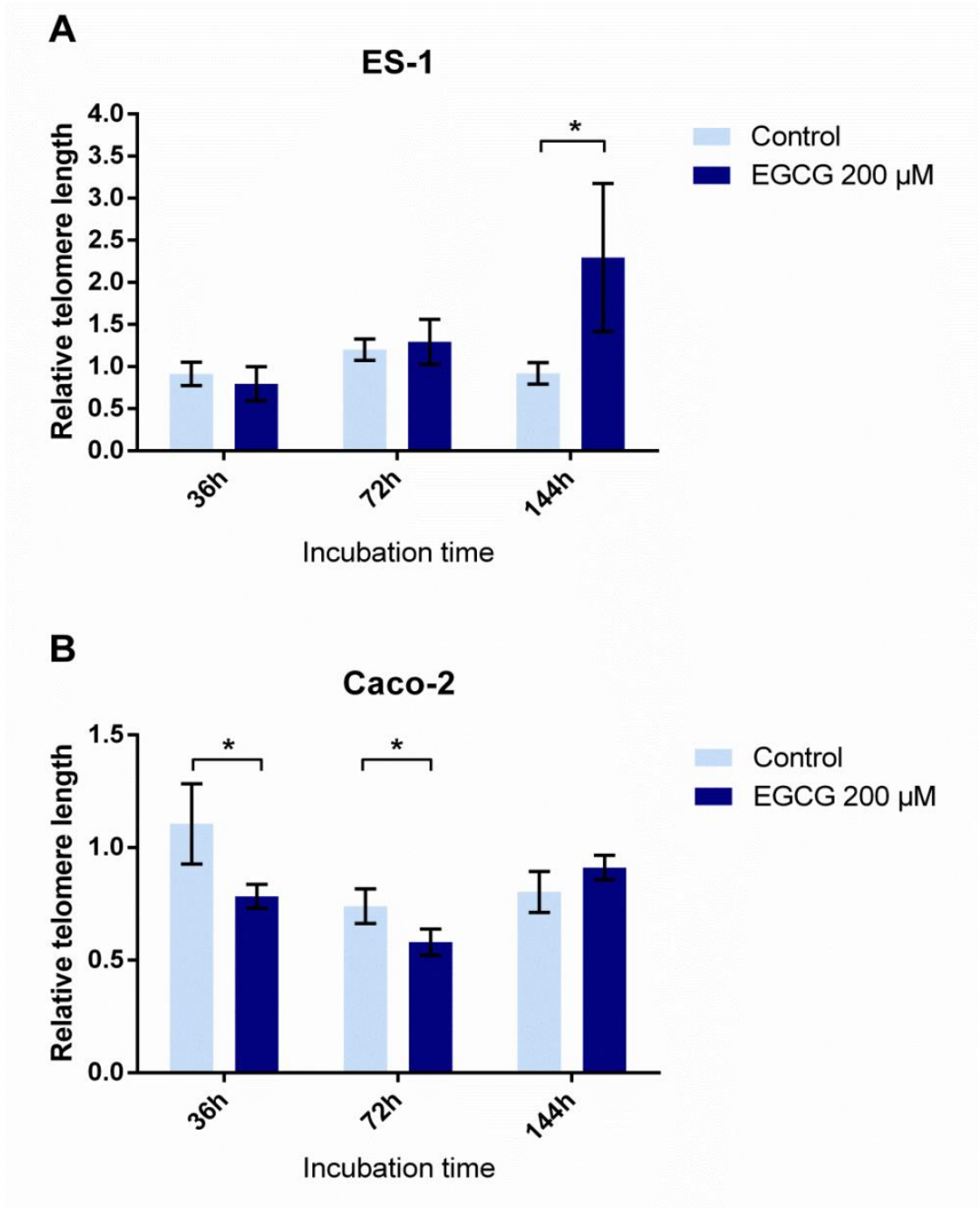

Figure 1. Relative telomere length in 2 different human cell lines with and without treatment with epigallocatechin gallate (EGCG). (A) and (B): Bar graphs show relative telomere length from human fibroblasts ES-1 and Caco-2 colorectal adenocarcinoma cells after 36, 72, and 144 h incubation with 200 µM EGCG compared to vehicle (DMEM) control. Relative telomere length was analyzed by qPCR and calculated based on the 2−ΔΔCT algorithm using the difference in cycle threshold between telomere- and single copy gene 36B4, normalized to an untreated positive control. Cell treatment with EGCG was carried out in quadruplicate in at least 3 independent experiments. Statistical significance was checked by two-way ANOVA followed by Sidak's test for multiple comparisons as well as two-samples t-Test using GraphPad Prism6 software. P-values of smaller than 0.05 are indicated by one asterisk.

Relative telomerase activity is affected after EGCG treatment in Caco-2 cells: Telomerase activity was assessed using the Real-Time Quantitative Telomeric Repeat Amplification Protocol Assay and expressed as percentage of telomerase activity of control cells (0 h, 100 %). Fibroblasts and other healthy somatic cells are reported to have undetectable or very low telomerase activity [40,41]. In agreement, we too observed minute levels of telomerase activity in ES-1 fibroblasts of just above detection limit. In untreated Caco-2 cancer cells telomerase activity decreased over time from 15.28 % ± 5.45 after 36 h, to 13.36 % ± 0.22 after 72 h and to 2.52 % ± 0.08 after 144 h incubation. Compared with untreated controls, we found a significant response to 200 µM EGCG in Caco- 2 cells resulting in reduced telomerase activity values of 1.04 % ± 0.05 (36 h, $p \leq 0.0001$), 0.86 % ± 0.03 (72 h, $p \leq 0.0001$) as well as 0.551% ± 0.06 (144 h, $p \leq 0.01$) (Fig. 2).

The methylation status of hTERT varies between test conditions and cell type:

Sodium bisulfite pyrosequencing was performed to assess the methylation status of nine auspicious CpG sites in the *hTERT*-region. Calculated mean percental (%) methylation levels of the *hTERT* promoter of Caco-2 untreated controls at 36, 72 and 144 h were 49.66 ± 2.21, 48.13 ± 1.86, and 48.73 ± 1.73, respectively (Fig. 3A and 3B). The *hTERT* methylation status of those Caco-2 cells receiving treatment remained unaffected irrespective of the duration or dosage of treatment with values (%) of 49.98 ± 2.63 or 47.82 ± 1.16 after 36 h or 72 h incubation with 200 µM EGCG (Fig. 3A).

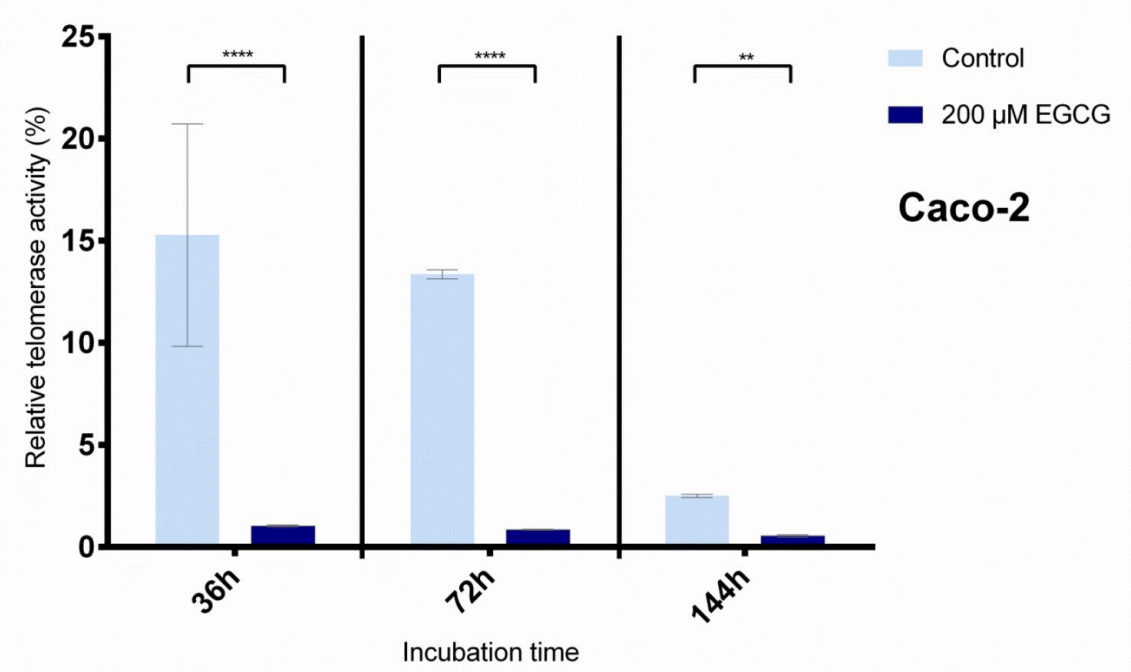

Figure 2. Effects of EGCG treatment on telomerase activity in Caco-2 colorectal cancer cells. Columns display telomerase activity in Caco-2 cancer cells after 36, 72, and 144 h incubation with 200 µM EGCG compared to an untreated control. Telomerase activity was measured using the Real-Time Quantitative Telomeric Repeat Amplification Protocol Assay combining a real-time PCR technique with a conventional TRAP method. Activity is expressed as percentage of the activity in the control cells (0h, 100%). Statistical significance was indicated by two-way ANOVA followed by Sidak's test for multiple comparisons as well as the two samples t-Test using GraphPad Prism6 software. P-values: < 0.05 (*), < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

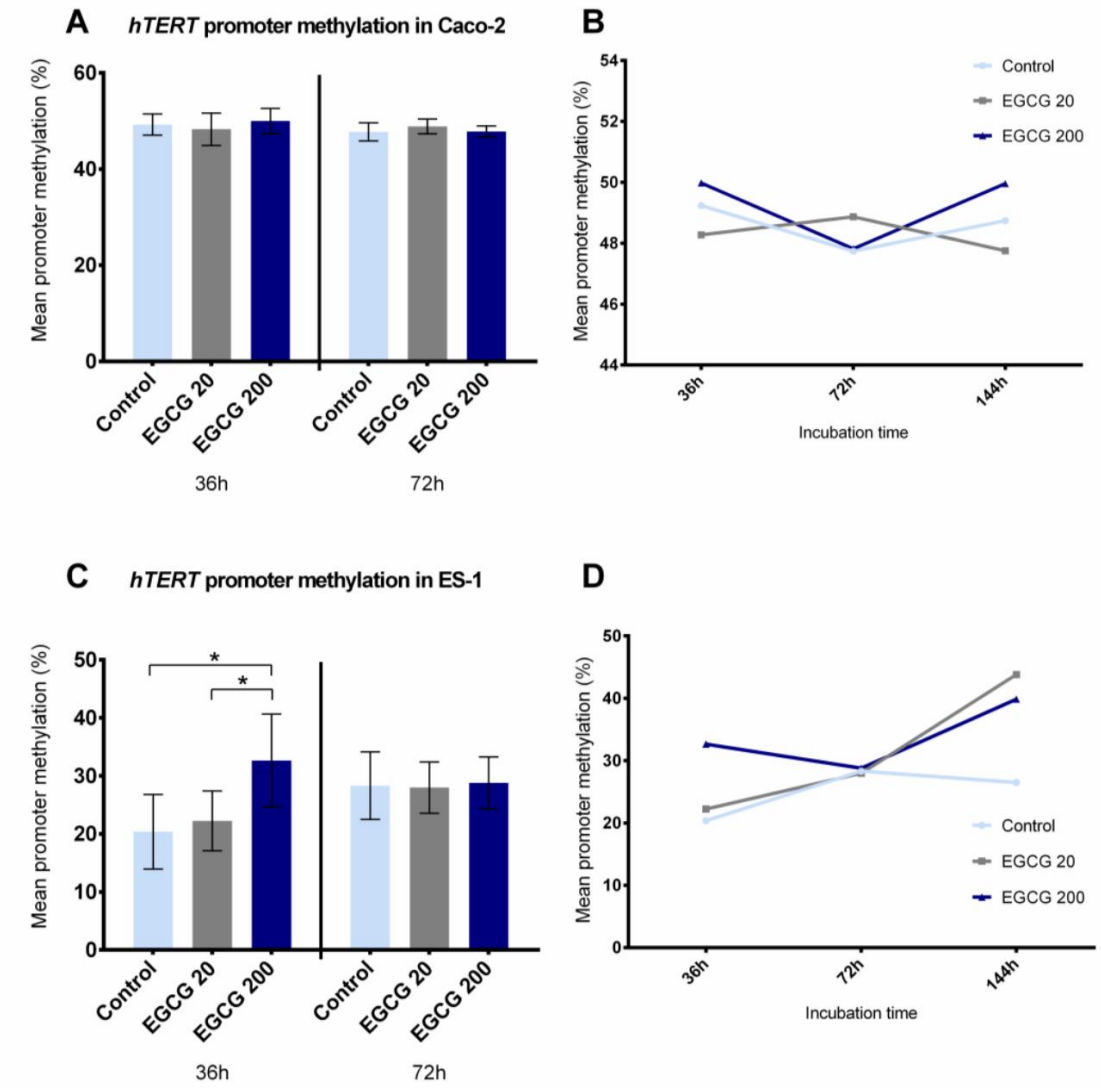

Figure 3. Mean promoter methylation of *hTERT* in Caco-2 cancer cells and ES-1 primary fibroblasts after incubation with different doses of EGCG. (A) and (C) show mean methylation percentages ± SD of *hTERT*, (B) and (D) depict mean values for each group after 36, 72 and 144 h incubation with 20 or 200 µM EGCG compared to untreated DMEM control. Methylation status was assessed by sodium bisulfite

pyrosequencing. Statistical significance was indicated by the two-samples t-Test using GraphPad Prism6 software. P-values less than 0.05 are indicated by one asterisk; (n=6 per group).

