

# **Calorie restriction affects human nucleotide excision repair and the expression of aging-associated genes**

**Dissertation zur Erlangung des  
akademischen Grades eines Doktors der Naturwissenschaften  
(Dr.rer.nat.)**

vorgelegt von  
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an der



Mathematisch-Naturwissenschaftliche Sektion  
Fachbereich Biologie

Konstanz, 2021

Tag der mündlichen Prüfung: 05.07.2021

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## Summary

Calorie restriction (CR) is the only intervention known to influence the lifespan of model organisms like worms, flies, yeast or rodents positively. The impact of CR on model organisms is extensively researched and molecular mechanisms have been hypothesized or even revealed. However, studies involving human test persons were scarce in the past and mostly dealt with the improvement of health parameters like body weight, insulin sensitivity, blood pressure or cholesterol. These are beyond doubt important parameters, whose improvements lead to an increased quality of life and a healthier aging. However, one of the most common aging-related diseases threatening a healthy aging is cancer. The emergence of cancer is closely linked to an increase in DNA mutations and impaired DNA repair. Human DNA is constantly exposed to damaging substances, which arise either intrinsically due to the physiologic cell metabolism or extrinsically as a result of the impact of environmental factors. In order to prevent the formation of DNA mutations out of DNA damages and hence cancer formation, functional DNA repair mechanisms are required to guard the integrity of the genome. Despite the positive effects of CR, complying with the restrictions a CR is accompanied with is rather difficult for most persons. A convenient approach to circumvent the restrictions of a CR partially are so-called calorie restriction mimetics (CRMs), as they could complement or support the positive effects of CR. Two CRMs researched and discussed at the moment are spermidine and resveratrol. Both CRMs have been shown to have positive influences on the occurrence of cardiovascular diseases in humans and thus have been proposed to act CR-mimicking.

Since the influence of CR on the human organisms is still not understood completely, this work aims at contributing to a better understanding of the impact of CR on human DNA repair and the mechanisms of aging. Furthermore, this thesis deals with the verification of the similar effects CR and the CRMs spermidine and resveratrol have on the expression of aging-associated genes. For this purpose, the influence of F.X. Mayr therapy on DNA repair capacity of nucleotide excision repair (NER) and the expression of the aging-associated genes *SIRT1*, *XPA*, *SIRT3*, *SOD2*, *FOXO3*, *AMPK $\alpha$* , *LC3B* and *BECN1* was analyzed in human peripheral blood mononuclear cells (PBMCs). The modified host cell reactivation assay (mHCRA) revealed a significant increase in DNA repair capacity due to CR. However, only individuals

displaying a low DNA repair capacity before CR experienced an increase, whereas no alteration was obvious for the individuals displaying a normal DNA repair capacity in the beginning of the study. In the end, DNA repair capacity of the “low” group was elevated to the level of the “normal” group. With regard to the expression of aging-associated genes, a significant increase in *SIRT1*, *AMPK $\alpha$*  and *BECN1* expression was verified as consequence of CR. Just like DNA repair capacity, the increase in mRNA expression was solely attributed to individuals with a low DNA repair capacity in the beginning. Furthermore, DNA repair capacity as well as mRNA expression were subject to donor variability. The CRMs spermidine and resveratrol were used to treat human PBMCs *ex vivo* and subsequently mRNA expression of the aging-associated genes *SIRT1*, *SIRT3*, *SOD2* and *FOXO3* was analyzed. Both CRMs significantly induced mRNA expression of *SIRT3*, *SOD2* and *FOXO3*, whereas only spermidine induced *SIRT1* expression significantly. The CRMs seem to particularly have an influence on anti-oxidative properties. Furthermore, spermidine increased NER capacity in human fibroblasts. Taken together, CR influences DNA repair capacity of NER positively, thus providing a better protection of DNA damages and their consequences. The increase in mRNA expression of aging-associated genes also hint at an improved DNA repair and autophagy as well as at better defense mechanisms against oxidative stress. Since not every person benefits from CR, CRMs might be a way to boost CR effects, but they surely cannot replace the holistic approach of a CR. In a wider sense the research conducted within the scope of this thesis contributes to a better characterization of the influence of CR and CRMs on human health and thus to finding ways to a healthier aging. Simultaneously, the results highlight the importance of studies involving humans in order to better characterize influences of CR and CRMs.

## Zusammenfassung

Kalorienrestriktion (CR) ist die einzige bekannte Intervention, welche die Lebensspanne von Modellorganismen wie Würmern, Fliegen, Hefen oder Nagetieren positiv beeinflusst. Der Einfluss von CR auf Modellorganismen ist Gegenstand vieler Studien, wobei bereits Wirkmechanismen vorgeschlagen oder sogar aufgedeckt wurden. Studien mit menschlichen Testpersonen existieren jedoch wenige und diese befassten sich hauptsächlich mit der Verbesserung von Gesundheitsparametern wie Körpergewicht, Insulinsensitivität, Blutdruck oder Cholesterin. Dies sind zweifellos wichtige Parameter, deren Verbesserungen zu einer höheren Lebensqualität und einem gesünderen Altern führen. Eine der häufigsten altersbedingten Krankheiten, die ein gesundes Altern bedrohen, ist jedoch Krebs. Das Auftreten von Krebs ist eng mit einer Zunahme von DNA-Mutationen und einer nachlassenden DNA-Reparatur verbunden. Die humane DNA erfährt täglich Schädigungen, welche intrinsisch durch den physiologischen Zellstoffwechsel oder extrinsisch durch schädigende Agenzien verursacht werden. Um die Bildung von DNA-Mutationen aus DNA-Schäden und damit die Krebsentstehung zu verhindern, sind funktionelle DNA-Reparaturmechanismen erforderlich, um die Integrität des Genoms zu schützen. Trotz der positiven Auswirkungen von CR ist es für die meisten Menschen schwierig, die mit einer CR verbundenen Einschränkungen zu akzeptieren. Ein Ansatz zur teilweisen Umgehung dieser Einschränkungen sind sogenannte Kalorienrestriktionsmimetika (CRMs), da sie die positiven Effekte einer CR ergänzen oder unterstützen könnten. Zwei derzeit häufig untersuchte CRMs sind Spermidin und Resveratrol. Es wurde gezeigt, dass beide CRMs einen positiven Einfluss auf das Auftreten von Herz-Kreislauf-Erkrankungen beim Menschen haben, daher wurde ihnen bereits eine CR-imitierende Wirkung attestiert.

Da der Einfluss von CR auf den Menschen noch nicht vollständig verstanden ist, soll diese Arbeit zu einem besseren Verständnis der Auswirkungen von CR auf die DNA-Reparatur und die Mechanismen des Alterns beitragen. Darüber hinaus befasst sich diese Arbeit mit der Überprüfung der ähnlichen Auswirkungen von CR und den CRMs Spermidin und Resveratrol auf die Expression von altersassoziierten Genen. Zu diesem Zweck wurde der Einfluss der F.X. Mayr-Therapie auf die DNA-Reparaturkapazität der Nukleotid-Exzisionsreparatur und die Expression der altersassoziierten Gene *SIRT1*, *XPA*, *SIRT3*, *SOD2*, *FOXO3*, *AMPK $\alpha$* , *LC3B* und *BECN1* in humanen peripheren Blutzellen (PBMCs) analysiert.



Unter Verwendung des modifizierten host cell reactivation assay (mHCRA) wurde ein signifikanter Anstieg der DNA-Reparaturkapazität aufgrund von CR festgestellt. Allerdings wiesen nur Personen, die vor CR eine geringe DNA-Reparaturkapazität zeigten, einen Anstieg auf, während bei Personen, die zu Beginn der Studie eine normale DNA-Reparaturkapazität zeigten, keine Veränderung offensichtlich war. Die DNA-Reparaturkapazität der "niedrigen" Gruppe erhöhte sich auf das Niveau der "normalen" Gruppe. Die Expression der altersassoziierten Genen *SIRT1*, *AMPK $\alpha$*  und *BECN1* war als Folge der CR signifikant erhöht. Genau wie die DNA-Reparaturkapazität war ein Anstieg der mRNA-Expression ausschließlich bei Personen mit einer anfänglich niedrigen DNA-Reparaturkapazität zu sehen. Darüber hinaus konnte sowohl bei der DNA-Reparaturkapazität als auch bei der mRNA-Expression eine Donorvariabilität festgestellt werden. Humane PBMCs wurden zudem *ex vivo* mit den CRMs Spermidin oder Resveratrol behandelt und anschließend die mRNA-Expression der alterungsassoziierten Gene *SIRT1*, *SIRT3*, *SOD2* und *FOXO3* analysiert. Beide CRMs induzierten die mRNA-Expression von *SIRT3*, *SOD2* und *FOXO3* signifikant, während nur Spermidin die *SIRT1*-Expression signifikant induzierte. Die CRMs scheinen insbesondere einen Einfluss auf die antioxidativen Eigenschaften zu haben. Spermidin beeinflusste zudem die NER-Kapazität humaner Fibroblasten positiv. Zusammengefasst beeinflusst eine CR die DNA-Reparaturkapazität positiv und bietet so einen besseren Schutz vor DNA-Schäden und deren Folgen. Die erhöhte mRNA-Expression von altersassoziierten Genen deutet auch auf eine verbesserte DNA-Reparatur und Autophagie sowie auf bessere Abwehrmechanismen gegen oxidativen Stress hin. CRMs könnten zudem eine Ergänzung zu einer CR sein, insbesondere für Personen, welche nicht oder nur schwach auf eine CR reagieren. Sicherlich können sie aber nicht den ganzheitlichen Ansatz einer CR ersetzen. Im weiteren Sinne trägt diese Arbeit dazu bei, den Einfluss von CR und CRMs auf die menschliche Gesundheit besser zu charakterisieren und damit Wege zum gesünderen Altern zu finden. Gleichzeitig unterstreichen die Ergebnisse die Bedeutung von Studien am Menschen, um die Einflüsse von CR und CRMs besser charakterisieren zu können.

## Abbreviations

°C	degree centigrade
μF	microfarad
μg	microgram
μl	microliter
<sup>1</sup> O <sub>2</sub>	singlet oxygen
6-4PP	pyrimidine (6-4) pyrimidone photoproduct
8-OHdG	8-hydroxydeoxyguanosine
8-oxoG	7-8-dihydro-8-oxoguanine
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
AP site	apurinic / apyrimidinic site
Atg6	autophagy-related gene 6
ATP	adenosine triphosphate
BECN1	Beclin-1
BER	base excision repair
cDNA	complementary DNA
CFS	cell free system
CPD	cyclobutane pyrimidine dimer
CR	calorie restriction / calorie reduction
CRM	calorie restriction mimetic
CS	cockayne syndrome
CSA	cockayne syndrome protein A
CSB	cockayne syndrome protein B
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA Pol δ, ε, κ	DNA polymerases δ, ε, κ
DR	direct reversal
DSB	double strand break
DSB repair	double strand break repair
DsRed	<i>Discosoma</i> red fluorescent protein
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ERCC1-XPF	excision repair cross-complementation group 1-XPF
F.X. Mayr	Franz Xaver Mayr
FACS	fluorescence activated cell sorting
FADU	fluorimetric detection of alkaline DNA unwinding
FBS	fetal bovine serum
FOXO3	forkhead box O3
g	gram

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G3PDH	glycerol-3-phosphate dehydrogenase
GGR	global genomic repair
GUSB	glucuronidase beta
h	hour
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HR	homologous recombination
kg	kilogram
kJ	kilojoule
kV	kilovolt
l	liter
LB medium	lysogeny broth medium
LC3B	microtubule associated protein light chain 3 beta
mg	milligram
mHCRA	modified host cell reactivation assay
ml	milliliter
MMR	mismatch repair
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NER	nucleotide excision repair
ng	nanogram
NHEJ	non-homologous end-joining
NOS	reactive nitrogen species
O <sub>2</sub> <sup>-</sup>	superoxide
OGG1	8-oxoguanine glycosylase 1
OH•	hydroxyl radical
PAH	polycyclic aromatic hydrocarbons
PARP1	poly(ADP-ribose) polymerase 1
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCNA	Trimeric proliferating cell nuclear antigen
PCR	polymerase chain reaction
PGC-1 $\alpha$	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
qPCR	quantitative realtime polymerase chain reaction
RFC	replication factor C
RNA	ribonucleic acid
RNA Pol II	RNA polymerase II
ROS	reactive oxygen species
RPA	replication protein A
RPLP0	ribosomal protein lateral stalk subunit P0
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute

SEM	Standard error of the mean
Sir2	silent mating type information regulation 2
SIRT1	silent mating type information regulation 2 homolog 1
SIRT3	silent mating type information regulation 2 homolog 3
SOD2	superoxide dismutase 2
SSB	single strand break
TCR	transcription coupled repair
TFIIH	transcription initiation factor IIIH
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet
UVA, UVB, UVC	ultraviolet A, B, C
x g	x-fold gravitational constant
XP	<i>xeroderma pigmentosum</i>
XPA	<i>xeroderma pigmentosum</i> complementation group A
XPB	<i>xeroderma pigmentosum</i> complementation group B
XPC	<i>xeroderma pigmentosum</i> complementation group C
XPD	<i>xeroderma pigmentosum</i> complementation group D
XPF	<i>xeroderma pigmentosum</i> complementation group F
XPG	<i>xeroderma pigmentosum</i> complementation group G

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# 1 Introduction

To get as healthy as possible as old as possible, that is what most people want to achieve. However, that is more easily said than done. Fact is that with increasing age, the probability to suffer from age-related diseases also increases. Although the average life expectancy of people continues to increase, it is important how many so-called healthy life years remain for a person in a given age. Per definition, healthy life years are the years a person of certain age lives without severe health restrictions. With ongoing age, the incidence of severe age-related diseases also rises. Among the most common age-related diseases are cardiovascular diseases, hypertension, neurodegenerative diseases, type 2 diabetes and cancer (Kumar *et al.*, 2017). These diseases often lead to limitations in everyday life and to a higher mortality of the persons concerned. In contrast to earlier, the life expectancy of people in industrialized countries is steadily increasing, due to a better medical care and improved and more specialized medicines. However, today's lifestyle, which involve less exercise, often mostly sedentary work and more stress at a professional and private level, is increasingly causing illnesses such as hypertension, hyperlipidemia and overweight. Studies on forced settlement of people have linked the change of lifestyle to an increased prevalence and incidence of overweight and obesity (McLennan and Ulijaszek, 2015; Ravussin *et al.*, 1994; Sahani, 2013). Overweight or obesity in turn can lead to secondary diseases such as type 2 diabetes, heart attack or stroke, all of which are also age-related diseases. One way to reduce the incidence of these diseases, to postpone the occurrence of age-related diseases and thus to contribute to healthy aging, is calorie restriction (CR). Extensive research has already been carried out on CR in model organisms such as yeast, *Drosophila melanogaster*, mice, rats and non-human primates (Lin *et al.*, 2002; Rogina *et al.*, 2002; Stuart *et al.*, 2004; Weindruch *et al.*, 1986; McCay *et al.*, 1935; Colman *et al.*, 2014), but relatively few studies involving humans have been conducted up to now. A second approach to achieve the positive effects of CR, without the limited calorie intake of a CR, are the so-called calorie restriction mimetics (CRMs). The hope is that CRMs could replace or at least support the effects of a CR in the future. Promising CRMs are for example spermidine and resveratrol (Eisenberg *et al.*, 2009; Timmers *et al.*, 2011). The effects of CRMs on model organisms have also been researched extensively, but effects of CRMs on human volunteers have been less frequently conducted in the past. For both approaches, the CR and CRMs, this is currently changing.



Practically all living organisms are prone to the complex multifactorial process of aging. In order to contribute to the mechanisms by which CR and CRMs in humans lead to positive effects on aging processes, this thesis deals with the impact of CR on DNA repair and mRNA expression of aging-associated genes in humans. In addition, studies on the influence of CRMs on DNA repair and mRNA expression of aging-associated genes were performed.

## 1.1 The origin of calorie restriction

Looking at human evolution, there have always been times when food was scarce and people, albeit involuntarily, were exposed to CR. From the beginning of human evolution, when the availability of food depended crucially on hunting success, to later occurring crop failures and famines, to wars and the resulting lack of food, people would be forced to survive with reduced calorie intake. As a result, evolutionarily, the human body is designed to cope with occasional food availability rather than the excess supply of food available to people in developed countries today. A temporary partial or total renunciation of food is deeply rooted in people's culture, originally often for religious reasons. In almost every world religion, for example Christianity and Islam, fasting is an integral part. In antique Rome it was assumed that the ingestion of more than one meal per day is not healthy (Paoli *et al.*, 2019). Likewise, the Greek physician and father of modern medicine Hippocrates of Kos recommended and promoted fasting (Bengmark, 2015). Considering this, fasting, which is an extreme form of CR, can be seen as one of the oldest therapeutic approaches of humanity. Nowadays, caloric restriction, also synonymously called calorie reduction, is commonly referred to as a 20 % to 40 % reduction of calorie intake compared to the *ad libitum* nutrition (Brandhorst and Longo, 2016) without being accompanied by malnutrition.

If CR predominantly serves the purpose of improving health and one's well-being, it is called therapeutic fasting or fasting cure. The difference between a therapeutic fasting and fasting is the intake of 200 – 500 kcal per day during therapeutic fasting (Michalsen and Li, 2013), whereas fasting means no intake of calories. Otto Buchinger is known as the founder of the fasting cure. He voluntarily underwent a three-week fasting cure in order to alleviate a rheumatoid arthritis. After the fasting period, the illness was cured and due to his experiences, he began to research the effects of therapeutic fasting and to developed new fasting approaches. (Buchinger, 2018) A second important personality in the field of fasting is the

Austrian physician Franz Xaver Mayr, founder of F.X. Mayr therapy, which is subject of this thesis. F.X. Mayr therapy mainly aims at regeneration of the gastrointestinal tract, the learning of a proper eating behavior and finally the promotion of health by doing so. Originally, F.X. Mayr diet consisted of stale bread rolls and milk, whereby one bite of bread was chewed approximately 40 times, mixed with a teaspoon of milk and then swallowed. This procedure assures a very slow way of eating. Essential for the success of F.X. Mayr therapy is to stop eating as soon as the feeling of satiety occurs. (Rauch, 2015) Finally, this special diet inevitably results in a CR. Via this monotonic nourishment the intestinal tract is cleaned, rested, detoxified and regenerated. This modified fasting procedure thus represents a form of CR for humans. Today, the original F.X. Mayr therapy is less frequently performed, but several modified procedures are carried out regularly. Modified forms include a different diet during the therapy, e.g. spelt buns, sheep yogurt, potatoes and root vegetables along with omega-3 fatty acids (Rupprecht, 2016). Furthermore, the duration of the therapy as well as the therapeutic procedure varies. (Witasek, 2019) Nevertheless, all approaches have in common that the intake of calories is reduced during the length of the therapy.

### 1.1.1 Calorie restriction in model organisms

The systematic research of the effects of CR on living organisms has already been carried out in 1935 by McCay and colleagues. Using rats as model organisms, McCay studied the effects of CR starting at the time of weaning and found that not only did the growth of animals slow down, but their lifespan was also prolonged. (McCay *et al.*, 1935) Mice fed an about 65 % reduced calorie amount per week after weaning show an up to 65 % increased lifespan compared to the *ad libitum* fed control animals. Furthermore, lymphoma incidence was reduced, whereas hepatoma incidence was not. (Weindruch *et al.*, 1986) When gradually introducing adult mice to CR, the maximum lifespan increased up to 20 % and the occurrence of spontaneous lymphoma was inhibited completely (Weindruch and Walford, 1982). The onset of CR after weaning or at adulthood does not seem to play any role in the positive effects on life span-extension and on the occurrence of age-related lymphoma in mice.

One of the first and most commonly used model organism for CR studies is the nematode *Caenorhabditis elegans*. The nematode's lifespan can be prolonged by nutritional restriction (up to 52 %), whereby the earlier the beginning of the restriction, the greater the extension of the

lifespan with the greatest lifespan extension after a commencement during growth phase (Klass, 1977). Additionally, a complete removal of bacterial food extends the nematode's lifespan more clearly than a partial reduction (Kaeberlein *et al.*, 2006). In *Saccharomyces cerevisiae*, lifespan can be increased up to 25 % by breeding the yeast in medium containing 0.5 % instead of 2 % glucose (Lin *et al.*, 2002). Moreover, Jiang *et al.* found that a reduction of glucose in the growth medium of *Saccharomyces cerevisiae* from 2 % to 0.001 % leads to an up to 59 % increased mean lifespan and that a reduction of amino acids also leads to lifespan extension. They attributed this result to the CR and not the reduction of a particular nutrient. (Jiang *et al.*, 2000) Similar results have been obtained in studies using *Drosophila melanogaster*. The average lifespan of calorie restricted flies, which were fed a medium containing 66 % less yeast and glucose than the medium fed to the control group, was almost twice the average lifespan of the corresponding control group (46.2 days vs 25.4 days in the control group). Pletcher and colleagues concluded that as a consequence of the alteration of age-related transcription levels, the extension of lifespan is attributed to a deceleration of the normal aging process. (Pletcher *et al.*, 2002) However, the lifespan extension seems not to be related to a reduction of calories, but by specific ingredients of the food administered to *Drosophila* (Mair *et al.*, 2005). These findings are not in accordance with the widely held belief that a reduction of calories and not the source of calories are crucial for lifespan extension. This in turn leads to the assumption that there are different mechanisms of lifetime extension in different species.

Due to the close genetic relationship of humans and non-human primates, the findings of studies involving these animals are of special interest for understanding the aging process in humans. Within the scope of a study initiated in 1989 the effects of a 30 % CR was researched in rhesus macaques over a period of 20 years. Amongst other beneficial consequences, CR slowed the aging process, led to a prevention of diabetes, a reduced incidence of cardiovascular diseases and finally to an increased number of monkeys not suffering from age-related diseases. (Colman *et al.*, 2009) A second long term study of caloric restricted rhesus macaques led to the conclusion that caloric restricted monkeys have a decreased risk of death compared to *ad libitum* fed animals of the same age (Bodkin *et al.*, 2003). In contrast to these findings, Mattison *et al.* report that CR does not lead to an increased survival of rhesus macaques, thereby suggesting that the consequences of CR are more complex in a long-living model organism like monkeys compared to rather short-lived organisms (Mattison *et al.*, 2012).

### 1.1.2 Calorie restriction in humans– a way to counteract aging?

Aging can be defined as a progressive process to which almost every living organism is exposed with increasing time. Aging is accompanied by an accumulation of cellular damages and dysfunctions, e. g. accumulation of damages to nuclear or mitochondrial DNA and deregulation of nutrient-sensing. In their review published in 2013 López-Otín and colleagues suggested nine hallmarks of aging: genomic instability, telomere shortening, mitochondrial dysfunction, epigenetic modifications, loss of proteostasis, impairment of nutrient-sensing, cellular senescence, stem cell exhaustion, as well as alterations of cell-cell communication. (López-Otín *et al.*, 2013) Thus, in order to postpone the aging process, interventions must address one or several of these hallmarks of aging. However, the process of immunoaging should be added to López-Otín's hallmarks of aging, since the aging-associated changes of the immune system leads to higher infection and cancer rates as well as lower protection of chronic inflammation (Weyand and Goronzy, 2016).

It has already been well documented that CR in different model organisms leads to an increase in lifespan and also delays or prevents the occurrence of age-related diseases. In contrast to the numerous animal studies, there are few studies on humans focusing on lifespan extension. One of the few studies on the influence of CR on the lifespan of humans researched the exceptionally high number of centenarians on the Japanese island of Okinawa. Kagawa *et al.* concluded that the low caloric intake is responsible for reaching an extraordinary high age. (Kagawa, 1978) Walford and colleagues hypothesized that due to the alterations concerning physiology, hormone status or hematology gained in a 2-year CR study on humans, the effects of CR on rodents or monkeys were similar to those on humans (Walford *et al.*, 2002). By extension, this could also mean lifespan-increasing properties of CR for humans. However, most often human studies on CR are focused on improvement of health parameters like blood pressure, weight, blood glucose, cholesterol and sometimes the integrity of DNA. A 1-year study of CR (approximately -330 kcal/day compared to nutrition before the study) showed that risk factors for coronary heart disease improved substantially and LDL-cholesterol as well as body fat decreased significantly due to the intervention (Fontana *et al.*, 2007). In non-obese individuals, one year of modest CR reduces abdominal adipose tissue (Racette *et al.*, 2006), thus reducing the risk of hypertension, cardiac infarction and type 2 diabetes. Furthermore, Most *et al.* found a reduced ten year risk for cardiovascular disease (- 30 %) due to significantly

reduced cholesterol, systolic blood pressure and body weight after 24 months of CR (Most *et al.*, 2018). A six months study on CR in humans found reduced fasting insulin level in the intervention groups, reduced core body temperature as well as reduced DNA damages (Heilbronn *et al.*, 2006). Glucose intolerance as well as type 2 diabetes are the result of an impaired insulin sensitivity. The human body tries to compensate early stage glucose intolerance by increasing the level of fasting insulin. (Kahn *et al.*, 2014) Therefore, a reduction of fasting insulin level by CR, as mentioned above, counteracts the emergence of type 2 diabetes and thus contributes to aging healthier.

With regard to preserving the integrity of DNA, CR can reduce oxidative damage to DNA and RNA in human white blood cells significantly (Hofer *et al.*, 2008). Reducing the risk of DNA damages also means a simultaneous reduction of the risk of DNA mutations. As a result, the risk of cancer formation is also diminished, since the accumulation of damages to the DNA is generally accepted as the first step in carcinogenesis (Hanahan and Weinberg, 2000). It is commonly known that the probability to be diagnosed with cancer increases with increasing age. CR might be a way to counteract the causes of carcinogenesis, promote important health parameters and thus contribute to a healthier aging of humans. However, CR can not only reduce the risk to develop cancer, but may also help to support cancer therapy. Cancer patients receiving chemotherapy report lesser side effect when fasting for 48 h to 140 h prior and 5 h to 56 h after the application of chemotherapeutics, but due to the small number of only ten patients surveyed a confirmation of this statement by clinical trials is pending (Safdie *et al.*, 2009). Short-term fasting (24 h prior to and 24 h after the application of chemotherapeutics) can lead to a decrease of toxicity to bone marrow, reduce DNA damages induced by the chemotherapeutic drugs in PBMCs and promote the recovery from DNA damages (de Groot *et al.*, 2015). In contrast to healthy cells, cancer cells tolerate nutrient shortages poorly. Healthy cells shut down their metabolism when nutrients are scarce, while cancer cells are heavily dependent on glycolysis (Warburg effect). They adapt poorly to the new nutritional conditions and show reduced proliferation, which may lead to a higher efficiency of anti-cancer treatments. (Szypowska and Regulska-Ilow, 2019) In animal studies it has already been demonstrated that tumor progression can be inhibited by short-term starvation. Additionally, murine cancer cell lines were sensitized to chemotherapeutic treatment by glucose and serum restriction. (Lee *et al.*, 2012) Furthermore, the toxicity of chemotherapeutics towards human

and rat cancer cell lines can be increase by short term starvation, whereas normal cells are protected from the harmful effects of chemotherapy (Raffaghello *et al.*, 2008). However, it remains to be seen, whether these results can also be transferred to tumor diseases in humans.

## 1.2 Calorie restriction mimetics

As described in the chapters before, CR can have a lot of beneficial effects in animal models as well as in humans. Despite the multitude of evidence supporting the health-promoting effects of CR, few people are willing to live on such a diet for longer periods. The limitations and waivers in everyday life probably discourage people to commit to a calorie restrictive lifestyle. Furthermore, malnutrition and undernourishment must be avoided in order to benefit from CR. In addition to research into the effects of CR on the human body, aging research is also increasingly studying alternatives that have the positive effects of a CR but are not accompanied by the limitations. Substances called calorie restriction mimetics (CRMs), as the name suggests, have at least some of the same health-improving effects as a calorie restrictive diet. However, they could also be health-beneficial in addition to a CR.

One of the best-characterized CRMs is the polyphenol resveratrol. It is found in various plants, such as red grapes, raspberries, peanuts and red wine (Burns *et al.*, 2002; Langcake and Pryce, 1976). All of these foods are very common in human nutrition, therefore health-promoting abilities are of particular importance. Extensive research on resveratrol and its health benefits has been carried out since it has first been mentioned in a scientific publication in 1939 and although no general consensus regarding the health benefits could be reached, general perception is that resveratrol has health-beneficial characteristics (Pezzuto, 2019). Resveratrol has also been suggested as the active ingredient in red wine, which is responsible for the cardio-protective properties (Das *et al.*, 1999). Moreover, resveratrol shows anti-oxidative properties for example by up-regulating mRNA expression of superoxide dismutase 1 (Spanier *et al.*, 2009) or by scavenging reactive oxygen species (Leonard *et al.*, 2003). Resveratrol also shows anticancer properties by inhibiting ovarian cancer cell proliferation via an induction of apoptosis as well as via an inhibition of glycolysis and thus activating AMPK. Activated AMPK in turn prevents cancer cell growth and induces apoptosis by inhibiting mammalian target of rapamycin (mTOR). (Liu *et al.*, 2018) Besides, there is further evidence

that resveratrol may play a role in therapy and chemoprevention of various cancer types (Ko *et al.*, 2017; Stagos *et al.*, 2012).