The findings were somewhat different for ES-1 fibroblasts. Mean percental promoter methylation of *hTERT* in ES-1 cells after 36, 72 and 144 h incubation were found to be 20.36 ± 6.45, 28.31 ± 5.82 and 26.49 ± 13.99. After incubation with 200 µM EGCG, a significantly higher methylation was found after 36 and 144 h compared to untreated controls with values of 32.64 ± 8.00 and 39.86 ± 4.75, respectively ($p \leq 0.01$) (Fig. 3C and 3D). Analyzing the methylation status of 9 CpG sites within the *hTERT* promoter region, revealed a significant result in fibroblasts concerning CpG 1-6 after 36 h incubation with 200 µM EGCG (Fig. 4B). This effect was also evident after 144 h, with significantly lower methylation values in the 200 µM EGCG treatment group in CpG 1, 2, 3, 4 and 6. While mean *hTERT* methylation in Caco-2 cells was significantly higher than in ES-1 fibroblasts ($p < 0.001$), and all CpG sites in ES-1 cells analyzed showed lower methylation than in Caco-2 cells ($p < 0.001$), no significant changes in the methylation status upon EGCG treatment were found in Caco-2 cells (Fig. 4A).

Methylation status of c-Myc in Caco-2 cells changes after incubation with EGCG: The percental methylation status of the *c-Myc* promoter region was analyzed by MS-HRM in Caco-2 cancer cells and ES-1 primary fibroblasts after 36, 72, or 144 hours incubation with 20 and 200 µM EGCG, respectively, nd compared to untreated controls. Significantly higher *c-Myc* promoter methylation was found in Caco-2 cells after 36 h treatment with EGCG at both concentrations compared to untreated control ($p \leq 0.01$) (Fig. 5A). In contrast, EGCG incubation hardly affected the *c-Myc* methylation status of ES-1 fibroblasts (Fig. 5B): Control fibroblasts had methylation levels (%) ranging from 6.89 ± 1.15 (at 36 h), over 7.52 ± 0.23 (at 72 h) to 3.45 ± 0.99 (at 144 h). After incubation, values reached 7.99 ± 0.86 (20 µM EGCG), 8.31 ± 1.53 (200 µM EGCG) after 36 hours, 7.56 ± 0.94 (20 µM EGCG), 6.35 ± 1.2 (200 µM EGCG) after 72 hours and 5.29 ± 0.82 (20 µM EGCG) as well as 3.6 ± 0.33 (200 µM EGCG) after 144 hours.

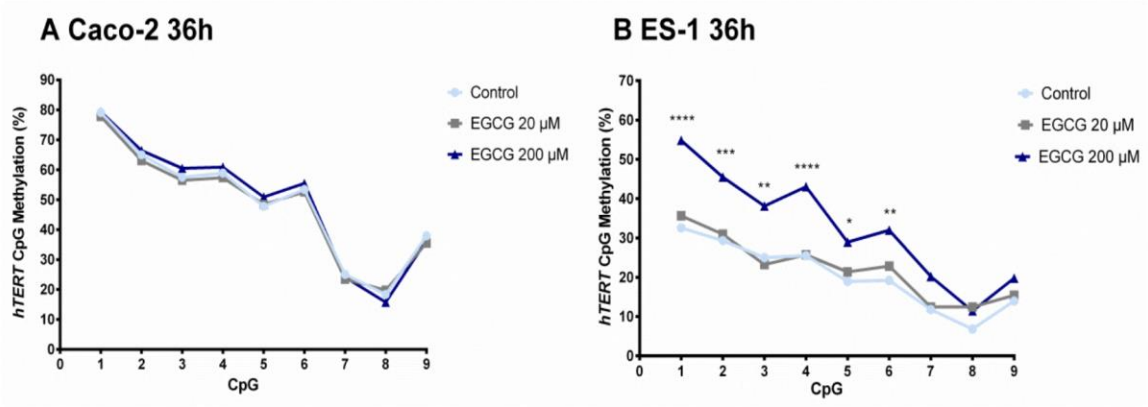

Figure 4. Effect of EGCG treatment on CpG promoter methylation of *hTERT* in Caco-2 cells and ES-1 fibroblasts. The line charts display the methylation status of 9 CpG sites within the *hTERT* promoter region analyzed by sodium bisulfite pyrosequencing in Caco-2 cells (A) and ES-1 fibroblasts (B) after 36 h incubation in 3 groups (non treatment control, 20

and 200 µM EGCG). Significant effects of EGCG treatment with at CpG 1-6 are labeled with asterisks. Statistical significance was indicated by two-way ANOVA followed by Sidak's test for multiple comparisons as well as the two-samples t-Test using GraphPad Prism6 software. P-values: < 0.05 (*), < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****); (n=6 per group).

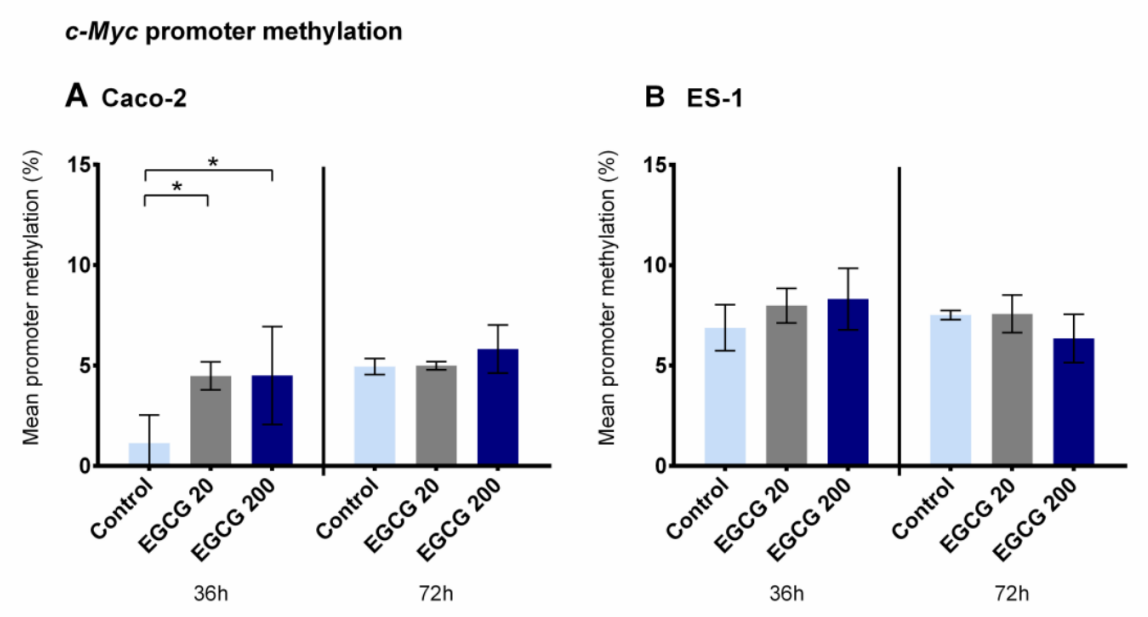

Figure 5. Changes in mean promoter methylation of *c-Myc* in Caco-2 cancer cells and ES-1 primary fibroblasts after incubation with EGCG. Methylation status of *c-Myc* promoter region in Caco-2 cells (A) and ES-1 fibroblasts (B) after 36h and 72 h incubation in 3 groups (non treatment control, 20 µM and 200 µM EGCG) analyzed by Methylation-Sensitive High Resolution Melting (MS-HRM). Statistical significance compared to untreated control was observed in Caco-2 cells after 36 h treatment with EGCG in both

concentrations. Significance was assessed by the two-samples t-Test using GraphPad Prism6 software. P-values of less than 0.05 are indicated by one asterisk (n=8 per group).

EGCG counteracts cellular oxidative stress: In Caco-2 cells sole treatment with 250 and 500 µM H2O2, respectively, significantly increased MDA levels versus the untreated control (Fig. 6, left panel). Similarly, two separately tested high EGCG concentrations (100 and 200 µM) increased MDA levels, but only by trend (Fig. 6, middle panel). Conversely, a low concentration of EGCG (20 µM) alone significantly decreased MDA levels versus the untreated control (Fig. 6, middle panel). The same was true for EGCG (20, 100 and 200 µM) in simultaneous combination with H2O2, attenuating lipid peroxidation, and resulting in decreased MDA levels (Fig. 6, right panel).