Apart from research on the health-promoting effects of resveratrol, its CR-mimicking properties have also been extensively researched. In 2003 Howitz *et al.* were the first ones to describe a CR-mimicking effect of this polyphenol. They demonstrated an activation of silent mating type information regulation 2 (Sir2), a homolog of SIRT1 in humans, in *Saccharomyces cerevisiae* and a subsequent the lifespan-extension (~ 70 %) similar to the effect CR has in yeast as well as an increased DNA stability. (Howitz *et al.*, 2003) Furthermore, resveratrol and other sirtuin activating compounds extend the lifespan of *Drosophila melanogaster* and *Caenorhabditis elegans* in a Sir2-dependent manner via a CR-related pathway (Wood *et al.*, 2004). In addition, mice on a high calorie diet show improved health, survival and increased insulin sensitivity due to resveratrol intake (Baur *et al.*, 2006). In non-human primates CR as well as a treatment with resveratrol improve glucose tolerance and thus affect insulin sensitivity positively (Marchal *et al.*, 2012). There are numerous other examples of the CR-mimicking effects of resveratrol in model organisms, but more interesting is, whether resveratrol is able to mimic CR in humans.

A study published in 2010 found a first evidence that elderly obese people with impaired glucose tolerance benefit from a 1 – 2 g dose of resveratrol per day and thus display an improved insulin sensitivity at the end of the four-week study (Crandall *et al.*, 2012). Timmers *et al.* treated obese humans with 150 mg resveratrol per day for 30 days and reported a significantly reduced metabolic rate, an increase of SIRT1 protein, an improved mitochondrial respiration in muscle as well as other health-improving consequences. They concluded that resveratrol has in fact CR-mimicking effects in obese humans. (Timmers *et al.*, 2011) In type 2 diabetes patients supplemented with 5 mg resveratrol twice a day for four weeks, an improvement of insulin sensitivity, presumably due to a reduction of oxidative stress and a subsequently more efficient insulin signaling, could be demonstrated (Brasnyó *et al.*, 2011). On the other hand, a combined daily treatment of overweight or obese test persons with the polyphenols resveratrol and epigallocatechin-3-gallate (80 mg and 282 mg respectively) for twelve weeks did not improve insulin sensitivity, but improved mitochondrial capacity and fat oxidation, two other health-beneficial parameters (Most *et al.*, 2016).

Another CRM, the polyamine spermidine, has only just caught the attention of gerontologists. Polyamines like spermidine fulfill a variety of tasks in the human organism. The role of polyamines has been reviewed profoundly, for example by Pegg *et al.*, who focused on the involvement of polyamines in growth and development of animals (Pegg, 2016) and Igarashi and Kashiwagi, who focused on the modulation of DNA and RNA functions by polyamines (Igarashi and Kashiwagi, 2010). Furthermore, polyamines like spermidine play an important role in cellular metabolism (Tabor and Tabor, 1984) and are involved in the synthesis of proteins, DNA and RNA (Konecki *et al.*, 1975). In different cells and tissues of humans and rats, the spermidine content decreases with increasing age (Cooper *et al.*, 1976; Jaenne *et al.*, 1964). This can result in an impairment of the functions and mechanisms mentioned above and underlines the importance of spermidine in aging processes. Moreover, Pucciarelli *et al.* analyzed whole blood of nonagenarians and centenarians and detected an enrichment of spermidine, hinting at an important role of this substance in aging processes of humans (Pucciarelli *et al.*, 2012).

Spermidine is known to act anti-tumorigenic, cardio and neuro protective as well as anti-inflammatory (Madeo *et al.*, 2018), thus has similar effects as a CR. However, one of the most important functions of spermidine is the induction of autophagy. Spermidine inhibits E1A-associated protein p300 (Pietrocola *et al.*, 2015), a histone acetyltransferase, which usually acetylates and thus deactivates autophagy-relevant proteins (Lee and Finkel, 2009). By inhibiting E1A-associated protein p300, spermidine induces autophagy (Pietrocola *et al.*, 2015). Autophagy, or more specifically a decrease in autophagy, is believed to be closely linked to aging, as studies in rats already showed (Donati *et al.*, 2001). Since the process of autophagy is evolutionarily conserved, it is likely that there is also a link between aging and a decline in autophagy in humans. Furthermore, CR is an effective inducer of autophagy (Bergamini *et al.*, 2003), suggesting that an autophagy-inducing substance like spermidine might have CR-mimicking properties. With regard to its potential as a CRM, spermidine has already been shown to have a positive effect on the lifespan and the induction of autophagy in model organisms like yeast, flies and worms as well as human immune cells (Eisenberg *et al.*, 2009). In a prospective, population-based study, researchers could correlate spermidine intake with a reduced risk of cardiovascular diseases like heart failure and also lower blood pressure. Since the researchers used questionnaires to evaluate spermidine intake rather than supplementing



test persons with defined amounts of spermidine, the assessment is to be seen as indirect. (Eisenberg *et al.*, 2016) Nevertheless, with increasing age, the occurrence of cardiovascular dysfunction and co-morbidities like obesity and diabetes type 2 increases and a higher spermidine intake might be able to prevent or postpone these diseases in a similar way as CR.

The question remains whether the many positive effects of a CR can also be triggered by CRMs, or whether CRMs are rather used in addition to support a CR.

### 1.3 Human blood cells in biomedical studies

Studies on DNA repair and aging in humans are often carried out using human *in vitro* cultured cells (Hart and Setlow, 1974; Burger *et al.*, 2007). On the one hand, this approach has certain advantages with regard to test planning and availability of the cells. On the other hand, *in vitro* cultured cells do not necessarily reflect the current status of the queried parameter in the human body, especially when it comes to systemic influences such as CR or food intervention studies. *Ex vivo* studies can circumvent this obstacle by using cells, which were isolated directly from the human body. A suitable sample material for this is human blood and the cells it contains. Human blood is an aqueous solution, which consists of plasma as a liquid component and the blood cells as solid components. Furthermore, it contains salts, proteins, low-molecular substances like monosaccharides, hormones, nutrients as well as metabolic products, which are transported to their place of action by circulation induced by the cardiovascular system. Blood sampling is relatively easy and painless, and the cells it contains, in particular peripheral mononuclear blood cells (PBMCs), can be purified quickly and easily. PBMCs consist of lymphocytes (T cells, B cells and natural killer (NK) cells), monocytes and dendritic cells, but neither erythrocytes nor platelets due to their lack of nuclei. Granulocytes are also not assigned to the PBMCs because of their multi-lobed nuclei. In humans approximately 70 % - 90 % of the PBMCs are lymphocytes (T cells: 70 - 85 %; B cells: 5 - 20 % and NK cells: 5 - 20 %), up to 10 % - 20 % are monocytes and approximately 1 % - 2 % are dendritic cells, although the frequencies vary across individuals (Kanof *et al.*, 2001; Kleiveland *et al.*, 2015). It should be noted that since the vast majority of PBMCs are lymphocytes, these naturally have a significant influence on the results obtained with PBMCs.

As mentioned above, studies of systemic influences on the human organism require test material, which is fast and easily accessible. PBMCs are therefore extremely suitable, as they do not require cell culture prior to their use in different experimental setups. In addition to examining immunological issues, PBMCs or lymphocytes have already been used to study DNA repair, research of gene or protein expression as well as *ex vivo* studies. Using an optimized protocol for the comet assay, DNA repair ability of PBMCs for oxidative DNA damages was determined for both frozen and freshly isolated blood cells. Within the terms of this study involving human volunteers, a positive effect of a short-time food intervention with the plant *Brassica carinata* on DNA repair of oxidative DNA damages could be reported. (Odongo *et al.*, 2019) Furthermore, a study using human PBMCs showed that the immunosuppressant cyclosporin A leads to a decrease of the repair of UV-induced DNA damages and an increased cancer occurrence in kidney transplant recipients treated with the immunosuppressant. The same was stated for human PBMCs, which were treated with cyclosporin A *ex vivo*. (Herman *et al.*, 2001) Moreover, Arimilli and colleagues used human PBMCs in order to research the influence of different tobacco products on the basis of alterations in gene expression, stating that cigarette consumption affects immune-related pathways (Arimilli *et al.*, 2017). Human PBMCs are also used in the context of studies on metabolic diseases. In PBMCs of insulin-resistant and metabolic syndrome individuals, protein and gene expression of the aging-associated Sirtuin 1 was significantly reduced compared to the control group (Kreutzenberg *et al.*, 2010). Furthermore, PBMCs, or more precisely lymphocytes, are suitable for analyzing nucleotide excision repair capacity of human, as different studies have already shown (Athas *et al.*, 1991; Qiao *et al.*, 2002).

The studies involving PBMCs shown here are certainly only a part of the actually existing studies. Nevertheless, they demonstrate that human PBMCs are suitable for the investigation of a wide range of scientific questions.

#### 1.4 Types DNA damages

All cells within the human body are constantly exposed to DNA damaging agents, either due to the physiologic cell metabolism or as a result of the impact of environmental factors. Hoeijmakers summarized the threats to DNA integrity into three categories: spontaneous reactions, the regular cellular metabolism and exogenous physical or chemical agents

(Hoeijmakers, 2009). Within the course of the normal cellular metabolism byproducts like free radicals such as reactive oxygen species emerge and lead to base oxidation and DNA strand breaks. Furthermore, an incorrect incorporation of nucleotides during replication leads to spontaneous DNA modifications. Additionally, spontaneous endogenous deamination and alkylation processes constantly endanger the integrity of the genome. (Lindahl and Barnes, 2000) It is estimated that up to  $10^4 - 10^5$  DNA lesions occur in each cell per day (Lindahl, 1993; Hoeijmakers, 2009), presenting a challenge for the maintenance systems of genome integrity and emphasizing the importance of a functional DNA repair.

Causes of endogenous DNA damages can be, for example, reactive oxygen species (ROS), reactive nitrogen species (NOS), deamination or depurination. Of the previously mentioned endogenous DNA damaging agents, ROS are considered to be the primary cause, leading to oxidized DNA bases, single strand and double strand breaks (de Bont and van Larebeke, 2004). Superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $OH\cdot$ ) and singlet oxygen ( $^1O_2$ ) are examples for ROS (Bayr, 2005). Within cells, ROS emerge from mitochondrial respiration in form of the byproduct  $O_2^-$  (Cadenas and Davies, 2000) or from inflammation, among others. An increased presence of ROS in cells, which exceeds the available antioxidant defenses, leads to oxidative stress, which in turn is closely linked to aging processes (Kudryavtseva *et al.*, 2016). The most common oxidative DNA lesion caused by ROS is 7-8-dihydro-8-oxoguanine (8-oxoG). Moreover, increased levels of 8-oxoG have also been associated with aging (Olinski *et al.*, 2007). The oxidized form of guanine is able pair with adenine, additionally to cytosine, leading to GC to TA transversions and thus to permanent DNA mutations after replication. On the other hand, ROS also fulfill important roles as signaling molecules and modulators of cellular functions (Devasagayam *et al.*, 2004).

In addition to endogenous causes of DNA damages, these can also result from exogenous influences. These exogenous agents can be ultraviolet (UV) radiation, ionizing radiation or alkylating agents. According to the wavelength, UV radiation is subdivided into UVA (315 – 400 nm), UVB (280 – 315 nm) and UVC (<280 nm) radiation. The major source of UV radiation on the earth's surface is the sun. UVA is the least energetic of the three and thus has poor ability to directly induce DNA damages due to the long wavelength and the resulting poor ability to interact with DNA directly. Nevertheless, it has been shown that UVA induces

cyclobutane pyrimidine dimers (CPDs), although at lower levels (Besaratina *et al.*, 2005). UVB interacts directly with DNA, because its spectrum of wavelengths is near the wavelength of maximum absorption of DNA of 260 nm. Consequently UVB is able to damage DNA directly, resulting in bulky DNA adducts like cyclobutane pyrimidine dimers (CPDs) by covalently linking adjacent pyrimidine bases or the pyrimidine adducts called pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) (Pfeifer, 1997). These DNA adducts can inhibit DNA replication as well as transcription and can lead to DNA double strand breaks at the locations where replication forks collapse (Rastogi *et al.*, 2010). Indirectly, UVA and UVB both can promote oxidization of bases, resulting for example in the formation of 8-oxoG (Cadet and Douki, 2011). Furthermore, UVA and UVB are both risk factors for developing skin cancer (Khan *et al.*, 2018; Mullenders, 2018). UVC is the most energetic of the UV subcategories, thus it is also able to induce DNA damages like CPDs and 6-4PPs directly (Rochette *et al.*, 2006). However, UVC is absorbed by the ozone layer and hence does not reach the earth's surface.

DNA damages can furthermore arise from exposure to pollutants like polycyclic aromatic hydrocarbons (PAHs) like benzo[a]pyrene (B[a]P), which can be contained in processed food like charcoal-grilled meat (Farhadian *et al.*, 2011), tobacco smoke or incomplete combustion of organic materials amongst others. PAHs are considered as carcinogens, causing for example lung, colorectal or pancreatic cancer (Straif *et al.*, 2005). After metabolic activation, they can react with the heterocyclic bases of DNA, resulting in distortion of base-pairing and helix-structure of the DNA (Skosareva *et al.*, 2013). If not repaired, the formation of these DNA adducts can lead for example to apurinic sites or point mutations, which contribute to cancer formation. Furthermore, mutagenic and cytotoxic properties are attributed to PAHs. (Błaszczuk and Mielżyńska-Švach, 2017)

Although ionizing radiation (alpha-, beta-, gamma-radiation, X-rays) is frequently used in medicine for example in radiology or for therapeutic approaches in cancer treatment, it is nevertheless able to introduce damages to DNA. There is a wide range of DNA lesions resulting from exposure to ionizing radiation, for example ROS, apurinic or apyrimidinic sites (AP sites), single and double strand breaks or base losses. Often two or more of these lesions appear jointly, generating so-called clustered DNA lesions, which are characteristic for an exposure to ionizing radiation. (Sage and Shikazono, 2017) Ionizing radiation can ionize DNA

directly and thus cause direct DNA damages. Moreover, DNA damages can be caused indirectly via an ionization of water, which leads to oxidative stress and subsequent formation of ROS.