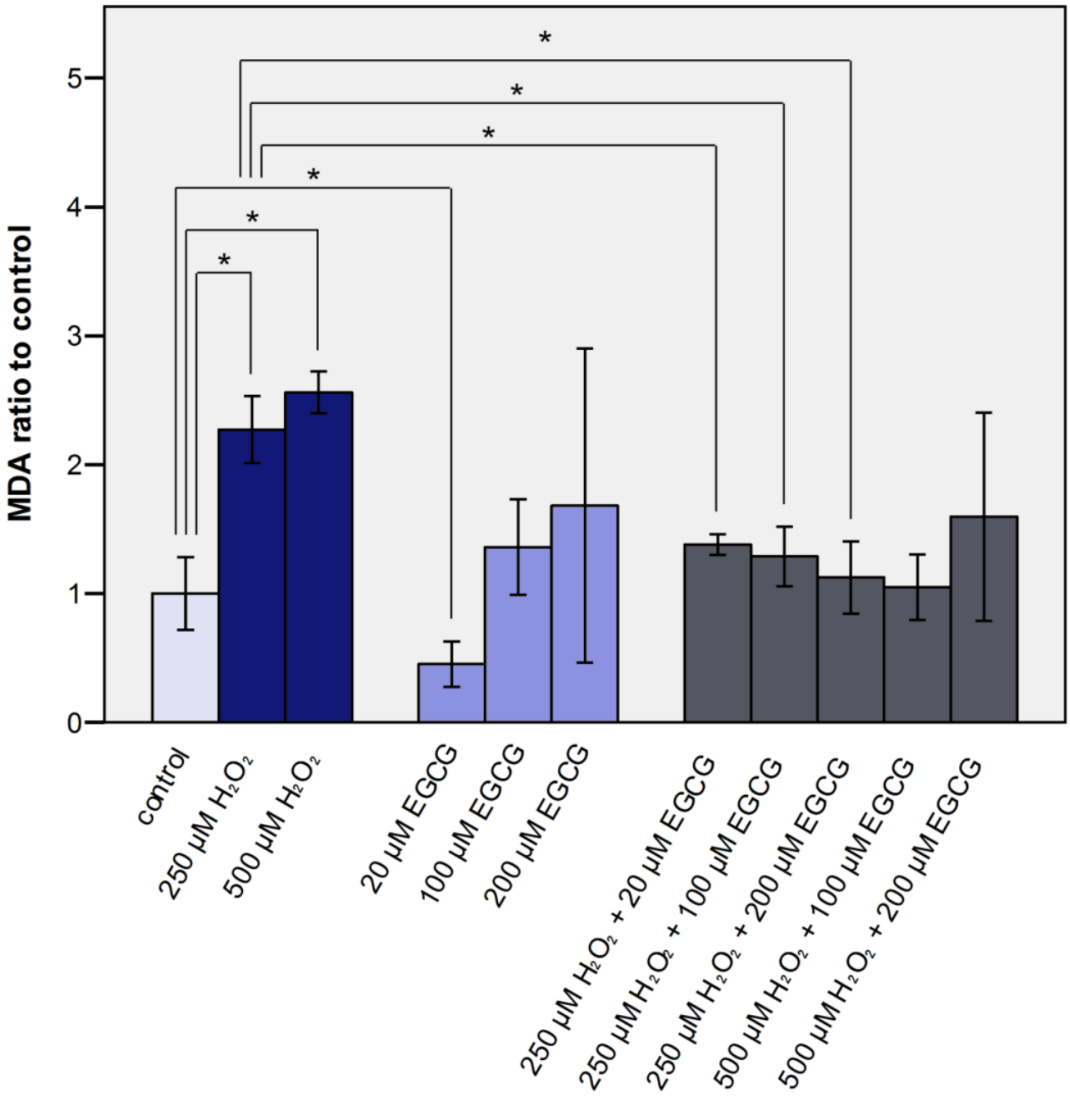

Figure 6. Pro- and antioxidative effects of EGCG in Caco-2 cells assessed by MDA levels as marker for lipid peroxidation. MDA levels in Caco-2 cells after incubation with 20, 100 and 200 µM EGCG analyzed by HPLC and fluorescence detection at 533 nm. For H2O2 induced oxidation 250 and 500 µM H2O2, respectively were used. Bar charts display mean MDA ± SD over respective untreated controls. Significance was assessed by the two-samples t-Test using GraphPad Prism6 software. Experiments were independently repeated. P-values of less than 0.05 are indicated by one asterisk.

DISCUSSION

Predicated on EGCG's reported *in vitro* antioxidant and chemo-preventive activities, recent topical research has increasingly focused on the compound's potential molecular role in cancer treatment. While EGCG has been reported to interact with over 300 proteins at a molecular level, stoichiometric information remains scarce [42]. However, studies suggest EGCG exerts its proposed anti-proliferative (i.e. "anti-cancer") effects in a highly targeted manner exclusively on cancer cells, while omitting their healthy counterparts, by selectively regulating carcinogenic signaling pathways [19,29,43,44]. These include a plethora of targets, as recently reviewed by Negri et al. [45]. Indeed, other mechanistic studies observed a dose-dependent association between EGCG and telomere length in normal cells and conversely, decreased telomerase levels and *hTERT* gene expression in cancer cells [24,29], supporting EGCG's proposed selective anti-proliferative effects. It is well accepted, that telomere shortening occurs in most human somatic cells with each DNA replication cycle, ultimately leading to cellular senescence or apoptosis. Cell division also increases the likelihood of replication mistakes to occur that might or might not be relevant in the pathogenesis of age-related diseases such as cancer. Indeed, a loss of telomeric integrity affects the replicative capacity of all cells of the body and triggers global epigenetic alterations impacting chromatin and transcriptional properties, accelerating cellular senescence and aging along with age-related health conditions. Conversely, cancer cells maintain their telomeres by expressing telomerase or activating the alternative lengthening of telomeres pathway [46], and moreover, recent studies have shown a strong correlation of long telomeres with different types of cancer, e.g. caused by mutations in the shelterin complex [47,48]. However, research has indicated that the mean telomere length of telomerase-positive cancer cells such as Caco-2 is stable [49]. Intriguingly, this finding supports and complements the phenomena of

telomereshortening and decrease in telomerase activity encountered upon EGCG treatment of Caco-2 cells in this study. To identify EGCG-dependent alterations in the epigenetic control of telomeres, we analyzed methylation of *hTERT* and the protooncogene c-*Myc,* due to its close link to *hTERT* gene expression. As the *hTERT* promoter is highly enriched in CpG dinucleotides, in the context of methylation analyses, different regions have been described. Though DNA methylation studies of *hTERT* have generated inconsistent data, hypermethylation of the upstream of the transcription start site (UTSS) region has been strongly associated with TERT expression incancers [50]. At -165 and +44 E-boxes are found, which are binding sites for c-Myc and Mad1 [51], therefore CpG methylation analysis was conducted in this area to target possible interactions with c-Myc. Through its demonstrated ability to inhibit 5- cytosine DNA methyltransferase (DNMT) [18] and histone deacetylases (HDAC) [30], a regulatory epigenetic role for EGCG has been revealed. Topical research found EGCG suppresses telomerase activity in cancer cells, ultimately halting tumor proliferation [29,52]. In that respect, hypomethylation of genomic DNA alongside gene-specific hypermethylation in methylation-sensitive CpG islands of promoter regions of relevant genes have been reported [53,54,55]. CpG hypermethylation in the CTCF binding region of the promotor region of *hTERT* results in a decreased binding affinity of the repressor CCCTCbinding factor (CTCF) and increased expression of the catalytic subunit of hTERT [20]. Interestingly, hypermethylation of the *hTERT* gene has been correlated with telomerase activity in both healthy and tumor tissues, while conversely, demethylation was not associated with increased *hTERT* expression [56]. Although a series of transcription factors and pathways have been implicated in the regulation of *hTERT* expression, CTCF appears to be a correlated key determinant of mortality [20]. While in the present study merely non consistent changes in the methylation status of nine auspicious CpG sites were found following EGCG incubation of Caco-2 cells, we located six significantly hypermethylated CpG sites in normal fibroblasts, as well as a significantly higher overall mean methylation status of *hTERT* after treatment with 200 µM EGCG, both after 36 h and 144 h. These findings emphasize a role for EGCG in regulating telomeres and ultimately cellular aging. Unexpectedly, at 72 h incubation, significant methylation differences of control and treated cells were not apparent in fibroblasts. We hypothesize, this finding might be ascribed to the proposition that the overall methylation status in cell lines is dynamic (i.e.

changes over time) depending on cell line and environmental factors [57,58]. c-Myc, for which the *hTERT* promoter contains a binding site, is considered a “master regulator” of tumorigenesis controlling many aspects of cell proliferation, differentiation and cellular metabolism [59]. Reportedly, activation of *c-Myc* is observed in more than half of human cancers and its dysregulation is a proposed marker for genomic instability [59,60,61]. The critical connection of *c-Myc* expression and EGCG was recently established [62], demonstrating a significant decrease of *c-Myc* expression along with reduced hTERT protein levels after treatment with EGCG in an immortal cell line. Signaling through NF-κB has been proposed as a possible underlying mechanism. Thus, we adopted methylation of *c-Myc* with regard to its indirect ability to regulate telomerase through *hTERT*. We found that, versus respective controls, *c-Myc* methylation was hardly affected in fibroblasts whereas significantly higher methylation, likely resulting in decreased c-Myc mRNA expression, was found in Caco-2 cells after 36 h treatment with EGCG. These results underline the multifaceted molecular effects of EGCG reported, emphasizing the compound’s highly specific possible chemotherapeutic relevance. As mentioned above, interestingly, the methylation status of *hTERT* in Caco-2 cells was but little affected, neither by time nor treatment with EGCG. However, similar to an earlier report on HeLa cervical cancer cells [19], a significant decrease in relative telomere length and a decline in telomerase activity was observed after EGCG treatment. Therefore, we suggest that telomere shortening might be indirectly further enhanced by EGCGmediated inhibition of telomerase, through generation of ROS. While the exact effects of ROS on telomerase are not well understood to date, oxidative-stress-induced functional inhibition of telomerase in cancer cells has been reported recently [63]. Telomeric DNA itself is thought to be particularly susceptible to ROS-mediated damage along with telomeric attrition, both of which are exacerbated by treatment with EGCG which furthermore has been shown to exhibit genotoxicity in a telomereindependent fashion [64]. Regardless of the exact underlying mechanisms, reportedly the biological effects of EGCG follow a concentration-dependent pattern [65]. Overall, concentrations of 10-200 µM EGCG exerted antiproliferative effects in human cancer cell cultures from different tissues [33,66,67,68,69]. In our study, dose-dependency was confirmed in that 200 µM EGCG proved to be more efficacious than 20 µM regarding telomeric regulation in Caco-2 cells.

While physiological concentrations of EGCG (< 10 µM) have been reported to stabilize metabolic function and assist in the management of oxidative stress [65,70,71], experimental evidence suggests cancer inhibition might require higher doses [69,72]. Despite this proposition, inversely, otherstudies have raised concern over possibleadverse effects of EGCG when used at high concentrations [73,74,75]. Therefore, potential future clinical applications must include strategies and techniques that effectively and safely deliver EGCG exclusively to target sites while limiting unwanted (systemic) side effects. Recently, research groups have successfully explored nanotechnology-inspired methods using encapsulated EGCG, which demonstrated increased bioavailability and functional selectivity at the target site, and ultimately a reduction in tumor growth [76,77]. Albeit, before EGCG as a compound with possible therapeutic effects can find its way into the clinic, several issues need to be resolved. For instance, EGCG has been shown to undergo a whole range of complex structural changes *in vivo* and oxidatively polymerize in cell culture. This results in cell-specific oxidative environments in hyperproliferative versus normal cells [78,79]. Autooxidation of EGCG is reported to produce ROS in a dose-dependent manner [80]. Low concentrations (< 5 µM) have been associated with the promotion of cell growth, whereas abundance of ROS resulting from the use of high EGCG concentrations (> 50 µM) provoked apoptotic and inhibitory effects including a reduction of telomerase activity [79,80,81], as was also demonstrated in the present study, and emphasized by the additional finding of telomere shortening. However, it remains to be fully clarified whether suchlike auto-oxidative properties of EGCG can also occur at organ sites *in vivo* [80], and which potential risk this might pose to macromoleculessuch as proteins or lipids. To shed some light on this issue, we assessed malondialdehyde (MDA) levels [73,74] in Caco-2 cells to obtain a first indication of potential pro-oxidative effects of EGCG in a physiologically relevant tissue model. MDA is a well-accepted and widely used surrogate marker of tissue lipid peroxidation (i.e. oxidative stress) [24,34,82,83,84]. Measuring MDA formation in our cell culture systems enabled us to assess possible adverse effects of high doses of EGCG on the one hand, but also identify the compound's potential to inhibit peroxidation induced by H202 on the other hand. In accordance with earlier literature [85], EGCG alone at low concentration (20 µM) significantly decreased MDA levels, while high concentrations (100 and 200 µM, respectively) were non-significantly associated with increased MDA levels. When combined with H2O2, EGCG

quenched H2O2-induced lipid peroxidation. Similar findings had been reported earlier for human dermal fibroblasts where EGCG decreased MDA levels [86]. Measurement of basal and H2O2-induced ROS production, glutathione (GSH/GSSH) or other related oxidative stress biomarkers could help evaluate the cellular antioxidant response and the extent and quality of EGCG's antioxidant potential in future research. However, there is consensus, that the antioxidant property of EGCG is mediated by many different mechanisms including the activation or inhibition of enzymes involved in the modulation of ROS levels. As regards the latter, the sirtuins enzyme family (SIRTs) has been intensively studied because of their increasing significance in cancer biology and other age-associated diseases [3]. SIRTs (SIRT1-SIRT7), are nicotinamide adenine dinucleotide (NAD+) dependent histone deacetylases, which modulate the regulation of a variety of inflammatory and metabolic pathways, including those associated with redox signaling [87,88,89]. Though both sirtuins and telomeres are heavily implicated in ageing related processes, their molecular interplay is not fully understood. However, recent studies support tightly intertwined mechanisms for different sirtuin subgroups in telomeric regulation in part because of their nuclear localization [88,90,91,92]. Furthermore, SIRT1is reported to interact with transcription factors related to the *hTERT* promoter [93] and deacetylates the C-terminus of c-Myc, additionally affecting *hTERT* promoter activity [94]. As regards the activation of sirtuins, there could be a role for polyphenols [87,95], as also demonstrated recently by us using 3T3-L1 preadipocytes [96]. Recently, rapidly emerging scientific findings about the interplay of microRNAs (miRNAs) with *hTERT* have shed new light on the regulation of telomeres [97]. Also in this context, polyphenols such as EGCG constitute potential modulators especially in cancer cells, as they have been shown to regulate miRNA expression [98,99].