The first chemotherapeutics used in cancer treatments were alkylating agents, for example mustard nitrogen (Gilman and Philips, 1946). Up to now they play an important role in the therapy of cancer types like malignant melanoma, non-Hodgkin's lymphoma or pancreatic cancer (Kaina *et al.*, 2007). Alkylating agents are defined as reactive chemical agents, which are able to transfer alkyl carbon groups to a variety of biomolecules, for example to DNA. They can be of exogenous origin (e. g. constituents of water, air or food, tobacco smoke or pollutants) or they emerge endogenously as a result of oxidative damage or within the course of biochemical reactions (DeMarini, 2004; Bartsch and Montesano, 1984; Marnett and Burcham, 1993). Alkylating agents can either be monofunctional, containing one reactive site modifying a single base in DNA, or bifunctional with two reactive sites, which are able to form inter- or intra-strand crosslinks of two DNA bases. N7-methylguanine is the predominant methylation adduct caused by monofunctional alkylating agents. Due to the probability of a spontaneous depurination and the subsequent formation of AP sites, N7-methylguanine can be regarded as indirectly mutagenic (Fu *et al.*, 2012). The generation of O<sup>6</sup>-methylguanine by alkylating agents harbors the danger of mispairing with thymine during DNA replication and thus leading to cytotoxicity and mutagenicity.

However, a low level of DNA damages may be advantageous, since DNA repair mechanisms may be triggered by them. It has been suggested that low amounts of persistent DNA lesions in active genes lead to a stalling of RNA polymerase II in transcription coupled repair of nucleotide excision repair (TCR), leading to an increased stress resistance and thus counteracts aging (Schumacher, 2009). This phenomenon called hormesis describes contradictory effects of one substance at different doses, mostly meaning a beneficial or stimulatory influence at low doses and a toxic or harmful effect at high doses (Calabrese *et al.*, 2007).

## 1.5 DNA repair pathways

Cells are constantly exposed to DNA damaging agents. Some of them emerge endogenously due to normal cell metabolism and some exogenously as a result of exposure to damaging noxae like chemicals or different types of radiation. Every cell within the human body experiences tens of thousands of DNA damaging events every day (Lindahl and Barnes, 2000). Since the preservation of the integrity of the genome is crucial for the survival of a cell, powerful defense mechanisms exist in both pro- and eukaryotes to counteract the development of DNA mutations. The term DNA damage response (DDR) summarizes mechanisms and pathways, which are activated upon DNA damage in order to sense and signal the damage and initiate a finely orchestrated series of actions leading to the protection of the cell. The complex network of DDR mechanisms include cell cycle checkpoints as well as DNA repair mechanisms (Jackson and Bartek, 2009). Some of the most important of these DDRs, also known as DNA repair pathways, are nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), double strand break (DSB) repair and direct reversal (DR). DNA repair mechanisms can be found in both pro- and eukaryotes and thus underline the importance of genome-preservation for all living organisms. **Figure 1** shows an overview of the main DNA damaging agents, their impact on the DNA and the corresponding DNA repair pathways.

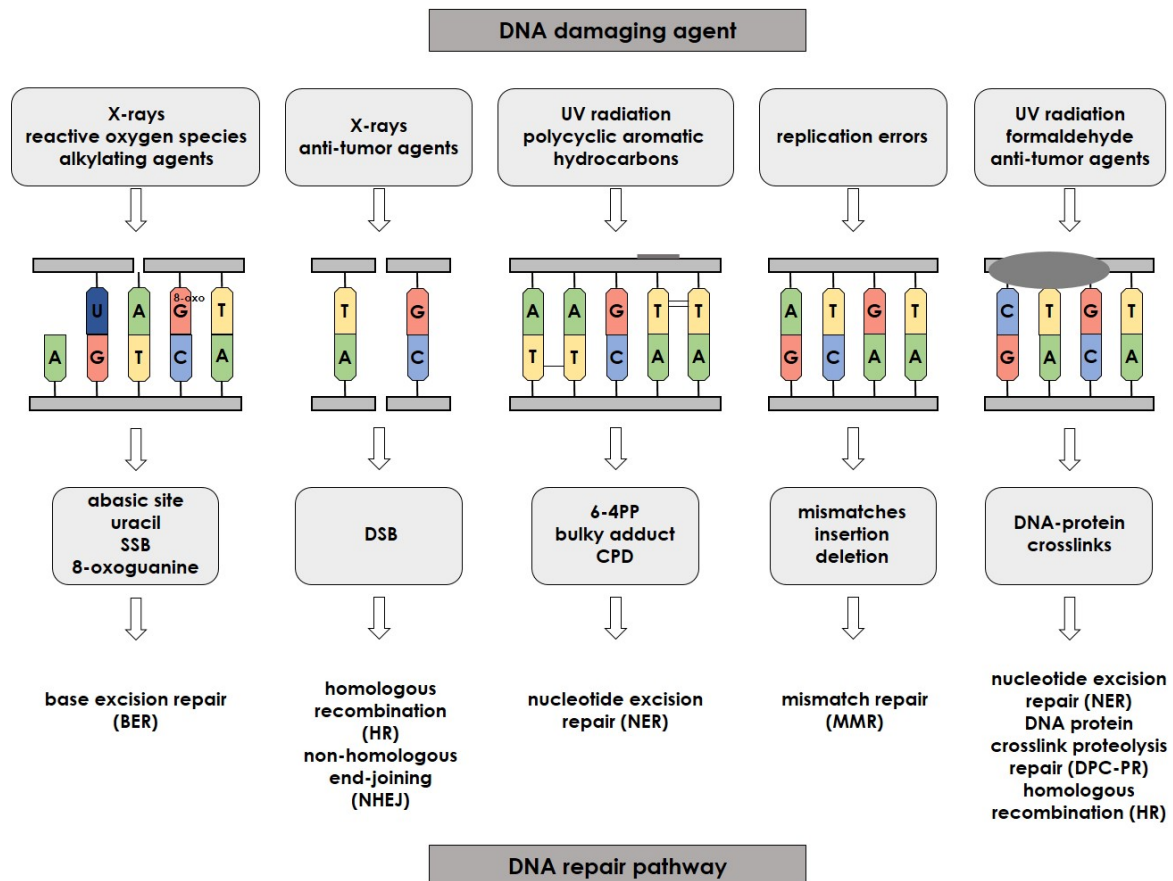


Figure 1: DNA damaging influences, resulting DNA damages and their corresponding DNA repair pathways.

There are numerous endogenous and exogenous causes for DNA damages. These harmful influences can result in characteristic DNA damages, for which there are special DNA repair pathways. Image adapted from Vaz (Vaz *et al.*, 2017).

In the following and due to the relevance for the present work, the pathway of the NER is presented in detail in the subsequent chapter 1.5.1. Additionally, a brief introduction of BER, MMR, DSB repair and DR is given in chapters 1.5.2, 1.5.3, 1.5.4 and 1.5.5, respectively. Several remarkable reviews on DNA repair pathways can be recommended at this point (Dexheimer, 2013; Hakem, 2008; Hsieh and Yamane, 2008; Krokan and Bjørås, 2013; Shuck *et al.*, 2008; Wallace, 2014).

### 1.5.1 Nucleotide excision repair

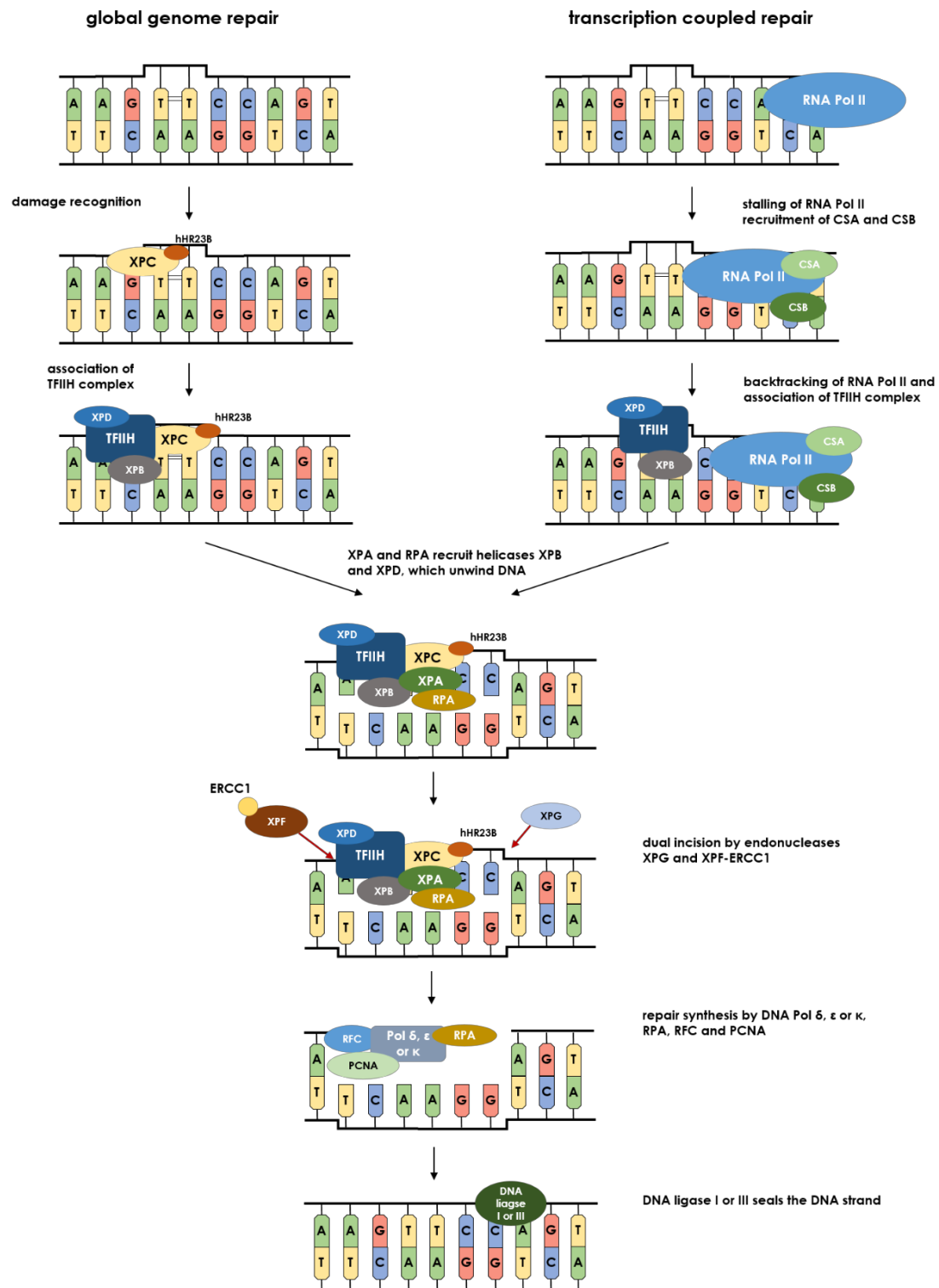
The task of nucleotide excision repair (NER) is the repair of structural anomalies of the DNA e.g. bulky, helix-distorting DNA damages, which include UV-induced DNA lesions or damages resulting from exposure to chemicals. Two types of DNA damages often caused by UV radiation are CPDs and 6-4-PPs, as described in chapter 1.4. The pathway of NER involves the interaction of about 30 proteins, which operate in an orchestrated manner in order to

restore genome integrity (Aboussekhra *et al.*, 1995). This multistep process is highly conserved and follows similar steps in a variety of species, starting with damage recognition, followed by a dual incision and the removal of the lesion-containing oligonucleotide, repair synthesis via copying of the opposite DNA strand and finally sealing of the DNA strand (Sancar, 1994a; Sancar and Reardon, 2004). NER can be subdivided into two subpathways, the transcription coupled repair (TCR) and the global genomic repair (GGR). TCR repairs DNA damages in the transcribed strands in active genes, whereas GGR operates throughout the genome and also repairs damages in the non-transcribed stand of active genes (Hanawalt, 2002). The two subpathways show differences regarding the recognition of the helix-distortion, but proceed via a common pathway to excise and replace the DNA lesion.

As mentioned above, NER consists of damage recognition, opening of an approximately 30 nucleotide denaturation bubble around the DNA lesion, dual incision of the lesion-containing DNA strand, removal of this oligonucleotide, re-synthesizing of the DNA strand and ligation. GGR is performed by the orchestrated action of six essential NER proteins: *xeroderma pigmentosum* complementation group C (XPC), transcription initiation factor IIIH (TFIIH), *xeroderma pigmentosum* complementation group A (XPA), replication protein A (RPA), *xeroderma pigmentosum* complementation group G (XPG) and excision repair cross-complementation group 1-XPF (ERCC1-XPF) (Aboussekhra *et al.*, 1995), some of which also play an important role in TCR as the two subpathways continue in the same manner at the step of damage verification. GGR is initiated by XPC, the main damage recognition sensor of the GGR subpathway of NER (Sugasawa *et al.*, 1998). The binding of XPC is required for the association of the TFIIH protein complex to the damage site and the subsequent verification of the lesion (Volker *et al.*, 2001). TCR in turn, is initiated by a stalling of RNA polymerase II (RNA Pol II), which recruits the cockayne syndrome proteins A and B (CSA and CSB) to the damage site. These two proteins are required for the assembly of TCR proteins (Fousteri *et al.*, 2006) and are furthermore specific for the TCR subpathway. The formation of a CSA-CSB protein complex presumably leads to backtracking of RNA polymerase II and leaving the damage site accessible for further repair proteins (Marteiijn *et al.*, 2014), amongst them TFIIH. In the following, TCR and GGR proceed via a sequential assembly of repair factors. The helicase subunits of TFIIH, *xeroderma pigmentosum* complementation group B and D (XPB and XPD) open the DNA in order to form a ~30 nucleotide bubble, which is stabilized by XPA and



RPA. XPA is thereby recruited by TFIIH, is taking part in the verification of the damage and is responsible for assembling the incision factors of NER (Li *et al.*, 2015; Schärer, 2013; Reardon and Sancar, 2003). The endonucleases XPG and ERCC1-XPF, which is recruited by XPA, cut the lesion-containing DNA strand 3' and 5' to the lesion, whereby XPG cuts 3' (O'Donovan *et al.*, 1994) and ERCC1-XPF cuts 5' to the lesion (Sijbers *et al.*, 1996). Thus a piece of 22-30 nucleotides, which includes the lesion, is excised. Repair synthesis of the resulting gap is carried out by DNA polymerases  $\delta$ ,  $\epsilon$  or  $\kappa$  (DNA Pol  $\delta$ ,  $\epsilon$  or  $\kappa$ ) (Ogi *et al.*, 2010), replication factor C (RFC), trimeric proliferating cell nuclear antigen (PCNA), RPA and DNA ligase I or III, which finally seals the DNA strand (Shivji *et al.*, 1995; Paul-Konietzko *et al.*, 2015). **Figure 2** illustrates the main steps of NER including the subpathways GGR and TCR.