CONCLUSIONS

Telomere regulating effects of EGCG are on account of several related mechanisms that include antioxidant properties, activation of sirtuins, miRNAs and the modulation of signaling pathways, such as NF-κB. Our study confirms EGCG's proposed antioxidative properties *in vitro* by exerting a protective effect against H2O2-induced lipid peroxidation, demonstrated by decreased MDA levels in Caco-2 cells. At the same time, pro-oxidant effects of high doses of EGCG could constitute a possible mechanism for the opposed

modulation of telomeres by EGCG in cancer versus normal cells. As EGCG is known to play an active role in modulating cell metabolism and apoptosis, selectively targeting the telomerase gene and telomeres in cancer cells, our observations are in line with earlier reports and emphasize a potential role for EGCG in reinforced novel anti-cancer drugs [100]. Despite those promising results it must be considered that effects are strongly affected by cell type characteristics, cell systems and culture conditions. Cell lines reflect their respective tissue origin in their different potential of DNA repair capacity and clearance of H2O2 and intracellular ROS generation, also resulting in a specific susceptibility to phyto-/chemicals such as dietary compounds [80,101,102]. Future research including but not limited to an in-depth characterization of active enzymes that are selectively expressed in EGCG treated cells will assist in understanding EGCG's mode of action in physiological and pathological conditions. Plantderived compounds like EGCG may eventually offer promising new treatment options for degenerative and hyperproliferative diseases. For this to happen, *in vivo* models are a crucial prerequisite to clarify EGCG's pharmacological potential, assess its bioavailability, ideal route/dosageof administration and safety.

List of Abbreviations: bp, base pairs; CTCF, CCCTCbinding factor; DMEM, Dulbecco's modified Eagle medium; DNMT, 5-cytosine DNA methyltransferase; EGCG, Epigallocatechin gallate; FBS, fetal bovine serum; GSH, glutathione (GSH/GSSH); HDAC, histone deacetylases; HPLC, High Performance Liquid Chromatography; *hTERT*, human Telomerase Reverse Transcriptase; MAP, mitogen activated protein; MDA, malondialdehyde; miRNAs, microRNAs; MS-HRM, methylation-sensitive high-resolution melting; NF-κB, Nuclear factor kappa B; NAD+, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; rTL, relative telomere length; SIRT, sirtuin; UTSS, upstream of the transcription start site.

Author's Contributions: Angelika Pointner, Alexander Haslberger and Ulrich Magnet conceived the presented idea. Angelika Pointner, Katja Zappe and Ulrich Magnet developed the theory, performed the experiments and verified the analytical methods. Angelika Pointner took the lead in writing the manuscript. Alexander Haslberger encouraged Angelika Pointner to investigate the topic and supervised the findings of this

work. Christine Mölzer, Anela Tosevska and Ulrike Krammer contributed to experiments regarding telomeres and cell lines. Angelika Pointner, Alexander Haslberger, Christine Mölzer, Berit Hippe, Elisabeth Dum and Stephanie Lilja contributed to editing and review. All authors discussed the results and approved the final version of the manuscript.

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12.2.2 Differences in host cellular microRNA expression of EBV positive and EBV negative participants and acute or chronic, in addition lytic and latent infection regarding immune response - a pilot study; submitted at MDPI diagnostics-1248195 20.May 2021

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Abstract

Epstein-Barr virus (EBV) is a lifelong persistence herpesvirus associated with severe pathologies. The virus developed strategies to overcome immune system, which are still not yet fully understood. Thus, we investigated whether there are differences in the host cellular miRNA expression in people infected with EBV and EBV-negative participants and in further subgroups. Whole blood and dried blood spots of 156 patients were collected. Based on 24 immune relevant markers, we divided the EBV-positive subjects into subgroups. Telomere length, IL-6 methylation and 9 host miRNAs were assessed. Cytokine receptors, namely IL1-r, IL12-r, IFNα-r, TNFα-r were decreased in EBV-infected participants compared to the study population without EBV, whereas CRP was upregulated. Additionally, miR328 and miR21 were upregulated in EBV-positive subjects. Between chronic and acute EBV infection miR877 was statistically significant. Differences in miR155, miR21, miR127 and miR877 expression were observed within the four subgroups chronic latent, chronic lytic, acute within last few months and acute early stage. No significant differences were obtained for miRlet7g, miRlet7a and miR151. EBV miRNAs have been investigated in the aspect of escaping host immune system via modulating host gene expression. These results show that host miRNAs can be used as biomarkers to represent different EBV-subgroups.

Keywords: EBV, chronic, acute, biomarkers, miRNAs, immune system

Introduction

Epstein-Barr virus (EBV) is a ubiquitous oncogenic human herpesvirus with an infection rate of 95% and persists in individuals during their lifetime, but immunocompetent individuals are not associated with clinical symptoms. Primary EBV infections that occur during childhood are mainly asymptomatic. Infections that occur in adulthood can lead to infectious mononucleosis in the acute phase. EBV is transmitted mainly via saliva and aerosols. In the oropharynx, EBV infects B cells via binding its glycoprotein gp350 to the complement receptor CD21 [138][139]. Binding of gp350 to CD21 activates several pathways, in particular NF-κB, which results in interleukin-6 (IL-6) upregulation [140]. EBV infection of B cells can exhibit latent or lytic infection for the persistent and lifelong infection in humans [141]. During the latent, nonproductive infection, the EBV viral DNA acts like its host chromosomal DNA. Nevertheless, different gene expression patterns are recognized and characteristic for the latent and lytic phase. Expression of latent membrane proteins (LMP1, LMP2A, LMP2B) and EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C) are specific for the latent phase. In contrary, BZLF1, BRLF1, BMRF1, BALF1 and BHRF1 expression initiates the EBV lytic phase, where the EBV infection reactivates and its genome is amplified up to 1000-fold by replication and infecting neighboring cells [138]. Impaired cellular immune and physiological stress, but not physical stress, are causes for EBV reactivation [142]. The EBV-encoded protein LMP1 is essential for EBV-mediated B cell transformation [143]. CD80 and CD86 are active antigens on B lymphocyte surfaces and can be upregulated by triggering B-cell receptors, whereas CD80 expression can be upregulated by a variety of stimuli, for example cytokines [143] [144]. EBV infection leads to an increase of CD86 and CD80 playing a role in immunity and its dysbalanced expression may lead to autoimmune disorders [144].

The host's immunity plays an essential role by recruiting cells such as neutrophils, macrophages and dendritic cells. These cells produce reactive oxygen species (ROS) and pro-inflammatory cytokines that help to fight the pathogen. Cells from the innate immune system detect microorganisms through their pattern recognition receptors (PPRs) which bind to pathogen-associated molecular patterns (PAMP). This shapes and controls the adaptive immune system, which leads to the development and activation of T and B cells, T helper cells and killer cells [145]. Additionally, miRNAs can regulate immune responses and play a modulatory role in the assembly of the inflammasome complex [146]. Studies

report that EBV-miRNAs target viral and cellular genes, which influence the immune response and contribute to malignancy of EBV infection. MiR-BHRF1-3, which are viral miRNAs, target genes, which contribute to the survival of EBV infected cells [147]. On the one hand, host cellular miRNAs upgulate the innate and adaptive immune system leading to the manifestation of inflammation or even the induction of EBV-associated cancer, but on the other hand can also protect against pathogens [148].

In our study we focused on host cellular miRNA expression as biomarkers to identify EBV-positive and EBV-negative individuals and to discriminate between four different subgroups. EBV infection can undergo lytic and latent stages. We therefore clustered the EBV-positive group into four different subgroups and examined host miRNA differences and functions.

Materials and Methods

Sample collection

Data and blood samples have been collected from 156 participants in total, all showing negative health symptoms. MiRNA gene expression analysis was done from capillary blood drops collected on Whatman® protein saver cards (Sigma Aldrich, St. Louis, MO, USA), further procedure described in the next section. Additionally, whole blood was taken from the participants for detection of EBV, immunoglobulins, different B-cell receptors and cytokines by the medical supervisor Dr. Jacob.

Markers for viral infection

Multiple biomarkers regarding the immune system, inflammation and viral infection, namely CD86, CD80, CD19, CIC-IgA, CIC-IgG, CIC-IgM, CIC-C3, CRP- high sensitive, TNF-α, IL6, IL8, IL1-r, IL3-r, IL6-r, IL7-r, IL9-r, IL12-r, IL13-r, IL15-r, IL17-r, IFNa-r, IFNb-r, TNFa-r and TNFb-r were analyzed of each study participant.

Detection of EBV and classification of the subgroups

EBV was detected via qPCR, thus participants were separated in EBV-positive and EBV-negative. In order to distinguish acute and chronic infection, several blood parameters that are characteristic for a virus infection have been categorized. Chronic EBV-positive participants were clearly distinguished by the decreased expression of CD80 and CD86 and increased in IgG. Some participants in this group additionally showed lytic patterns

with increased GP350 and IgM. We therefore distinguished the group further into chronic latent and chronic lytic [140] [143] [144].

The acute infected participants had high IgM and CD80 or CD86. Some participants were clearly detected for EBV and GP350, but Igs and CDs were still in the range of a healthy individual. For this reason, all acute subjects were distinguished between an infection within last months and an infection at the very early stage [140] [143] [144].

Total DNA/RNA extraction and miRNA expression

DNA and RNA were sequentially extracted using the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermofisher, Waltham, MA, USA) and the KingFisher Duo Prime System (Thermofisher, Waltham, MA, USA). cDNA synthesis and qRT-PCR were performed using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Thermofisher, Waltham, MA, USA), the Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermofisher, Waltham, MA, USA), and the ExpressionSuite Software (Thermofisher, Waltham, MA, USA), The protocol, TaqMan® Advanced miRNA Assays Single-tube assays, Catalog Number A25576, (Thermofisher, Waltham, MA, USA), was used without exceptions. miR-24 was used as a housekeeping and reference gene for the other target miRNAs, which were miRlet7g, miRlet7a, miR877, miR155, miR127-3p, miR151a, miR328 and miR21-5p (Thermofisher, Waltham, MA, USA). These miRNAs were selected in a pilot study upon a broad miRNA Array from Qiagen analyzing 360 miRNAs out of 4 EBV infected samples and four healthy controls.

Telomere length and methylation status

Relative telomere length was determined in genomic DNA, isolated from the dried blood spots using a StepOne Plus real time PCR Detection System (Applied Biosysteme). For PCR, single-copy gene primers, telomere primers (Biomers, Germany) and a LightCycler® 480 Sybr®Green I master mix (Roche) were used.

Methylation status, qPCR and high-resolution melt analysis were applied. Bisulfit conversion was done using the EpiTect bisulfite kit (Qiagen) following the manufacturer's protocol and using a maximum of 2 µg of genomic DNA. EpiTect HRM PCR kit (Qiagen) and primers for IL-6, was carried out in a Rotor Gene Q (Qiagen). Standards were generated with REPLI-g Mini Kit and mixed accordingly, to generate standards with 0, 25, 50, 75 and 100% methylation and compared to the samples.

Results

Study population characteristics and differences between EBV positive and EBV negative participants

In total 156 participants were enrolled in this study, consisting of 72 males and 84 females with a mean age of 48.98 years (Table 1). Most of the study subjects were positively detected for EBV in the blood via qPCR (Table 1). In the next step, EBV-positive population was divided into 49 chronic and 46 acute EBV infected participants depending on the immunoglobulins (Igs) and B-cell receptor patterns. This resulted in 20 males and 29 females in the chronic group and 22 males and 24 females in the subpopulation of EBV positive group. (Table 2). Each subgroup was divided into a next dimension, namely chronic latent, chronic lytic, acute within the last months and acute at the early stage, depending on the blood parameters characteristic for virus infections. 29 subjects were assigned into the chronic latent group and 20 subjects were assigned into the chronic lytic infection state (Table 2). 26 patients were assigned into the acute within last months subgroup and 20 patients were assigned into the acute early stage subgroup (table 2).

Table 1: population characteristics between EBV positive and EBV negative study population

	Total study population	EBV positive	EBV negative
Total N/%	156 (100%)	95 (60.9%)/ (100%)	61 (39.1%)/ (100%)
Male N/ %	72 (46.15%)	42 (44.21%)	30 (49.18%)
Female N/ %	84 (53.85%)	53 (55.79%)	31 (50.82%)
Mean ± SD			
Age	48.98 ± 14.638	47.90 ± 13.85	48.67 ± 16.827
Age male	49.43 ± 15.176	49.54 ± 12.562	48.14 ± 16.615
Age female	48.66 ± 14.282	46.64 ± 14.764	49.16 ± 17.282

Table 2: subgroup characteristics: characteristics of the populations between chronic and acute subgroups and population characteristics between chronic latent, chronic lytic, acute last months and acute early stage subgroups

N=94	EBV positive		EBV positive	
	Chronic		**acute**	
N/ %	49 (51.58%)/100%		46 (48.42%)/100%	
Male N/ %	20 /40.8%		22 / 47.8%	
Female N/ %	29 /59.2%		24 /52.2%	
Mean ± SD				
Age	49.14± 13.454		46.56 ± 14.298	
Age male	51.70 ±10.219		45.75 ± 14.471	
Age female	47.38 ±15.216		47.48 ± 14.397	
	Chronic latent	**Chronic lytic**	**Acute last months**	**Acute early stage**
N/ %	29 (59.18%)/100%	20 (40.82%)/100%	26 (56.52%)/100%	20 (43.48%)/100%
Male N/ %	11 /37.93%	9 /45%	10 /38.46%	12 /60%
Female N/ %	18 /62.07%	11 /55%	16 /61.53%	8 /40%
Mean ± SD				
Age	49.31 ±12.104	48.90± 15.532	47.35± 15.189	45.47 ± 13.310
Age male	52.27 ± 8.545	51 ±12.48	45.40 ± 14.569	49.36 ± 14.672
Age female	47.50 ±13.755	47.18 ±18.071	48.56 ± 15.908	40.13 ± 9.568

Differences in inflammation markers between EBV positive and EBV negative

Multiple inflammation markers were analyzed in each study participants, where most of them were not statistically significant between EBV-positive and EBV-negative participants. Nevertheless, statistically significant differences in IL-1 and IL-12 receptors were seen between EBV-positive and EBV-negative (Figure 1a & b), additionally to IFN-α and TNF-α (Figure 1c & d), which were all decreased in EBV-positive patients. In contrast, CRP was higher in EBV positive study population (Figure 1e) (all $p<0.05$). Additionally, we observed a negative correlation of telomere length with age within the study population ($p<0.0001$) (Figure 1f), but did not observe differences in EBV-positive and EBV-negative participants.

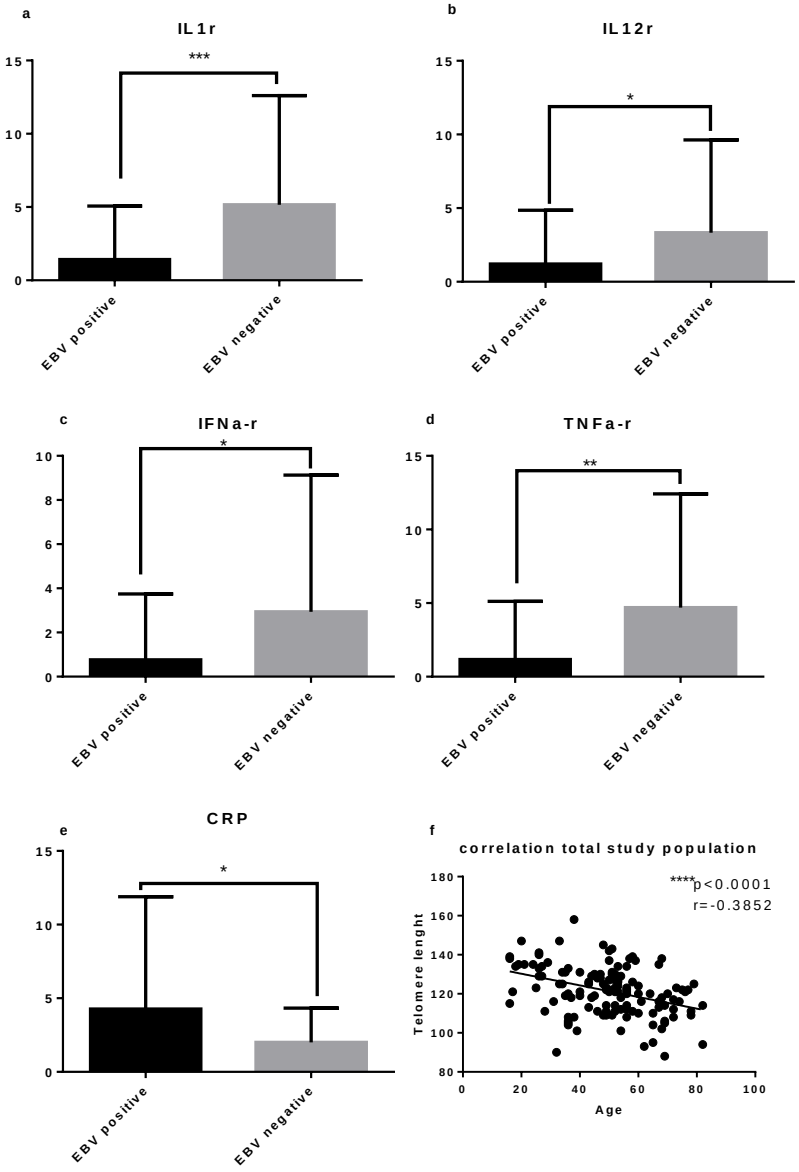

Figure 1: Inflammation: significant differences in EBV-positive and EBV-negative group regarding inflammation markers (Figure 1a-e). Pearson correlation of Age and telomere length (Figure 1f). The results are expressed as mean +/- SD. Statistical significance was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

miRNA expression in EBV-positive vs EBV-negative

EBV was detected via qPCR in the blood. Participants were divided into an EBV-positive and an EBV-negative group. Eight different miRNAs were chosen with the aspect to classify EBV-positive and EBV-negative participants. The EBV-positive and EBV-negative group could be statistically significant distinguished via expression of miR328 and miR21

(Figure 2c & d). The EBV-positive group showed higher miR328 and lower miR21-5p expression levels compared to participants detected negatively for EBV.

No significant differences were detected between miR155, miRlet7a, miRlet7g and miR151 (data not shown). MiR127 and miR877 were higher expressed in EBVpositive subjects (Figure 2a & b), but only miR877 showed a statistical trend.

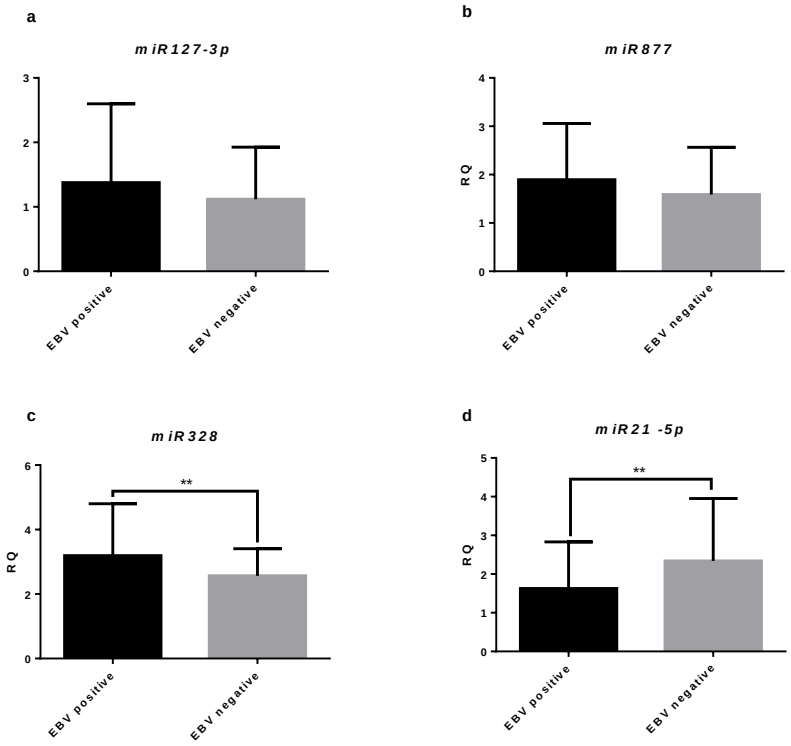

Figure 2: Gene expression EBV-positive versus EBV-negative: miRNAs differ between the EBV-positive and the EBV-negative group. The results are expressed as mean +/- SD. Statistical significance was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Differentiation of EBV positive chronic and acute infection regarding miRNA and cytokines

MiR155, miRlet7a, miRlet7g and miR151 could not be differentiated between patients with chronic or acute EBV infection (data not shown). EBV-positive patients had significantly higher miR328 expression compared to EBV-negative patients (Figure 2c), but did not differ between chronic and acute EBV infections (Figure 3c). MiR21-5p expression showed significant differences between the EBV-positive and the EBV-negative group (Figure 2d) with the highest expression levels in the acute vs. chronic infection group,

although not statistically significant tested (Figure 3d). MiR127-3p expression was slightly higher in EBV-positive participants (Figure 2a) and higher in acute EBV infection group (Figure 3b). The same result is observed for miR877, the expression was increased statistically significant in acute EBV patients (Figure 3a) ($p<0.05$). Moreover, cytokine secretion did not differ between the chronic and the acute EBV groups, as it did between EBV-positive and EBV-negative participants. Only secretion of TNF-α showed a statistical trend in higher secretion during acute EBV infection (data not shown).

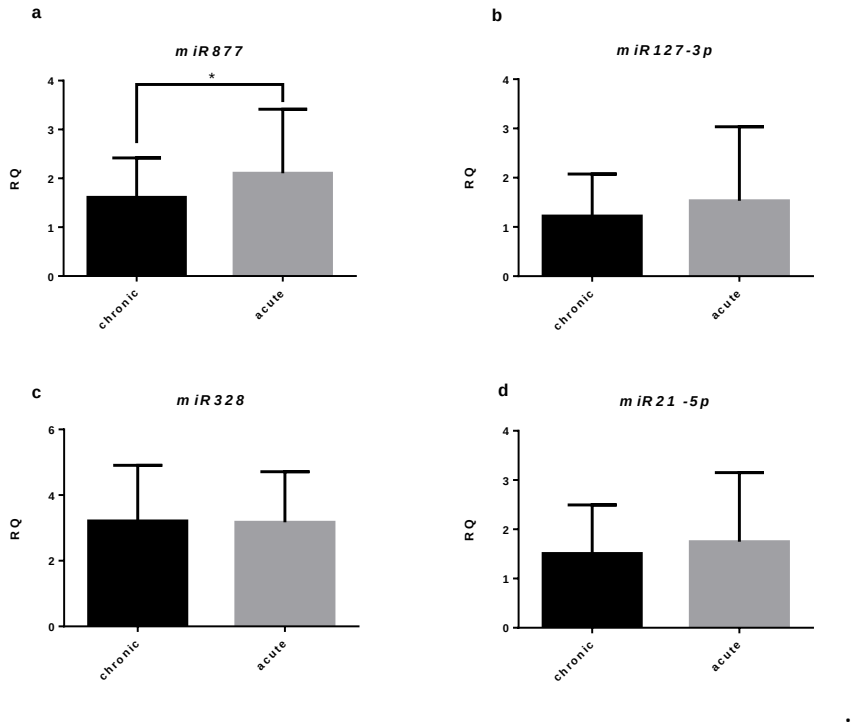

Figure 3: Gene expression pattern chronic versus acute EBV positive: miRNAs expression between the EBV positive chronic and acute infection groups. The results are expressed as mean +/- SD. Statistical significance was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Differences in host miRNA expression and cytokines within the subgroups chronic latent, chronic lytic, acute last months and acute early stage

Within the subgroups there are no differences seen for miRlet7a, miRlet7g (data not shown) and miR151 (Figure 4e). Similar to the EBV chronic versus acute groups, miR328 showed no differences when clustering more subgroups (Figure 4f), indicating miR328 as a maker for distinguishing EBV-positive and EBV-negative patients (Figure 2c). In contrast to the results before, we observed differences in miR155 expression between acute within last months versus acute early stage ($p<0.05$) (Figure 4d). Chronic latent and early stage

EBV-positive patients have higher miR155 expression than patients with chronic lytic or acute infection within the last months, although the expression between the two chronic subgroups showed only a strong trend (Figure 4d). MiR127-3p expression is still increased in the acute compared to the chronic subgroups, more precisely in the early onset of the infection (p=0.0507) (Figure 4c). Almost the same pattern was observed for miR21, but statistically significant (p<0.05) (Figure 4a). Interestingly, miR877 showed higher expression in the acute within last months infected group compared to the early stage (p=0.06) and in addition to the chronic subgroups (p<0.05) (Figure 4b). No differences in the cytokine secretion were observed within the four groups.