**Figure 2: Nucleotide excision repair and its two subpathways global genome repair (GGR) and transcription coupled repair (TCR).**

XPC is the main damage-recognition factor of GGR. In the following, TFIIH complex is recruited to the damage site. TCR is initiated by stalling of RNA Pol II. CSA and CSB are recruited, followed by backtracking of RNA Pol II and association of TFIIH complex. Subsequently the same repair steps are executed: XPA and RPA recruit the helicases XPB and XPD, which unwind the DNA in order to open a ~30 nucleotide bubble. Dual incision is carried out by XPG and XPF-ERCC1 and a piece of 22-30 nucleotides is excised. DNA Pol  $\delta$ ,  $\epsilon$  or  $\kappa$ , RFC, PCNA and RPA perform repair synthesis and DNA Lig I or III finally seals the DNA strand.

The importance of an intact NER is shown by the rare, but severe hereditary syndromes *xeroderma pigmentosum* (XP), cockayne syndrome (CS) and trichothiodystrophy (TTD) caused by NER deficiencies. XP patients show mutations in XP-NER genes resulting in a hypersensitivity to sun exposure, a 1000-fold increased risk for developing skin cancer and a 50 years reduced age of onset of skin cancer (Kraemer, 1994). CS patients show cutaneous photosensitivity, no predisposition for skin cancer, but postnatal failure of brain growth and progressive hearing as well as visual loss. Furthermore, they show many signs of the normal human aging process. As mitochondrial pathologies are the cause of many aging-associated features, a participation of mitochondrial pathologies as a cause for CS is also discussed (Karikkineth *et al.*, 2017). TTD patients show various clinical characteristics like brittle hair, severe neurological abnormalities, skeletal abnormalities and sun sensitivity but no increased occurrence of skin cancer (Kraemer *et al.*, 2007).

### 1.5.2 Base excision repair

The pathway of base excision repair (BER) is responsible for the correction of damaged DNA bases or single strand breaks, which often arise from spontaneous occurring DNA damages like deamination or hydroxylation of bases or from exposure to alkylating agents (Wallace, 2014; Duclos *et al.*, 2012). In contrast to NER, which repairs helix-distorting DNA damages, BER is responsible for the elimination of non-helix-distorting DNA damages. BER starts with the recognition of the modified base by a damage-specific DNA glycosylase, e.g. 8-oxoguanine glycosylase 1 (OGG1), followed by cleaving of the N-glycosidic bond, which is catalyzed by the appropriate DNA glycosylase. This step eliminates the modified base and generates an AP site. Subsequently, an AP endonuclease (APE1) cleaves the AP site, resulting in a single strand break (SSB). The SSBs are detected by poly(ADP-ribose) polymerase 1 (PARP1), which recruits further components of the BER complex in the following. At this point, the BER pathway is subdivided into short- and long-patch BER. During short-patch BER a gap of one nucleotide is generated and afterwards filled and ligated, whereas long-patch BER generates and refills a gap of 2-10 bases. A DNA polymerase (short-patch BER: DNA polymerase  $\beta$  with deoxyribose phosphate (dRP) lyase activity; long-patch BER: DNA polymerase  $\delta/\epsilon$ ) fills the gap(s) with the correct nucleotide(s) and finally a DNA ligase (short-patch: DNA ligase III; long-patch: DNA ligase I) seals the nicks. (Robertson *et al.*, 2009; Krokan and Bjørås, 2013; Mangerich and Bürkle, 2012; Krokan and Bjørås, 2013)

### 1.5.3 Mismatch repair

Mismatched DNA bases as well as insertion or deletion mispairing, which can all occur sporadically during replication or as a consequence of translesion polymerase activity, are repaired via the highly conserved mechanism of mismatch repair (MMR). The importance of MMR is shown by the correlation of increased cancer incidence and impaired MMR. (Li, 2008) The process of MMR starts with damage recognition, followed by a strand-specific incision 5' to the mismatched base. The DNA strand is excised up to marginally past the mismatch, subsequently DNA polymerase  $\delta$  re-synthesizes the DNA strand and DNA ligase I finally seals the remaining nick. (Liu *et al.*, 2017)

### 1.5.4 Repair of DNA double strand breaks

Although quite common in eukaryotes, double strand breaks (DSBs) are probably the most hazardous damages a cell can be faced with. They can lead to the loss of larger chromosomal regions or chromosomal translocations and thus represent the initial step in cancer formation. DSBs can be the result of ionizing radiation, chemical substances, replication errors, ROS or unintended actions of enzymes. In order to repair DSBs and minimize the loss of genetic information, two main pathways exist: homologous recombination (HR) and non-homologous end joining (NHEJ). (Ranjha *et al.*, 2018)

NHEJ rejoins the DNA strand directly without the use of a template, typically involving small deletions or insertions and hence is rather error-prone. The mechanism of NHEJ takes place throughout the cell cycle. (Chang *et al.*, 2017) Ku70/80 heterodimer is the first factor to bind to the DSB and stabilizes the following enzyme activities. The binding of the Ku heterodimer recruits the DNA-dependent protein kinase (DNA-PKcs), polymerases  $\mu$  and  $\lambda$  fill in the missing nucleotides, and DNA Ligase IV finally ligates the DNA ends. (Lieber, 2008)

For the process of HR, in contrast, a homologous sequence (e.g. the sister chromatid) is obligatory as template. This enables the restoration of missing genetic information and minimizes error-proneness, but restricts HR to the S/G2-phase of the cell cycle due to the availability a template. (Ranjha *et al.*, 2018) In comparison to NHEJ, HR is slower, but more accurate, the process is more complicated and involves larger numbers of enzymes (Chang *et al.*, 2017). The resection of DNA at the site of the DBS represents the first step of HR, leading

to an exposed single strand DNA, which is used for the search of a homologous DNA sequence. The following steps involve unwinding of DNA's secondary structures, invasion of the homologous DNA strand, synthesis of DNA by polymerase  $\eta$  and relegation. This process can result in Holliday junctions (4-way junctions), which can be resolved by several enzyme complexes, so that finally two DNA double strands exist. (Cerbinskaite *et al.*, 2012)

### 1.5.5 Direct reversal of DNA damages

Some DNA damages can be reversed directly after their formation by a mechanism called direct damage reversal. Undeniably, this is the most simple and sophisticated way to remove DNA damages and restore DNA to its original state. Alkylating substances, for instance, lead to the formation of O<sup>6</sup>-methylguanine, which is highly mutagenic due to its ability to pair with either thymine or cytosine during the process of DNA synthesis (Swann, 1990). The repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase transfers the methyl group of O<sup>6</sup>-methylguanine to an active cysteine, restoring the native guanine and resulting in an irreversible inactivation of the repair enzyme (Pegg *et al.*, 1995; Lindahl and Wood, 1999). Furthermore, photolyases reverse UV-induced DNA damages like CPDs and 6-4PPs directly by using visible light as energy source in order to split the dimer into the two bases (Sancar, 1994b). Additionally, *Escherichia coli* displays an increased resistance to alkylating and oxidizing agents when challenged by low doses of the same agent before. This adaptive response is carried out by the proteins AlkA (functions as glycosylase) and Ada (functions as alkyltransferase). (Volkert, 1988)

## 1.6 DNA repair measurement - the modified host cell reactivation assay

There are numerous assays, which can be used to detect DNA damages or to measure DNA repair. DNA strand breaks, for example, can be detected by polymerase chain reaction (PCR) followed by agarose gel electrophoresis or by single cell gel electrophoresis (comet assay), which is by the way also used for researching oxidative DNA damages. For the detection of DSBs, the characteristically phosphorylated histone 2AX ( $\gamma$ H2AX) is stained using immunofluorescence. Furthermore, immunohistochemistry or enzyme-linked immunosorbent assay (ELISA) using DNA damage specific antibodies are used to quantify DNA damages. (Figuerola-González and Pérez-Plasencia, 2017) For the detection of formation and repair of SSBs, an automated fluorimetric detection of alkaline DNA unwinding (FADU)

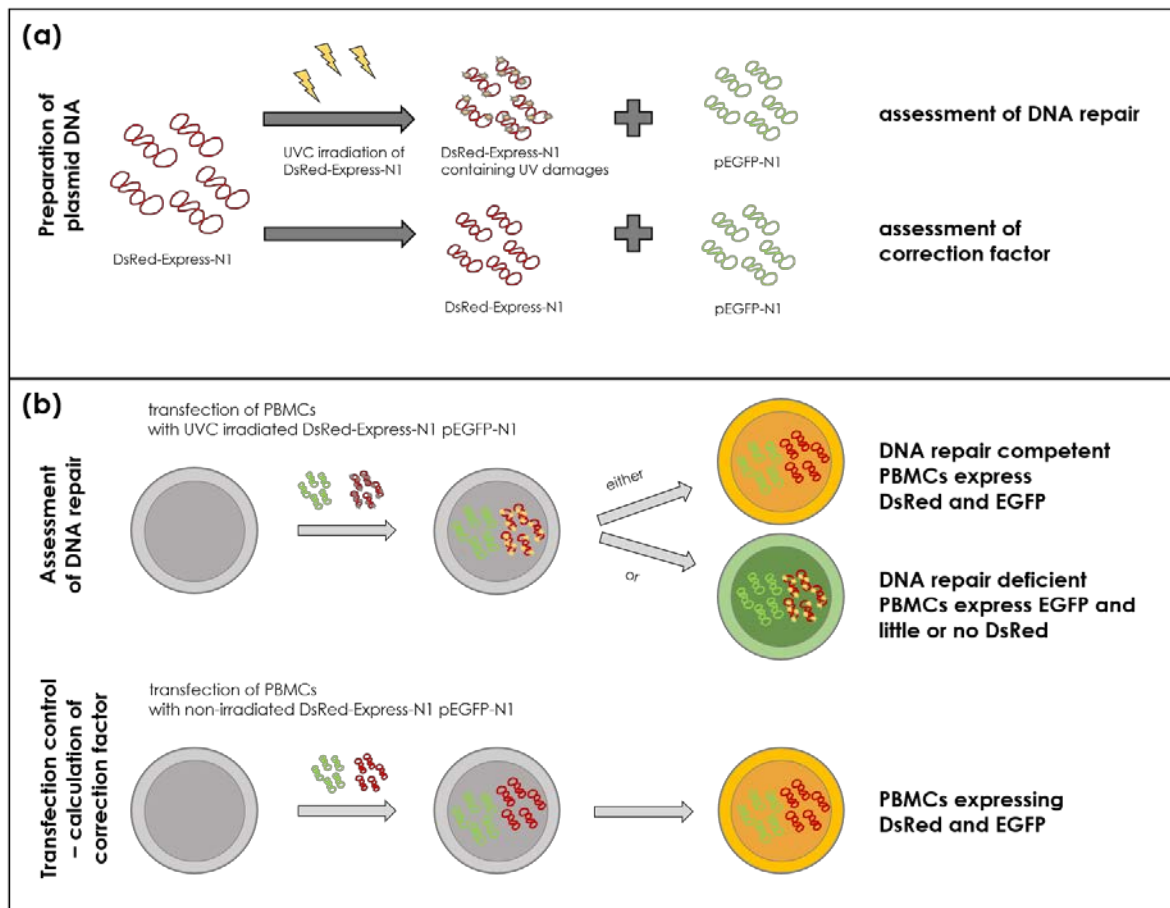
assay is available (Moreno-Villanueva *et al.*, 2009). Besides, DNA repair can be determined by the elimination products of the damage, for example measurement of the urinary level of 8-hydroxydeoxyguanosine (8-OHdG), which, in turn, is a biomarker for oxidative stress (Cooke *et al.*, 2009). The cell free system (CFS) allows the detection of DNA repair synthesis via radiolabeled nucleotides in a repair competent cell extract (Wood *et al.*, 1988). A similar principle represents the unscheduled DNA synthesis (UDS) assay, which determines the repair activity of cells by incorporation of labelled nucleotides (Rasmussen and Painter, 1964). The above-mentioned methods for detection of DNA damages and DNA repair represent only a part of the methods available. Since it is of major importance for this thesis, the modified host cell reactivation assay (mHCRA) will be explained in detail below.

A method to research the ability of cells to repair DNA damages is the mHCRA. The advantage of this assay is the detection of the functional repair of a previously damaged reporter plasmid, after its transfection into cells. Furthermore, the induction of the DNA damage is carried out extracellularly, the damaged DNA is introduced into the cells and an influence on DNA repair can be measured independently of the DNA damage event. Depending on the type of DNA damage induced, theoretically different DNA repair pathways can be investigated. The first proof of this functional repair was provided by Protić-Sabljić in 1985 in experiments using normal human fibroblasts and repair deficient *xeroderma pigmentosum* fibroblasts (Protić-Sabljić *et al.*, 1985). The method of transfecting a reporter plasmid into human cells and analyze its reactivation in order to research DNA repair was hereinafter modified so that human lymphocytes could be analyzed. Furthermore, UVC radiation was used to introduce DNA damages to the reporter gene chloramphenicol acetyltransferase. (Athas *et al.*, 1991) Since UVC radiation was used as DNA damaging agent, the occurring damages were mainly CPDs and 6-4-PPs. NER is responsible for repairing this UV-specific DNA damage, so the NER capacity of the cells was measured in this assay. An alternative of conducting HCRA using human lymphocytes and only one reporter plasmid was presented by Qiao *et al.* in 2002. They compared two different reporter genes (chloramphenicol acetyltransferase vs. luciferase) and concluded that luciferase is as suitable as reporter as chloramphenicol acetyltransferase for the measurement of UV-induced damages. (Qiao *et al.*, 2002)

Roguev and Russev took a new approach to being able to run HCRA independently of transfection efficiencies and reporter gene expression rates. They transfected two reporter plasmids coding for a green fluorescent protein and a yellow fluorescent protein (pEGFP-N1 and pEYFP-N1) at once into the cells. One of the plasmids was damaged by UVC prior to transfection (pEGFP-N1) so that its repair could be measured, and the other served as a transfection control and remained intact (pEYFP-N1). (Roguev and Russev, 2000) Again, the repair capacity of cell to restore UV-induced damages was measured. The next step in the further development of HCRA was the simultaneous transfection of primary human skin cells with two reporter plasmids (pEGFP-N1 as transfection control and UV-damaged pDsRedExpress-N1) and an analysis of the fluorescent proteins, which was conducted by the sensitive method of fluorescence activated cell sorting (FACS) (Burger *et al.*, 2007; Burger *et al.*, 2010). pEGFP-N1 codes for an enhanced green fluorescent protein (EGFP) and pDsRedExpress-N1 codes for *Discosoma* red fluorescent protein (DsRed). Although HCRA can be conducted using two reporter plasmids instead of only one, as described above, Mendez and colleagues used only a pmaxGFP plasmid and thus no transfection control in their study on DNA repair capacity of cryopreserved human lymphocytes (Mendez *et al.*, 2011).