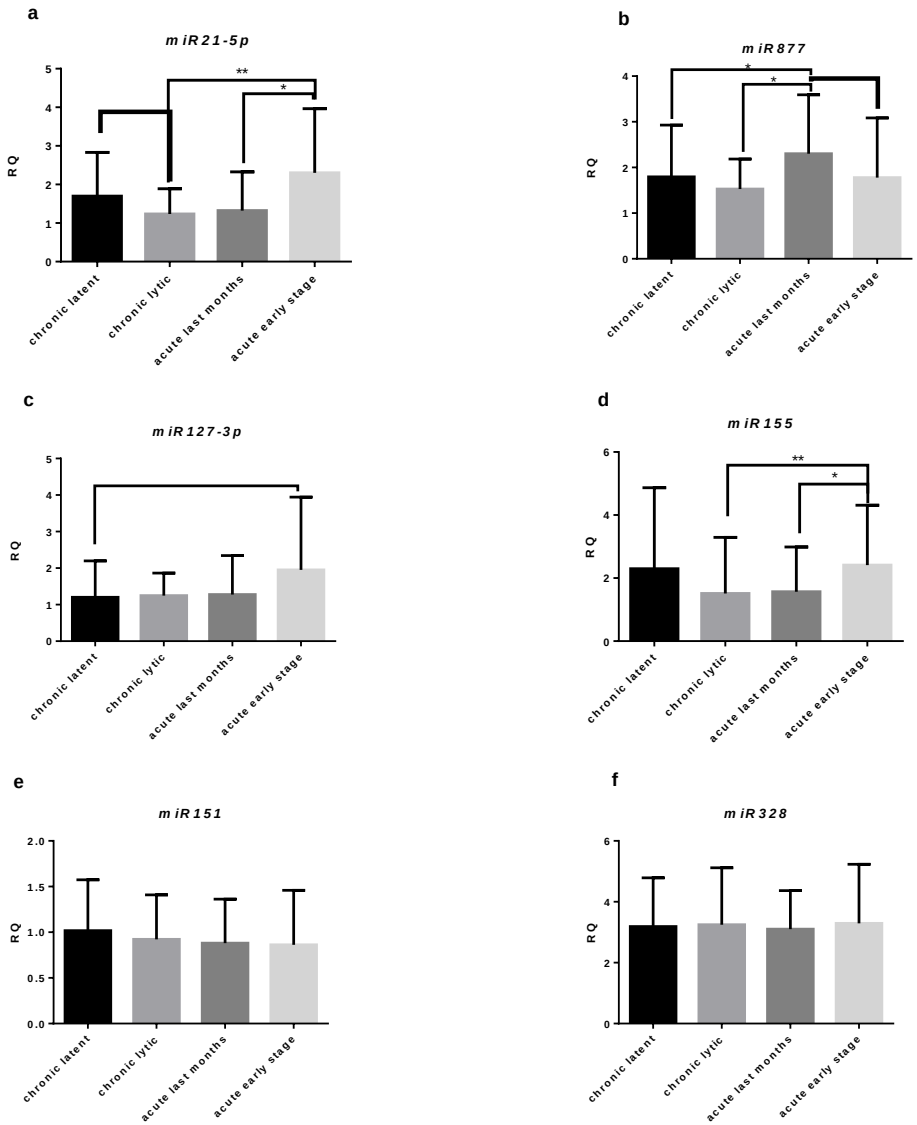

Figure 4: Gene expression pattern in EBV positive subgroups: miRNAs expression between EBV positive chronic latent, chronic lytic, acute last months and acute at the early-stage infection groups. The results are

expressed as mean +/- SD. Statistical significance was determined using paired t-test for parametric values and Wilcoxon test for nonparametric values.

Correlations

Different Pearson correlations between miRNA expression were obtained, especially miR328 and miR877 positively correlated in all EBV positive subjects (Figure 5a), but also in the acute and the chronic EBV infection group (Figure 5b and c). Highlighting the correlations obtained in the lytic subgroups (Figure 5d & e). Additionally, miR328 negatively correlated with IL6 methylation, meaning a higher secretion of this proinflammatory cytokine (Figure 5f). All results showed statistically significance with a p-value below 0.05.

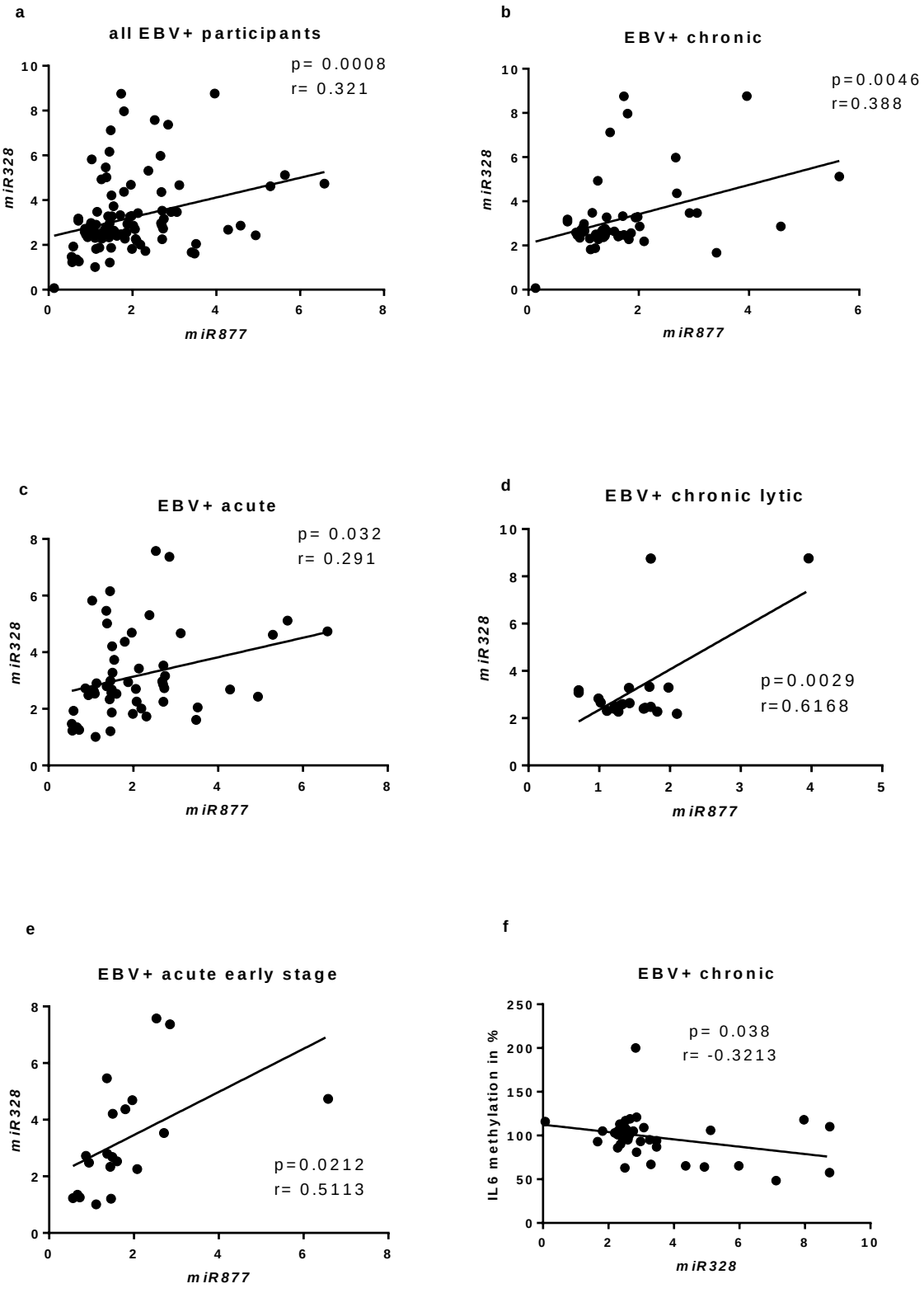

Figure 5: Pearson's correlation in EBV positive and subgroups: miRNA correlations in all EBV positive but also different subgroups and correlation of miR328 and IL6 methylation in chronic EBV participants (all $p<0.05$).

Discussion

Oncogenic virus infections reprogram host gene expression, driving proliferation, ensuring survival, and impacting the immune response [149]. In addition, they rely on the host cellular machinery for the amplification of their genome. Innate immunity is the first line of defense. Pathogen recognition induces interferon expression, which in turn triggers proinflammatory cytokine secretion [150]. Altered cytokine expression regulates cell-mediated immunity, thus IL-1 signaling serves as a key initiator of inflammation [151]. Interestingly, our results demonstrate that during an EBV infection receptors of different cytokines are downregulated, namely IL1-r, IL12-r, TNFa-r and INFa-r. The reason might be that viral miRNAs, namely miR-BHRF1-2-5p target IL-1 receptor 1 (IL1R1) and block NF-κB activation [151]. Furthermore, EBV miRNAs and proteins modulate adaptive host immune system via cytokines. They suppress proinflammatory IL12 secretion, which is necessary for differentiation of naive CD4+ T cells into antiviral Th1 cells. Additionally, IL-12 enhances IFN-y and further CD8+ T-cell production. Thus, inhibition of IL12r, which was observed in EBV positive participants with lower cytotoxic T-cell activation, promotes the survival of EBV-infected cells [151]. In EBV-infected B lymphocytes, viral proteins, like LMP1, activates NF-κB signaling and host miR155, as it inhibits CD8+ T cell activation [152]. Additionally, miR155 targets suppressor of cytokine signaling 1 (SOCS1), leading to a high expression of the latter in EBV infected cells, which suppresses JAK-STAT signaling [152]. Interestingly, our results did not show any differences in miR155 expression between EBV-negative and EBV-positive, thus also EBV negative participants having different health conditions. In addition, no differences were seen in acute and chronic EBV infection, but in chronic latent we saw a higher miR155 expression compared to chronic lytic and acute within last months, but not with acute early onset. This leads to a higher inflammatory immune response at the very onset of a virus infection, like reported by Goa et al [147]. In contrary, a higher miR155 expression in chronic latent infection represents EBV's potential to escape from the immune system [152].

Although Zuo et al. [153] reports a higher miRlet7a expression by EBNA1, a viral protein expressed in the latent stage, no differences in miRlet7g and miRlet7a were observed in our results for EBV-positive versus EBV-negative, neither in acute and chronic, nor in the four subgroups [153]. Same results were obtained for miR151, which was slightly higher in EBV-positive and chronic EBV-infected patients, like the expression of our selected

miRlet7 family, but not statistically significant. MiR151 was found to promote cancer development, meaning that our EBV-infected participants neither have a higher risk in cancer development via this miRNA comparing EBV-negative detected subjects, nor between the different subgroups [154]. Nevertheless, EBV is associated with many cancer types and its progression via reprogramming host gene expression, driving proliferation, ensuring survival, and impacting the immune responses. EBV exploits host miRNAs to escape from the immune system [138]. EBNA2, expressed during late latency, increases miR21 expression [152] [149]. This oncogenic miRNA is the most overexpressed miRNA during cancer, especially during B cell malignancies [155]. MiR21 downregulates toll-like receptor, myeloid differentiation factor 88, IL-1 receptor-associated kinase 1 and miR146a, all essential in pathogen recognition and antiviral immune response [152] [156] [157]. Moreover, it downregulates p21, thus increasing proliferation of infected cells [155]. Interestingly, this oncogenic miRNA was significantly downregulated in our EBV-positive study population. Acute EBV-positive participants had slightly higher expression but were clearly increased at acute early stage of EBV versus acute EBV infection within the last months. Chronic infection with lytic reactivation showed decreased expression compared to chronic latent and acute early stage. MiR21 is not only increased via tumorigenesis, it also controls the balance between pro- and anti-inflammatory responses [153]. This suggests that the EBV-negative study population could be fighting against inflammatory diseases. Nevertheless, the biggest problem in EBV-infected people is the poor immune response and developing memory cells. Sheedy et al. [158] reviewed the expression of miR21 in different cells, diseases, and immunity [158]. MiR21 expression induces the activation of T-cells and further co-ordinates T-cell memory [158]. Thus, high miR21 levels are a marker for immune cell activation, which is impaired in EBV-infected people, like we see in our results, which is reflected by an decrease in miR21 [158][159]. Nevertheless, in primary infection CD8+ T cells can increase 5-10 fold compared to asymptomatic individuals going along with pro-inflammatory stimulus of NfKB and increased miR21 expression [158] [159] [160][161].

Also, B cell differentiation is impaired in EBV infected participants. Viral products like EBNA1 regulate human miRNA like miR127. Its overexpression leads to persistence of BCL6 expression, thereby blocking germinal center exit and consequently the B cell differentiation process in malign lymphoma cells induced by latent EBV infection [149].

Our results show slightly higher expression in this miRNA in the EBV-positive and acute infection group. In the subgroups the highest expression is seen in the acute early beginning of the infection, indicating an early mechanism of the herpesvirus to escape from the immune system. In other cancer cells miR127 upregulation blocks BCL6 and inhibits tumor cell proliferation [162].

Like Navari et al. [163], we see a upregulation in miR877 in EBV-positive participants, a tumor suppressive miRNA [164]. Liang et al. [165] found that miR877 targets IL1ß. Its overexpression inhibits the cytokine production [165]. Additionally, miR877 is often mentioned in Sjögrens syndrome, where it favors to target transforming growth factor *β* (TGF*β*) signaling pathway, rather than pro-inflammatory IL-12 and toll-like receptor/NFkB pathways [166]. Sjögren syndrome has been linked with chronic and reactivated EBV infections [167]. Our results indicate that this is also the case in EBV infection, but further research is needed. Expression of miR877 positively correlated with miR328 expression in the EBV positive group, also in the lytic stage of chronic and acute EBV infected participants.

MiR328 is involved in cancer, autoimmune and neuronal diseases [168]. Tay et al. [169] reports miR328 is a key regulator in the innate immune system. A downregulation of miR328 facilitates bacterial clearance in non-typeable *Haemophilus influenzae via phagocytosis, reactive* oxygen species production and microbicidal activity [169]. According to our correlation results, all these mechanisms, important for the pathogen defense, would be impaired in EBV-positive participants, especially in the lytic reactivation of the virus. Furthermore, we obtained a negative correlation for miR328 and IL6 methylation in the chronic and latent EBV groups. EBV increases secretion of a pro-autoimmune interleukin IL17 [170]. Latter is produced by Th17 cells, a subgroup of T helper cells, which are associated with autoimmune diseases. In turn, IL6 secretion is stimulated [170]. This correlation was also observed in coronary heart disease [171]. In addition, autoimmune disorders in EBV-infected people are often seen in chronic stages [167].

The virus impacts several aspects in hosts immune response, which is demonstrated by our results. Thus, being one of the first study investigating differences in host miRNA expression in EBV-positive and EBV-negative participants and further subgroups, the outcome reflects EBV's modulation in host immune-relevant miRNAs and cytokines.

Further studies are needed, but therapies targeting the host's cellular miRNAs to treat EBV infections would be considered.

Conclusion and Limitations

EBV is a lifelong persistent human herpes virus with a major impact on immune system and severe health pathologies. Its miRNAs and proteins have been investigated in the aspect of escaping host immune system via modulating host gene expression. This study shows that there are differences in host immune-relevant miRNA expression, comparing EBV-positive to EBV-negative subjects and further differentiating between subgroups of EBV-positive patients. In the future, connections between these miRNAs and EBV gene expression would be highly interesting.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence their work in this paper.

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Author Contributions

U.J, S.L., B.H. methodology. B.E, V.B, U.K, B.H., investigation project administration. S.L., B.H. and U.K. analysis, validation and software. S.L. data curation. U.J, S.L., writing—original draft preparation, U.J, S.L., B.H, A.H., J.K. writing review and editing. B.H. supervision. All authors have read and agreed to the published version of the manuscript.

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12.2.3 Personalized nutrition and classification of metabolic types based on genetics, epigenetics and gut microbiota

Personalisierte Ernährung und Einteilung/ Klassifizierung von metabolischen Typen basierend auf genetischen, epigenetischen und mikrobiologischen Analysen

Personalized nutrition and classification of metabolic types based on genetics, epigenetics and gut microbiota

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Abstracts

Humans vary in their need and response to diet. Genetic dispositions, such as single nucleotide polymorphisms (SNPs) are frequently used for clustering consumers in metabolic types (metabotypes), according to individual characteristics of energy extraction from food or risk for metabolic diseases. Results are then used for individualized concepts for weight management or weight loss. However, SNPs explain only a minor part of metabolic variability whereas epigenetic regulation of metabolic enzymes, GI microbiota and lifestyle are of ample importance. In a pilot study enrolling 37participants under nutritional advice we analyzed results from a panel of SNPs, epigenetic markers and microbiota as well as food frequency questionnaires. The results of this study clearly indicate that epigenetic and microbiota markers need to be integrated in the definition of metabotypes. Such improved metabotypes may then enable an improved guidance for a personalized nutrition.

Keywords: personalized nutrition, metabolic types, SNPs, epigenetic, gut microbiota

Menschen unterscheiden sich in ihren Ernährungsbedürfnissen und deren Stoffwechsel. Genetische Veranlagungen, wie zum Beispiel Einzelnukleotidpolymophismen (engl. Single nucleotide polymorphisms SNPs) werden häufig verwendet um Patienten in verschiedene metabolische Typen (metabotypes) einzuteilen, passierend an den individuellen Eigenschaften wie zum Beispiel Energieextraktion aus verschiedenen Nahrungsmitteln oder das genetische Risiko für metabolische Erkrankungen. Diese Einteilungen können weites für ein individuelles Konzept für Gewichtsmanagement und Gewichtsverlust verwendet werden. Nichtsdestotrotz können SNPs nur einen kleinen Teil der metabolischen Variabilität erklären, weshalb epigenetische Regulation von Enzymen, die gastrointestinale Mikrobiota und der Lebensstil von selber Bedeutung sind. In einer Pilotenstudie mit 37 Teilnehmern und Ernährungsberatung wurden SNPs, epigenetische Marker, gastrointestinale Mikrobiota sowie Ernährungsfragebogen analysiert. Die Ergebnisse der Studie zeigen deutlich, dass epigenetische Marker als auch Mikrobiota zu den Analysen der Metabotypes integriert werden sollte. Diese verbesserten Metabotypes könnten eine bessere personalisierte Ernährungsberatung ermöglichen.

Schlüsselwörter: Personalisierte Ernährung, metabolische Typen, SNPs, Epigenetik, Darmmikrobiota

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INTRODUCTION

Dietary preferences and habits are controlled by socioeconomic, psychological, behavioral and in particular biological determinants such as hunger, satiety and sensory aspects (5). Body weight and composition, as well as metabolic rate are affected by nutrient intake and biochemical pathways regulating nutrient absorption, distribution, metabolism, excretions and other cellular energy processes (4). Genetic and epigenetic mechanisms act as key regulators and predispositions may even forecast the response to a weight loss intervention (3,23). The field nutrigenetics offers a new opportunity to evaluate the role of genes, which determine metabolism, disorders and further use the predisposition of genes for a personalized nutrition. Genome wide association studies (GWAS) indicate that particular gene polymorphisms such as single nucleotide polymorphisms (SNPs), the most common type of genetic variation, are related to obesity. SNPs within fat mass and obesity associated genes (*FTO*) were shown to increase the Body Mass Index (BMI) by 0.4kg/m²/allele, caused by an increased intake of fat (2, 11). For example, variants in *Melanocortin 4 receptor (MC4R)*, a gene activating stress neuropeptides, can be linked with lifestyle, food intake and eating habits and as well stress. Carriers of the risk allele have a significant higher intake of processed food and fruits according to recent studies (12). *Transcription factor 7-like 2 (TCF7L2)* is a key regulator of glucose homeostasis and has been most consistently associated with Diabetes Mellitus 2 (DM2).SNP rs7903146 has been reported to have the highest effect on development of DM2 (11).

The gene *Peroxisome proliferator-activated receptor gamma (PPARG)* encodes a regulator of adipocyte differentiation. Galbete et al. showed that subjects consuming a high amount of carbohydrates and carrying the risk allele had an increased obesity risk (13,14). Fatty acid desaturases (FADS) are enzymes involved in the metabolism of polyunsaturated fatty acids (PUFAs). It has been reported that individuals with a polymorphism in rs174547 within the FADS1 gene have increased triglyceride (TG) levels, decreased high density lipoproteins (HDL), cholesterol and an increased coronary artery disease risk (10). Leptin is an adipocyte-secreted hormone and regulates energy homeostasis, blood pressure and food intake. Polymorphisms at the *leptin receptor (LEPR)* decrease the beneficial effects of leptin, like reducing appetite and food intake, and moreover lead to an increased energy metabolism (19). *Angiotensin-converting enzyme (ACE)* gene variants are associated with endurance performance, like swimming, cycling and running, based on lower *ACE* activity and increased bradykinin. This mutation results in more oxygenated blood delivered to the working muscles (8). The SNP *transcription factor AP-2ß (TFAP2B)* rs987237 has a significant association with waist to hip ratio (3). Martinez et al. showed a higher weight loss in wildtypes with a low fat and low caloric diet (20,21).

Epigenetics. The main epigenetic mechanisms are DNA methylation, histone modifications and non coding RNAs (3). DNA methylations occur mainly in cytosines followed by guanines (CpGs) by the addition of methyl groups to the pyrimidine ring in position 5 of cytosine (1). Influenced by internal and external factors such as diet, lifestyle and environment, DNA methylations at CpGs are specific and vary over time within an individual and further may act transgenerational (1,3,22). CpG methylation can change the activity of a gene and therefore is able to repress or promote its expression. Epigenetic markers such as DNA methylation of specific CPGs are used as predictors for metabolic risks and predictors for the success of a diet related treatment, like weight loss or weight maintenance (7). For instance, an elevated Interleukin 6 (IL6) release in blood is linked with a decreased gene promoter methylation. High *IL6* blood levels are associated with several inflammatory diseases (8). Moreover, studies showed a higher *IL6* methylation in obese individuals. Aumueller et al. reported that low *IL6* methylation is associated with a better weight loss (25).

Another promising epigenetic marker associated with metabolism constitutes the *long interspersed element 1 (LINE1)*. *LINE1* is a retrotransposon, which is widely expressed in the human genome (3) and is associated with genetic instability and chromosomal abnormalities (22). Usually assessed to estimate global DNA methylation, *LINE1* methylation is related to BMI, DM2, insulin resistance, cardiovascular disease, inflammatory response and cancer (3,9) as well as obesity and metabolic syndrome (MetS).