The latest further development of mHCRA was conducted within the scope of this thesis. It is based on the method described by Burger *et al.* (Burger *et al.*, 2010), but was further modified and adapted to an application with human PBMCs in order to measure human DNA repair capacity *ex vivo* (Matt *et al.*, 2016).

The principle of the mHCRA for measuring NER capacity using two reporter plasmids, as is was conducted within the framework of this thesis is presented in **Figure 3**.



**Figure 3: Principle of the modified host cell reactivation assay.**

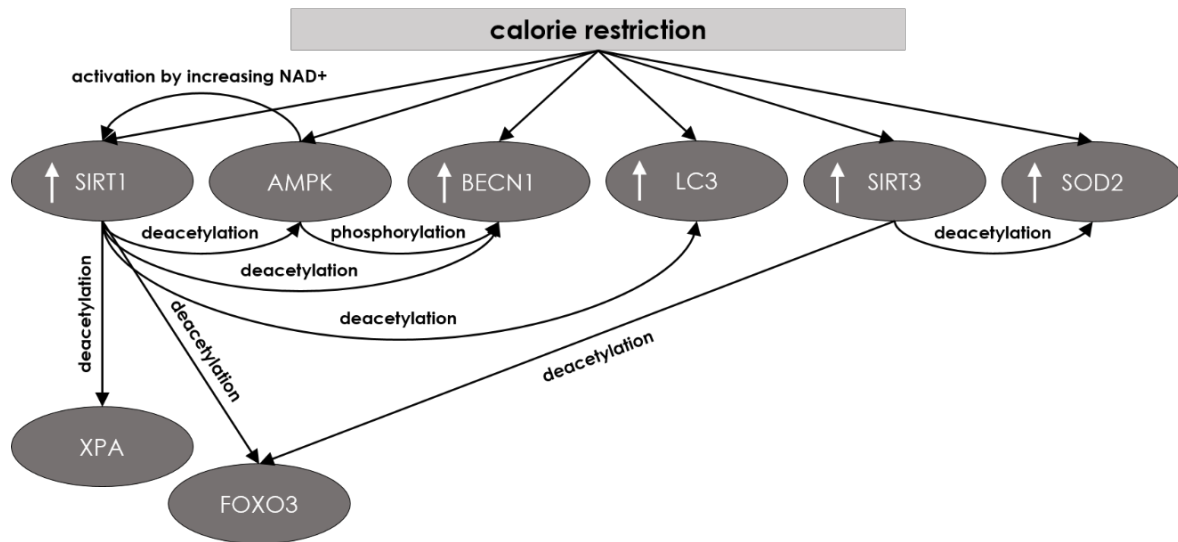
(a) Preparation of plasmid DNA prior to transfection. One part of DsRed-Express-N1 is irradiated with UVC in order to introduce UV-specific DNA damages and serves for assessment of DNA repair. The other part of DsRed-Express-N1 remains undamaged thus serving for the calculation of the correction factor. pEGFP-N1, which serves as transfection control, also remains non-irradiated. (b) Conduction of mHCRA. Measurement of DNA repair capacity requires the transfection of UV-irradiated DsRed-Express-N1 as well as non-irradiated pEGFP-N1. Subsequently the transfected cells express both DsRed and EGFP, in case they are able to repair the DNA damages introduced to the reporter plasmid previously or they only express EGFP, in case they are repair deficient. The calculation of the correction factor, which is used to correct transfection efficiencies, requires a separate transfection of non-irradiated DsRed-Express-N1 and non-irradiated pEGFP-N1. These cells express both fluorescent proteins.

## 1.7 Aging-associated genes

Aging can be defined as an ongoing physiological process that gradually leads to the loss of healthy functions of body and organs. The aging process ends with the death of the organism. So far there is no way to reverse aging. However, there are genes whose expression have a positive influence on the aging process. These genes can be referred to as aging-associated genes. In the context of this work, the focus was on eight potential aging-associated genes and their alteration of expression as a consequence of CR: Sirtuin 1 and 3 (*SIRT1* and 3), *xeroderma pigmentosum*, complementation group A (*XPA*), superoxide dismutase 2 (*SOD2*), forkhead box



O3 (*FOXO3*), AMP-activated protein kinase (*AMPK*), microtubule associated protein 1 light chain 3 beta (*LC3B*) and Beclin-1 (*BECN1*). **Figure 4** displays these genes, their connections to each other and their relation to CR. More detailed information about the genes can be found in chapters 1.7.1 - 1.7.8.



**Figure 4: Aging-associated genes, their interactions and their link to CR.**

CR induces gene expression of *SIRT1* and *SIRT3*, *BECN1*, *LC3B* and *SOD2*. *SIRT1* can deacetylate XPA, FOXO3, AMPK, Beclin-1 and LC3. AMPK can phosphorylate Beclin-1. *SIRT3* is able to deacetylate SOD2. Furthermore, *SIRT1* and AMPK activate each other bidirectionally. *SIRT1* deacetylates AMPK, Beclin-1, LC3, FOXO3 and XPA. ↑ indicates an induction of mRNA expression as a consequence of CR.

### 1.7.1 Sirtuin 1 – *SIRT1*

Sirtuins are highly conserved nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases. In mammals the sirtuin family consists of seven isoforms. The silent mating type information regulation 2 homolog 1 (*SIRT1*) is the best characterized sirtuin, which also shows the highest homology with the predominant sirtuin Sir2 of yeast (Frye, 1999). Moreover, *SIRT1* is the predominant sirtuin mainly localized in the nucleus and to a smaller extent in the cytosol. It is commonly accepted to be an important epigenetic regulator involved in a wide variety of cellular processes. A few examples are the modulation of chromatin structure by deacetylation of lysine residues in histones, alteration of gene expression, mediation of deacetylation of the tumor suppressor p53 and subsequent protection of cells from senescence, deacetylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) and thus modulation of glucose metabolism, regulation of Forkhead box O (FOXO) transcription factors or inflammation (Michan and Sinclair, 2007). Furthermore, a close

interaction of SIRT1 and the metabolic sensor AMP-activated protein kinase (AMPK) has been documented. SIRT1 activates AMPK by deacetylating AMPK kinase LKB1 (Lan *et al.*, 2008) and AMPK, in turn, activates SIRT1 presumably by indirectly increasing NAD<sup>+</sup> levels (Cantó *et al.*, 2009).

The association of SIRT1 with aging and longevity is illustrated by the example of *Saccharomyces cerevisiae*. An overexpression of *Sir2* leads to a significant extension of the yeasts' lifespan, whereas a deletion reduces lifespan (Kaeberlein *et al.*, 1999). A way to activate *Sir2* and prolong the replicative lifespan of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* is CR (Lin *et al.*, 2000; Rogina and Helfand, 2004; Wang and Tissenbaum, 2006). Since sirtuins are highly conserved, it is not surprising that they also play a role in the effects of CR in mammals. An increased SIRT1 level contributes to health-promoting effects of caloric restriction, e.g. postponing the onset of aging-associated diseases like cancer or diabetes (Cohen *et al.*, 2004; Herranz *et al.*, 2010). SIRT1 expression is also assumed to play a protective role against neurodegenerative diseases like Alzheimer's or Parkinson's disease (Wang *et al.*, 2013; Singh *et al.*, 2017). Additionally, SIRT1 plays a role in the regulation of autophagy by deacetylating autophagy-related components (Lee *et al.*, 2008). mRNA expression of *SIRT1* can be induced by CR in human blood cells, as Crujeiras and colleagues showed by conducting a 8 week CR study (Crujeiras *et al.*, 2008). Furthermore, the CR mimetic resveratrol also induces *SIRT1* expression in human monocytes (Tsuchiya *et al.*, 2017). As described above, the repair of DNA damages and thus the preservation of genome integrity is crucial for a healthy aging process. SIRT1 also plays a major role in the DNA pathway of BER, NER and the repair of DSBs (Lagunas-Rangel, 2019). Due to the relevance for the present work, the role of SIRT1 in NER is worth emphasizing. XPA, the main damage recognition sensor of the GGR subpathway of NER, can be deacetylated by SIRT1 and thus promote DNA repair (Fan and Luo, 2010).

### 1.7.2 *Xeroderma pigmentosum*, complementation group A – XPA

Since XPA plays an important role in the repair of DNA damages and thus helps to maintain the integrity of the genome, it can be described as an aging-associated gene. Amongst others, genomic instability is thought to be a hallmark of aging (López-Otín *et al.*, 2013). Consequently, maintaining genome stability, to which XPA contributes, is crucial for the healthy aging

process. XPA can be deacetylated by SIRT1, which identifies SIRT1 as a regulator of DNA damage repair carried out by NER (Jarrett *et al.*, 2018). Up to now, XPA mRNA expression has not been researched in context of CR in humans. However, due to the close association with SIRT1, its expression may also be affected by CR.

### 1.7.3 Sirtuin 3 – SIRT3

The mitochondrial-localized silent mating type information regulation 2 homolog 3 (SIRT3) is associated with an accelerated aging process and aging-associated diseases like for example cardio-vascular diseases or insulin resistance (Hafner *et al.*, 2010; Lantier *et al.*, 2015). Furthermore, SIRT3 mRNA expression is decreased in human breast tumors leading to the hypothesis that SIRT3 is able to act as a tumor suppressor by preserving the integrity of mitochondria (Kim *et al.*, 2010). The accumulation of oxidative stress is believed to contribute greatly to the aging process (Balaban *et al.*, 2005). As a consequence of CR, SIRT3 contributes to reducing oxidative stress by inducing superoxide dismutase 2 (SOD2) via deacetylation and thus contributes to delaying the aging process (Qiu *et al.*, 2010). In addition, Wegman *et al.* reported a mild induction of both SIRT3 and SOD2 mRNA expression in humans due to intermittent fasting (Wegman *et al.*, 2015). The stimulation of SIRT3 gene expression could thus also be a promising way to promote healthy aging.

### 1.7.4 Superoxide dismutase 2 – SOD2

Due to its antioxidant properties SOD2 can also be considered as aging-associated gene. The basic task of SOD2 is the regulation of the redox-status of cells by converting harmful superoxide ( $O_2^-$ ) into hydrogen peroxide ( $H_2O_2$ ). In muscle tissue of rats a 36-week CR led to significantly increased SOD2 mRNA expression levels compared to the control group (Sreekumar *et al.*, 2002). As mentioned above, SOD2 expression can be induced by intermittent fasting in humans (Wegman *et al.*, 2015). Again, the influence of CR on SOD2 in humans is poorly understood, but the hypothesis is, that an induction of SOD2 supports antioxidant defense mechanisms and thus supports a healthy aging process.

### 1.7.5 Forkhead box O3 – FOXO3

The transcription factor FOXO3 regulates a multitude of aging-associated cellular processes like autophagy, apoptosis, DNA repair or defense mechanisms against oxidative stress, particularly when cells are exposed to stress (Morris *et al.*, 2015). A detailed description of all processes would be beyond this thesis, therefore focus is on the relation of FOXO3 to CR and healthy aging. FOXO3 is clearly associated with aging, as confirmed by the positive influence of a human FOXO3 homologue on lifespan and metabolism of *Caenorhabditis elegans* (Lin *et al.*, 1997). Beyond that, FOXO3 polymorphisms have been suggested to be associated with aging in humans (Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). Furthermore, it triggers the process of autophagy in mice (Mammucari *et al.*, 2007). A decrease in autophagy, in turn, has shown to be associated with increasing age (Uddin *et al.*, 2012). In response to oxidative stress, FOXO3 is deacetylated by SIRT1, which reduces apoptosis, leads to cell cycle arrest and pushes cellular reactions towards stress resistance and repair (Brunet *et al.*, 2004). Additionally, SIRT3 can also deacetylate FOXO3 and thus lead to an increased protection of mitochondria of oxidative stress (Tseng *et al.*, 2013). Since FOXO3 is playing an important role in stress response, an indirect influence of nutrient stress in form of CR is possible.

### 1.7.6 AMP-activated protein kinase - AMPK

The metabolic sensor AMP-activated protein kinase (AMPK) is composed of a catalytic subunit  $\alpha$  and two regulatory subunits  $\beta$  and  $\gamma$ , which together form a heterotrimeric complex. Homologues have been confirmed in a variety of species ranging from mammals to *Drosophila melanogaster* (Carling, 2004). Its physiological role is the regulation of energy balance by promoting catabolic pathways and simultaneously switching off biosynthetic and other adenosine triphosphate (ATP) consuming pathways when AMP:ATP levels in the cell increase. AMPK plays major roles in reprogramming metabolism and cell growth, but also in autophagy. It can be activated by different types of stress like nutrient scarcity or extended exercise, but also by pharmaceutical agents like metformin or the CRM resveratrol. (Mihaylova and Shaw, 2011) As mentioned above, an increased AMP:ATP level activates AMPK, which finally leads to an increase of NAD<sup>+</sup> (Wang *et al.*, 2017). This, in turn, stimulates SIRT1. An activation of SIRT1 leads to an activation of AMPK. Taken together, AMPK and SIRT1 can be activated by each other bidirectionally. (Hou *et al.*, 2008) Moreover, AMPK can activate autophagy by phosphorylation of Beclin-1, which is an important regulator of autophagy (Kim

*et al.*, 2013). By phosphorylating and thus suppressing the mTOR complex 1 subunit raptor, AMPK is involved in the regulation of the important nutrient sensor mTOR (Gwinn *et al.*, 2008). Due to its involvement in metabolic pathways, it is likely that CR has an influence on AMPK. Considering the positive influence of CR on aging processes, AMPK can be referred to as an aging-associated gene.

### 1.7.7 Microtubule Associated Protein 1 Light Chain 3 Beta - LC3B

The highly conserved process of autophagy is an essential recycling process for maintaining cellular homeostasis by lysosomal degradation of old or damaged organelles, protein aggregates or pathogens. The best characterized protein family, which is involved in autophagy in mammals, is the microtubule associated protein 1 light chain 3 (LC3). In humans, the LC3 gene family consists of LC3A, -B and -C. (Schaaf *et al.*, 2016) LC3 exists in two forms, whereby LC3-I is located in the cytoplasm and LC3-II in membranes of autophagosomes. LC3 is described as one of the key regulators of autophagy, since it is involved in the control of the formation of autophagic membranes and the fusion of autophagosomes with lysosomes. (Kabeya *et al.*, 2000) However, LC3 is commonly considered as a marker for autophagy (Weidberg *et al.*, 2010). In humans, a significantly increased LC3 gene and protein expression could be demonstrated in individuals, who participated in long-term CR (Yang *et al.*, 2016). As a consequence of nutrient depletion, SIRT1 has been proposed to be involved in autophagosome formation by deacetylating nuclear LC3 and enabling its transport to the cytoplasm, where autophagosome formation takes place (Huang *et al.*, 2015). Autophagy is decreasing with increasing age (Uddin *et al.*, 2012). Consequently, an induction of autophagy may counteract the aging process.

### 1.7.8 Beclin-1 - BECN1

Another gene of interest in context of autophagy is *BECN1*, which is the mammalian orthologue of the autophagy-related gene 6 (*Atg6*) in yeast and whose gene product Beclin-1 mediates initial phases of autophagosome formation (Kihara *et al.*, 2001). The involvement of Beclin-1 in aging-associated diseases like cancer or neurodegenerative diseases is researched intensively. Mutations in *BECN1* or an altered expression have been associated with different types of cancer, for example breast cancer (Li *et al.*, 2010). Furthermore, acetylation of Beclin-1 inhibits autophagosome maturation and thus promotes tumor growth, whereas SIRT1 can

deacetylate Beclin-1 (Sun *et al.*, 2015). In turn, malfunctions or deficiencies of Beclin-1 are associated with neurodegenerative diseases like Alzheimer's (Pickford *et al.*, 2008). An association of Beclin-1 with aging is demonstrated, for example, by a decreased expression in aged human brains (Shibata *et al.*, 2006). An induction of Beclin-1 protein expression in humans can be achieved by CR, as demonstrated by Yang *et al.* in 2016 (Yang *et al.*, 2016). Additionally, nutrient deprivation for 48 h resulted in significantly increased mRNA expression of *BECN1* in human epithelial cells (Pan *et al.*, 2019). Apart from the studies mentioned above, few studies researching the influence of CR on *BECN1* mRNA expression in humans have been published. Due to its association with aging and aging-associated diseases, a better understanding of the link between *BECN1* and CR might lead to additional ways to support healthy aging.

## 1.8 Aims of this work

Life expectancy of people continues to increase and with that, incidences of aging-associated diseases also increase. However, people not only want an increased lifespan, but they want to age as healthy as possible. One way to support healthy aging is CR. The influence of CR on the human organism and the aging process still harbors many questions. Crucial for postponing or preventing many aging-associated diseases are intact DNA repair mechanisms. In order to be able to research the influence of CR on DNA repair, a reliable assay to determine DNA repair is required. Furthermore, the expression of aging-associated genes can contribute to a better understanding of the underlying mechanisms of the influence of CR on aging in humans. Lastly, CRMs may provide a possibility to support or boost the positive effects of CR in humans.

This thesis firstly aims at contributing to a better understanding of the impact of CR on human DNA repair and, in a broader sense, on the mechanisms of aging. The second aim was to provide first insights whether CRMs (spermidine and resveratrol) are able to influence aging-associated genes similarly to CR. Lastly, first insights whether spermidine influences DNA repair capacity should be gained. To achieve this, the following objectives were set:

- further advancing mHCRA in order to be able to study NER in human PBMCs as *ex vivo* studies
- researching the influence of CR on NER capacity of human PBMCs *ex vivo*
- analyzing the influence of CR on mRNA expression of aging-associated genes
- gaining first insights whether *ex vivo* treatments with CRMs can stimulate mRNA expression of human PBMCs similarly to CR
- conducting first experiments in order to research the influence of spermidine on NER capacity in human fibroblasts

## 2 Materials and Methods

### 2.1 Materials

**Table 1:** Chemicals and Reagents

Chemical / Reagent	
Dimethyl sulfoxide (DMSO) $\geq 99.5\%$ , Bioscience Grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
di-Sodium hydrogen phosphate heptahydrate ( $\text{Na}_2\text{HPO}_4 - 7 \text{H}_2\text{O}$ ) $\geq 98\%$ , p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dulbecco's Modified Eagle's Medium (DMEM), high glucose	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Ethanol ( $\text{C}_2\text{H}_6\text{O}$ ), ROTIPURAN® $\geq 99.8\%$ , p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Fetal bovine serum (FBS)	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Gentamicin 10 mg/ml	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Ham's F12 nutrient mix	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Kanamycin sulfate, $\geq 750$ I.U./mg	Sigma-Aldrich Chemie GmbH, Munich, Germany
Potassium chloride (KCl) $\geq 99.5\%$ , p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) $\geq 99\%$ , p.a., ACS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Resveratrol $\geq 99\%$	Sigma-Aldrich Chemie GmbH, Munich, Germany
RPMI 1640 without phenol red	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Sodium acetate trihydrate ( $\text{NaCH}_3\text{COO} - 3 \text{H}_2\text{O}$ ) Ph. Eur.	Riedel-de Haën AG, (now Honeywell Specialty Chemicals Seelze GmbH), Seelze, Germany
Sodium chloride ( $\text{NaCl}$ ) $\geq 99.5\%$ , p.a., ACS, ISO	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Spermidine $\geq 98\%$	Sigma-Aldrich Chemie GmbH, Munich, Germany
Tryptone/ peptone ex casein, pancreaticaly digested, granulated, for microbiology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany



Trypsin/EDTA (0.5 %), 10 x	Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany
Water (H <sub>2</sub> O), PCR Grade	Roche Diagnostics GmbH, Mannheim, Germany
Yeast extract, powdered, for bacteriology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

**Table 2:** Buffer and media

Buffer / Medium	Composition / manufacturer
Cryo medium	90 % FBS 10 % DMSO
Fibroblast medium	DMEM, high glucose 10 % FBS 50 µg/ml Gentamicin (all Gibco® by Life Technologies)
LB medium, 1 l	5 g yeast extract 10 g NaCl 10 g tryptone/ peptone (all Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in dH <sub>2</sub> O, autoclave solution add Kanamycin sulfate, final concentration 50 µg/ml
PBMC medium	RPMI 1640 without phenol red 20 % FBS (both Gibco® by Life Technologies)
Phosphate buffered saline (PBS), 10 x 500 ml	40 g NaCl 1 g KCl 13.4 g Na <sub>2</sub> HPO <sub>4</sub> – 7 H <sub>2</sub> O 1.2 g KH <sub>2</sub> PO <sub>4</sub> (all Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in ultrapure water before usage dilute solution 1:10 in ultrapure water and autoclave solution
Physiological saline solution	0.9 % NaCl (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in ultrapure water sterile-filter solution before usage (0.2 µm filter)

Sodium acetate, 3 M 100 ml	40.82 g NaCH <sub>3</sub> COO – 3 H <sub>2</sub> O in 100 ml dH <sub>2</sub> O
Stopping solution	90 % 1x PBS 10 % FBS

**Table 3:** Laboratory equipment

Description	Manufacturer
-86 °C freezer HERAFreeze™ HFU 600	Fisher Scientific GmbH, Schwerte, Germany
BD FACSCalibur™ Flow Cytometry System with 488 nm laser	Becton, Dickinson and Company, Franklin Lakes, USA
BD FACSVerser™ Flow Cytometry System with 488 nm, 405 nm and 640 nm laser	Becton, Dickinson and Company, Franklin Lakes, USA
Centrifuges Centrifuge 5804 R with swinging bucket rotor Centrifuge 5425 Centrifuge 5417 R	Eppendorf AG, Hamburg, Germany
CO <sub>2</sub> Incubator Hera cell 150	Kendro Laboratory Products GmbH, Langenselbold, Germany
Gene Pulser II with Capacitance extender plus and Pulse controller plus	Bio-Rad Laboratories, Inc., Hercules, CA, USA
Laminar flow clean bench BDK-SK 1800	BDK Luft- und Reinraumtechnik GmbH, Sonnenbühl, Germany
LightCycler® 480 qPCR platform	Roche Diagnostics GmbH, Mannheim, Germany
Luna-FL™ automated cell counter	Logos Biosystems, Inc., purchased via Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Microscopes Axiovert 25 AxioObserver Z1 fluorescence microscope	Carl Zeiss GmbH, Oberkochen, Germany
NanoPhotometer®	Implen GmbH, Munich, Germany
Neubauer counting chamber improved	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Pipette aids Pipetbboy acu 2	INTEGRA Biosciences GmbH, Biebertal, Germany

Pipettes, Eppendorf Research® Plus 10 µL, 20 µL, 200 µL, 1,000 µL	Eppendorf AG, Hamburg, Germany
QIAvac 24 Plus vacuum manifold with QIAvac Connecting system and vacuum pump	QIAGEN GmbH, Hilden, Germany
Shaking incubator	GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany
Stratalinker® UV Crosslinker Model 1800	Stratagene, La Jolla, CA, USA
Thermal cycler Biometra TRIO	Analytik Jena AG, Jena, Germany
Ultrapure water unit Pureflex 3	ELGA LabWater, High Wycombe, UK
Vortex mixer Vortex-Genie® 2	Scientific Industries, Inc., Bohemia, NY, USA
Water bath	GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany

Table 4: Consumables

Description	Manufacturer
0.4 cm electroporation cuvettes	Molecular BioProducts, Inc., purchased via Thermo Fisher Scientific GmbH, Karlsruhe, Germany
Cell culture material, sterile 6-well plates, Cell+ Cell culture dish 100 mm Cell culture dish 150 mm, Cell+	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Centrifuge tubes 15 ml and 50 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
CryoPure tube 1.8 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
FACS tubes 5 ml and 0.5 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Leucosep™, with porous barrier, 50 ml, pre filled with Leucosep™ separation medium	Greiner Bio-One GmbH, Frickenhausen, Germany
Multiply®-Pro cup 0.2 ml, Polypropylene	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Petri dishes	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Pipette tips 10 µl, 20 µl, 200 µl, 1,000 µl	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Pipette tips with filter 10 µl, 20 µl, 200 µl, 1,000 µl	SARSTEDT AG & Co. KG, Nuembrecht, Germany

SafeSeal tube 1.5 ml and 2 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Safety-Multifly®-needle 21G with multi adapter	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Serological pipettes 5 ml, 10 ml and 25 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
S-Monovette® K3 EDTA 4.9 ml, 7.5 ml and 9 ml	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Sterile filter Filtropur S 0.2 µm	SARSTEDT AG & Co. KG, Nuembrecht, Germany
Syringe Norm Ject® 10 ml and 20 ml	Henke-Sass, Wolf GmbH, Tuttlingen, Germany
LightCycler® 480 Multiwell Plates 96, white	Roche Diagnostics Deutschland GmbH, Mannheim, Germany
LightCycler® 480 Sealing Foil	Roche Diagnostics Deutschland GmbH, Mannheim, Germany

**Table 5:** Plasmids for DNA repair capacity measurements

Plasmid	Manufacturer
pEGFP-N1	Clontech Laboratories Inc., Mountain View, CA, USA
pDsRed-Express-N1 (GFP cassette of pEGFP-N1 was replaced with the DsRed cassette of pDsRed-Express)	

**Table 6:** Kits and ready-to-use mixes

Kit	Manufacturer
High Pure RNA Isolation Kit	Roche Diagnostics GmbH, Mannheim, Germany
LightCycler® 480 SYBR Green I Master	Roche Diagnostics GmbH, Mannheim, Germany
QIAGEN Plasmid <i>Plus</i> Giga Kit	Qiagen GmbH, Hilden, Germany
Transcriptor First Strand cDNA Synthesis Kit	Roche Diagnostics GmbH, Mannheim, Germany

**Table 7:** Primers for gene expression analyses

<b>Gene</b>	<b>Sequence forward primer 5'-3'</b>	<b>Sequence reverse primer 5'-3'</b>
<i>SIRT1</i>	gcagattagtagggcgttg	tctggcatgtcccactatca
<i>SIRT3</i>	catgagctgcagtgactggt	gagcttgccgttcaactagg
<i>FOXO3</i>	gcaagcacagagttgatga	caggtcgtccatgaggtttt
<i>SOD2</i>	caccagcactagcagcatgt	ggtgacgttcagggtgtca
<i>XPA</i>	gcagcccaagataattga	tggcaaatcaaagtgggtca
<i>AMPK<math>\alpha</math></i>	aaagtcggcgtctgttcaa	tgggtgagccacaactgtt
<i>LC3B</i>	ggtgagaagcagcttctgt	agattggtgtggagacgctg
<i>BECN1</i>	ggctgagagactggatcagg	ctgtccactgtccagatgt
<i>RPLP0</i>	cccgagaagacctctttt	agaagggggagatgttgagc
<i>G3PDH</i>	cgaccacttgtcaagctca	aggggtctacatggcaactg
<i><math>\beta</math>-Actin</i>	ggacttcgagcaagagatgg	agcactgtgttggcgtacag
<i>GUSB</i>	tcagaggaagtgatgcaga	agcagattctaggtgggacg

**Table 8:** Fibroblast cell lines

<b>Designation</b>	<b>Passage</b>	<b>Age</b>	<b>Gender</b>
F130522 Ast72	4-6	8 years	male
F131122 K25	4-9	63 years	male

**Table 9:** Test persons participating in F.X. Mayr therapy

	<b>Test person</b>	<b>Age</b>	<b>Gender</b>
First study	1	59	w
	2	58	w
	3	52	w
	4	54	m
	5	59	w
	6	47	m
	7	49	w
	8	48	w
Second study	1	60	w
	2	48	m
	3	44	w
	4	47	w
	5	53	w
	6	60	w
	7	53	w
	8	55	m
	9	55	w
	10	50	m
	11	48	m
	12	48	m
	13	38	w
	14	59	w
	15	49	w
	16	63	w
	17	25	w
	18	32	w

**Table 10:** Software

<b>Software</b>	<b>Publisher</b>
BD CellQuest Pro™ software , version 4.0.2	Becton, Dickinson and Company, Franklin Lakes, USA
BD FACSuite™ software, version 1.0.6	Becton, Dickinson and Company, Franklin Lakes, USA
Citavi 6	Swiss Academic Software GmbH, Waedenswil, Switzerland
GraphPad Prism for Windows, Version 7.04	GraphPad Software, San Diego, USA
Microsoft Office Professional Plus 2013	Microsoft Corporation, Redmond (Washington), USA

## 2.2 Methods

### 2.2.1 Isolation of plasmid DNA

Both plasmids, pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA) as well as a modified pDsRed-Express-N1 (replacement of the GFP cassette of pEGFP-N1 with the DsRed cassette of pDsRed-Express) were augmented in *Escherichia coli* K12. Prior to plasmid DNA isolation, cryopreserved *Escherichia coli* K12 containing either pEGFP-N1 or pDsRed-Express-N1 were streaked onto kanamycin containing agar plates and incubated at 37 °C over night. Preculture was prepared as follows. 20 ml of kanamycin containing LB medium were inoculated with *Escherichia coli* K12 from the agar plate grown over night. Preculture was incubated at 37 °C and 130 rpm for 4 h under aerobic conditions. When reaching an optical density at 600 nm ( $OD_{600}$ ) between 0.5 and 0.6, 0.6 ml the preculture were transferred into 300 ml of kanamycin containing LB medium and incubated at 37 °C and 130 rpm for approximately 14 h under aerobic conditions. When reaching an  $OD_{600}$  between 0.6 and 1.0, *Escherichia coli* K12 solution was centrifuged ( $6,000 \times g$ , 7 min and 4 °C) in order to harvest bacteria. All pellets were stored at -20 °C until further use. Purification of plasmid DNA was conducted with Plasmid *Plus* Giga Kit (Qiagen GmbH, Hilden, Germany) and the vacuum manifold QIAvac 24 Plus (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. In order to increase the yield of plasmid DNA, columns were incubates 5 min at room temperature (instead of 1 min as recommended in the manufacturer's instructions). Plasmid DNA was eluted in EB buffer (Qiagen GmbH, Hilden, Germany) and concentration of the DNA solution was determined by NanoPhotometer® (Implen GmbH, Munich, Germany) at 260 nm.