Microbiota. The microorganisms in the gut are a highly metabolic active community and are regarded as a regulator of its host homeostasis. The gut microbiota contains 100 times more genes than human cells. The composition of the microbiota varies over lifetime with diet as strongest impact factor (15). Indigestible complex carbohydrates are a major source for carbon, the main substrate for the gut microbiota. After their fermentation short chain fatty acids (SFAs), like acetate, propionate and butyrate are produced and absorbed via the colon mucosa. SFAs show multiple health promoting activities and have beneficial effects in appetite regulation, lipid and glucose metabolism (15,16). However, an imbalanced gut microbiota affects metabolites like butyrate and lipopolysaccharides (LPS), which interfere with the host's epigenetic mechanism and may trigger pro-inflammatory processes (24,29). GI microbiota have been grouped in enterotypes according to main bacterial groups with relevance for metabolic characteristics and discussed critically (30-32) .

OBJECTIVES

The Metabotype-Study was initiated to evaluate a clustering of participants into four different metabotypes based on differences in genetics as well as epigenetics and GI – microbiota. Analysis of cluster of SNPs as described scientifically and already used commercially should be complemented with analysis of epigenetic CpG methylation of metabolic relevant genes and analysis of gut microbiota composition. The hypothesis that a solely SNP based categorisation of metabotypes misses important aspects was supported by the outcome of a comparison of a SNPs analysis and an integrated analysis of SNPs including epigenetic and microbiota marker.

METHODS

The study population included 37 healthy men and women from 30 to 60 years of age. Exclusion criteria were chronic diseases, colitis ulcerosa, supplementation of pre- or probiotics, antibiotic intake and BMI over 30. Blood spots were used for sample collection of capillary blood. DNA extraction was conducted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). SNP analysis was performed with the StepOne Plus (Thermo Fisher, Massachusetts, USA) using TaqMan Mastermix and TaqMan SNP Genotyping Assays

from Thermo Fisher (Massachusetts, USA). For epigenetic analyses DNA was bisulfite converted, using the EpiTec bisulfite kit (Qiagen, Hilden, Germany). High resolution melting curve analysis was conducted to assess *LINE1* and *IL6* methylation. For quantification analysis of microbiota real-time polymerase chain reaction (PCR) was applied using TaqMan qPCR and SYBER Green qPCR in a Rotorgene 3000 after DNA extraction of stool samples using QIAamp Fast DNA Mini Stool Kit.

Metabolic types. For our study we chose 12 SNPs in total. *MC4R* rs17782313, *TCF7L2* rs7903146, *IL6* rs1800795, *SLC6A14* rs2011198, *FTO* rs9939609, *PPARG2* rs1801282 have been associated either with BMI and obesity or DM2 (2). Moreover *MC4R* rs17782313 and *LEPR* rs9436740 are linked with satiety, *IL6* rs1800795 with weight regain and *SLC6A14* rs2011198 with eating disorder development. Others like *TFAP2B* rs987237, *FADS1* rs174547, and *ADRB3* rs4994 as well as *FTO* rs993609 and *TCF7L2* rs7903146 are reported to correlate to different metabolic types. The *ACE* gene is associated with different sport types (19,26). For the classification of the different metabotypes we focused solely towards SNPs linked with nutrition and metabolism (18). As already described by Martinez et al. we gave points from zero to two for each SNP (2).

Verarbeitete Fälle

	Fälle					
	Gültig		Fehlend		Gesamt	
	N	Prozent	N	Prozent	N	Prozent
ACE * Körperliche_Bewegung_t0	37	100,0%	0	0,0%	37	100,0%

ACE * Körperliche_Bewegung_t0 Kreuztabelle

Anzahl

		Körperliche_Bewegung_t0					
		mehrmals täglich	täglich	4-6x/W	1-3x/W	1-3x/M	Gesamt
ACE	strenght	6	6	2	2	0	16
	balanced	4	3	2	2	2	13
	endurance	0	3	0	5	0	8
Gesamt		10	12	4	9	2	37

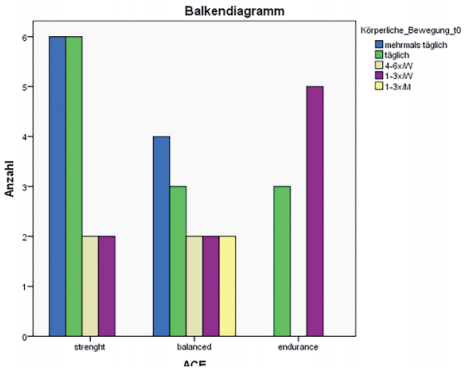

Figure 2: Sport types and physical activity

RESULTS

Figure 1 shows the contribution of the different metabotypes using SNP analysis.
In total we found 23 balanced types, 7 glyco, 2 protein and 5 fat types. In **figure 2** the distribution of different sport types and physical activity in our study population is shown. **Figure 3** shows the connection between IL-6-methylation and the amount of Cluster IV. The higher the methylation of IL-6, the higher the amount of Cluster IV bacteria. **Figure 4** shows the correlation between the different forms of the TCF7L2-SNP and the amount of Bacteriodetes. The wild type shows the highest quantity of Bacteriodetes.

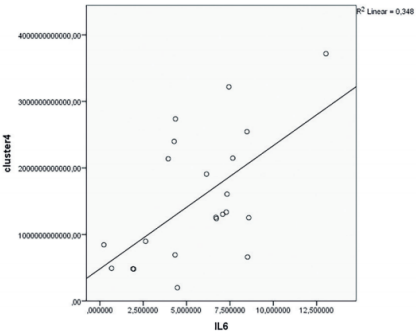

Figure 3: *IL6* Methylation and *Cluster IV*

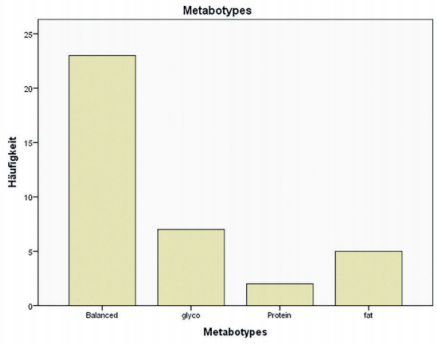

Figure 1: Different Metabotypes

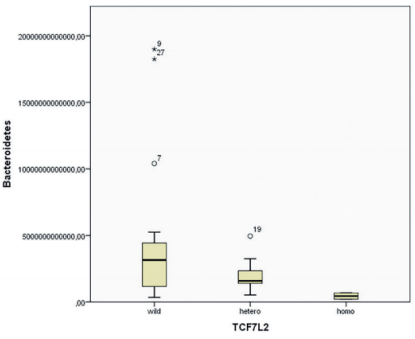

Figure 4: *TCF7L2* and *Bacteroidetes*

DISCUSSION

Our main findings identify an association between genetics, epigenetics and gut microbiota variations. Considering the outcome for the strength sport type, individuals in this group have a higher amount of *Firmicutes* in general as well as *Cluster IV* and a higher methylation of *IL6*. A high abundance of *Cluster IV* or *Firmicutes* which is mostly seen in humans with high BMI (27) is therefore suggested to result in a low weight loss. Latter was previously reported to be correlated with a high methylation of *IL6*. Further, *Firmicutes* and *Cluster IV* are associated with increased inflammatory and stress levels as reported in FFQ. Remely et al. showed that *Cluster IV* and *Cluster XIVa* decreased in people with DM2 after weight loss suggesting that people with higher BMI exhibit a higher distribution of these bacterial groups. Our results could additionally demonstrate this outcome with *Cluster IV* (27,28). Furthermore, we observed that the wildtype forms of *TCF7L2* and *LEPR* were correlated to higher amounts of *Bacteroidetes*, which were shown to be higher abundant in lean individuals. Both SNPs can be associated with obesity as well as diabetes: Carrying no risk allele portends persons are more likely lean, thus have a lower risk for obesity and DM2. This could be underlined by showing that a high amount of *Bacteroidetes* tends to correlate with a low risk for obesity, which has also been reported by previous studies (17). *LINE1* is considered to be highly methylated in participants with high BMI (33), which also emerges in our study. Moreover we observed a possible interaction of *LINE1* methylation with *PPARG2*, where heterozygotes were higher methylated, and consequently had a higher BMI. Controversially, the wildtype for *PPARG2* showed a positive correlation with methylation of IL6, which could indicate that wildtype carriers have a less efficiency to lose weight but a lower risk to develop DM2. This study demonstrates that interactions between genetic as well as epigenetic variations, the gut microbial composition and their influences through diet and lifestyle but also physical activity are relevant for genotype based interventions bearing an enormous potential in developing personalized diets based on the genotype (20).

Humans differ in height, weight, activity, cognition, strength, endurance and their preference for food, due to a wide range of biological variables. These variables include allelic polymorphisms and changes in the epigenomic and also metabolomic landscape due to environmental influences (6). DNA methylation changes are directly correlated to dietary interventions, weight loss and regain and further are associated with the development of diseases e.g. metabolic disorders (3). With increasing knowledge of gene-diet interactions for macro-and micronutrients it will be possible to give recommendations based on the (epi) genetic make-up (11,23).

CONCLUSION

Metabolic diseases are a central burden for public health and heath care. There is increasing evidence that genetic, epigenetic and microbiota aspects contribute to individual mechanisms, which result in individual pathways for metabolism and energy extraction from food. Genetic dispositions, such as SNPs are under scientific investigation but already in commercial use for defining metabolic types (metabotypes). These metabotypes define the risk for metabolic diseases, preferences for energy extraction from food and individualized concepts for weight management or weight loss. To our knowledge there is no other study focusing on SNPs and their classification into metabotypes. Furthermore, other nutritional recommendations based on genetic disposition do not consider environmental and nutritional effects on gene regulation. Results show that SNPs can be clearly attributed to metabotypes. Analysis of DNA methylation strengthens the outcome. Furthermore gut microbiota composition shows significant correlation with SNP and methylation according to metabotype clustering.

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12.3 Talks and poster presentations

12.3.1 *Senescent cells in adipose tissue and a possible senolytic compound*- Stephanie Lilja at 3rd LIMIOR Leipzig International Meeting for Interdisciplinary Obesity Research- epigenetics in Metabolic Diseases (eGMD)

12.3.2 Fasting and fasting mimetic supplementation address sirtuins expression, miRNA and microbiota composition- Stephanie Lilja at Metabesity conference 2021

12.3.3 *Fasting and fasting mimetic supplementation address sirtuins expression, miRNA and microbiota composition*- Stephanie Lilja at the 14th International Scientific conference on Probiotics, prebiotics, gut microbiota and Health- IPC 2020

12.3.4 *Personalisiertes Gesundheitsmanagement - Einführung in die Genetik und Epigenetik*- Prof. Alexander Haslberger, Dr. Berit Hippe, Ulrike Krammer MSc., Stephanie Lilja, MSc- August 2020 Sigmund Freud PrivatUniversity; Wien 1020; Freudplatz 3; Raum 47

12.3.5 *Neue Studie: Buchinger Fasten verändert Darm Mikrobiom*; Biomes Pressemitteilung 22.03.2021

12.3.6 Fasten ohne zu hungern? Webinar, Stephanie Lilja, Die Wiener Volkshochschulen 12.03.2021

12.3.7 *Stimmt es, dass Heilfasten die Darmgesundheit fördert*?, Wienerin 07.04.2021

12.3.8 *Der Darm auf Achse*, Fokus Mikrobiom, Ärzte Krone 18/2019

12.3.9 *Mit epigenetischen Marker Zellschäden Früh aufspüren*; Stephanie Lilja, Diana Gessner, Ulrike Kramer, Berit Hippe und Alexander G. Haslberger, Ärtze Woche Nr 10. 07.03.2019

12.3.10 Aktuelle Forschungsprojekte – ein Wissenschafts-Update; *Metabolisches Syndrom: Auswirkungen von Fasten und pflanzlichen Inhaltsstoffen auf epigenetische Mechanismen und Zellalterung*; Veoe; Einblicke Ausgabe 2/2019

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