### 2.2.2 Preparation of plasmid DNA for the modified HCRA

mHCRA requires the damage of one of the plasmids used. With the purpose of introducing UV-specific damages to the plasmid DNA, pDsRed-Express-N1 (1422 ng/ $\mu$ l, 900 ng/ $\mu$ l or 778 ng/ $\mu$ l) was irradiated with UVC (254 nm) using a Stratalinker® UV Crosslinker (Model 1800, Stratagene, La Jolla, CA, USA). 40  $\mu$ l drops of plasmid solution were exposed to 5 kJ/m<sup>2</sup> of UVC in a petri dish without lid so that the plastic of the lid does not shield the plasmid solution and the shortwave UVC reaches and damages the plasmid DNA. Subsequently pEGFP-N1 and pDsRed-Express-N1 (either irradiated or not irradiated) were mixed in a ratio

of 1:4 (10 µg pEGFP-N1 and 30 µg pDsRed-Express-N1 per transfection) for transfection of PBMCs and stored at -86 °C until further use. This procedure ensured the use of one plasmid mixture for all studies involving the F.X. Mayr test persons conducted during this thesis. Prior to the transfection of human fibroblasts, the same irradiation protocol was used for pDsRed-Express-N1 (900 ng/µl). After irradiation pDsRed-Express-N1 was stored at 4 °C until further use. No plasmid mixture was prepared for the transfection of fibroblasts.

### 2.2.3 Isolation of PBMCs

Human venous blood was withdrawn using K-EDTA Monovettes® (Sarstedt AG & Co., Nuembrecht, Germany). 12 ml anticoagulated blood were used to isolate PBMCs for the mHCRA and parallel gene expression analyses. *Ex vivo* treatments with CRMs were conducted using 9 ml K-EDTA blood. In both cases PBMCs were isolated using Leucosep™ tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). Anticoagulated blood was diluted 1:2 with physiological saline solution and transferred into Leucosep™ tubes. After centrifugation at 1,000 x g for 10 min in a swinging bucket rotor with brakes switched off, the PBMC-containing layer was transferred into a new centrifuge tube. PBMCs were subsequently washed with 10 ml 1 x PBS and centrifuged at 250 x g for 10 min. Washing of the cells was repeated twice using 5 ml 1 x PBS. In the last step, PBMCs were collected in 5 ml 1 x PBS and cell count was determined using either Neubauer-improved counting chamber (mHCRA and simultaneously gene expression analyses) or by using Luna-FL™ automated cell counter (Logos Biosystems, Inc., purchased via Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for the CRM experiments. CRM treatment as well as the mHCRA were carried out immediately after isolation of the PBMCs. For the analyses of mRNA expression during CR PBMCs were pelletized and stored frozen (-86 °C) until further use.

### 2.2.4 Establishing mHCRA for an application with human PBMCs

The basis for the transfection of human PBMCs was a Bachelor's thesis conducted by M. Seid (Seid, 2013), which resulted in the following protocol.  $2 \times 10^6$  PBMCs were resuspended 250 µl of Ham's F10 medium containing 40 % EB buffer (Qiagen GmbH, Hilden, Germany) and 20 - 40 µg plasmid DNA (in this case pEGFP-N1 only). This cell suspension was transferred into 0.4 cm electroporation cuvettes, electroporation was carried out using a voltage of 320 V and a capacitance of 750 µF. After applying of the pulse, the cell suspension was immediately



transferred into a 6-well plate prefilled with 3 ml RPMI medium containing 20 % FBS and FACS analysis was carried after an incubation at 37 °C and 5 % CO<sub>2</sub> for 24 h. (Seid, 2013) In order to optimize this procedure within the course of the thesis presented here, freshly isolated human PBMCs were transfected by electroporation also only with pEGFP-N1. Thereby different settings were tested regarding transfection efficiency. 20 µg of pEGFP-N1 were used in each case as well as 2x10<sup>6</sup> PBMCs and a total volume of 250 µl. Ham's F10 medium, Ham's F12 medium or RPMI medium without phenol red (all Gibco® by Life Technologies) and a final concentration of 24 % to 40 % EB buffer (Qiagen GmbH, Hilden, Germany) were tested. Voltages were set from 200 V to 500 V, capacitance was set between 500 µF and 1000 µF and combinations of different voltages and capacitances were used. Once transfection with only pEGFP-N1 was optimized, mHCRA was conducted in order to optimize the setting for this assay, too. Thereby 10 µg pEGFP-N1 in combination with 30 µg pDsRed-Express-N1 (either irradiated or non-irradiated) was used. Again, Ham's F10, Ham's F12 or RPMI medium without phenol red (all Gibco® by Life Technologies) and a final concentration of 15 % to 40 % EB buffer (Qiagen GmbH, Hilden, Germany) were tested in a total volume of 250 µl containing 2x10<sup>6</sup> PBMCs. Voltages were set from 320 V to 500 V, capacitance was set between 500 µF and 1000 µF and combinations of different voltages and capacitances were used.

Results of preliminary mHCRA experiments using different concentrations of pDsRed-Express-N1 gave rise to the assumption that the concentration of plasmid DNA solution influences DNA repair capacity. In order to verify this assumption, mHCRA was conducted using a pDsRed-Express-N1 solution with a concentration of 1422 ng/µl as well as a 1:2 dilution (778 ng/µl) of this solution in EB buffer (Qiagen GmbH, Hilden, Germany). UVC irradiation and preparation of plasmid mixes was carried out for both concentrations as described in chapter 2.2.2. mHCRA was subsequently conducted three times according to chapter 2.2.5 for both plasmid mixes using human PBMCs. Experiments were conducted as a part of M. Weber's bachelor thesis (Weber, 2015). Statistical analyses were carried out as unpaired t-tests with Welch's correction.

### 2.2.5 Modified HCRA for transfecting human PBMCs

The previously described steps during establishing and optimizing mHCRA for an application with human PBMCs resulted in the following final protocol. Transfection of human PBMCs

was carried out by electroporation (400 kV and 500  $\mu$ F) and 0.4 cm electroporation cuvettes (Molecular BioProducts, Inc., purchased via Thermo Fisher Scientific GmbH, Dreieich, Germany). In brief,  $2 \times 10^6$  freshly isolated PBMCs were resuspended in 150  $\mu$ l RPMI 1640 without phenol red (Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany) and mixed with 100  $\mu$ l of EB buffer (Qiagen GmbH, Hilden, Germany), which contained the plasmid solutions described in chapter 2.2.2 (10  $\mu$ g pEGFP-N1 and 30  $\mu$ g pDsRed-Express-N1), resulting in a total volume of 250  $\mu$ l. Subsequently electroporation was carried out using Gene Pulser II electroporator (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Afterwards, cell solution was transferred into 6-well cell culture dish (Sarstedt AG & Co., Nuembrecht, Germany) containing 3 ml PBMC medium and incubated at 37 °C and 5 % CO<sub>2</sub> for 24 h in an incubator. All experiments were carried out in triplicates using the mixture of plasmids consisting of UVC-irradiated pDsRed-Express-N1 and pEGFP-N1 for the determination of DNA repair capacity and the plasmid mixture of non-irradiated pDsRed-Express-N1 and pEGFP-N1 for calculation of the correction factor F (correction of DNA repair capacity with regard to transfection efficacy).

24 h after electroporation analysis of the transfected PBMCs was carried out using BD FACSCalibur™ flow cytometer and BD CellQuest Pro™ software (both Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Following formula allows for the calculation of DNA repair capacity:

$$DNA\ repair\ capacity\ [\%] = \frac{\text{percentage red fluorescent cells irradiated}}{\text{percentage green fluorescent cells}} * F * 100$$

and

$$F = \frac{\text{percentage green fluorescent cells}}{\text{percentage red fluorescent cells non - irradiated}}$$

The protocol for carrying out the mHCRA using human PBMCs, which was established within the scope of the present work, was published in the peer-reviewed protocol journal Bio-protocol (Matt and Bergemann, 2019).

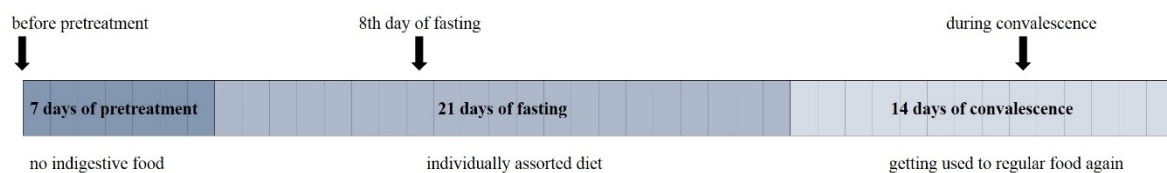
### 2.2.6 Reproducibility studies

During the adaptation of mHCRA to an application with human PBMCs, reproducibility studies were carried out to ensure the reliability of this newly adjusted assay. Four apparently healthy donors aged between 32 and 54 years were included into the first of these studies. For this purpose PBMCs were isolated out of 12 ml of venous blood withdrawn using K-EDTA Monovettes® (Sarstedt AG & Co., Nuembrecht, Germany) as described in 2.2.3 and mHCRA was conducted according to chapter 2.2.5 with FACS analysis after 24 h. This procedure was carried out on three consecutive days at the same time of day using one batch of pDsRed-Express-N1 and pEGFP-N1 plasmids as described in chapter 2.2.2. Subsequently, DNA repair capacities measured on the three days were compared to each other. In order to make a statement about reproducibility, DNA repair capacities of one donor were compared to each other in each case. Reproducibility studies were repeated independently two more times throughout ten months using the blood of two donors aged 32 and 35 years. In summary, three independent tests for reproducibility were conducted for two of the donors. These experiments were conducted using three different batches of pDsRed-Express-N1 and pEGFP-N1 plasmids. Statistical analyses for all reproducibility studies were conducted as two-way ANOVA with Tukey's multiple comparisons test.

### 2.2.7 Calorie restriction

Test persons aged between 25 and 63 years participated in a F.X. Mayr therapy within the center for Traditional Chinese Medicine (TCM) in Sigmaringen. This 6-week intervention consisted of one week of pretreatment, in which test persons were not allowed to eat indigestive food, followed by three weeks of diet food intake and a two-week convalescence, in which test persons re-adjust to regular food. The three weeks of diet food traditionally consist of stale bread rolls and milk. However, in this case a diet consisting of eupeptic food like spelt buns, sheep yogurt, potatoes, root vegetables, protein-containing side-dishes and omega-3 fatty acids was provided. Essential for a successful intervention is that the participants pay special attention to chewing and stop to eat when the feeling of satiety first occurs. For each of the individuals the diet is individually assembled. During these six weeks participants lost an average of 5 kg of their initial body weight, ranging between 3 kg and 8 kg due to the reduced calorie intake. For this reason F.X. Mayr therapy constitutes a CR for humans.

In the first study of the influence of CR on humans eight individuals (six female and two male individuals) were included, the second study consisted of 18 test persons (13 female and five male test persons). Venous blood was withdrawn at the center for Traditional Chinese Medicine (TCM) in Sigmaringen using K-EDTA Monovettes® (Sarstedt AG & Co., Nuembrecht, Germany) before pretreatment started, on the eighth day of diet food intake as well as during convalescence (on the ninth day on average). PBMCs were isolated according to chapter 2.2.3 and mHCRA was conducted as described in 2.2.5. Within the scope of the first study, the influence of CR on DNA repair capacity was analyzed. In context of the second study, DNA repair capacity as well as the influence of CR on the expression of aging-associated genes were researched. Following **Figure 5** shows the schematic illustration of the timing of blood withdrawal during F.X. Mayr therapy.



**Figure 5: Timing of blood withdrawal during F.X. Mayr therapy.**

Sampling of venous blood was carried out before the seven days of pretreatment, on the eighth of 21 days of fasting and during the 14 days of convalescence (on the ninth day on average).

Depending of their initial DNA repair capacity, test persons were grouped into the upper 50 % and the lower 50 % of individuals for statistical analyses. During establishment of mHCRA for the usage of human PBMCs, most of the test persons displayed NER capacity values between 85 % and 90 %. For this reason, the group containing the upper 50 % of test persons were defined as the “normal pre-existing DNA repair” group, whereas the lower 50 % of test persons were defined as the “low pre-existing DNA repair capacity” group. Statistical analyses were conducted as two-way ANOVA with Tukey’s multiple comparisons test for analyses of DNA repair capacity, as repeated measure one-way ANOVA with Dunnett’s multiple comparisons test for grouped analyses, as two-way ANOVA with Sidiak’s multiple comparisons test for comparing gender and as linear regression analyses for researching the correlation of DNA repair capacity and age of the test persons as well as for researching the correlation of mRNA expression of aging-associated genes. Furthermore, Pearson’s correlation coefficient was determined.

### 2.2.8 Treatment of PBMCs with CRMs

In order to research the influence of the two CRMs spermidine and resveratrol, freshly isolated PBMCs were incubated with the two substances in different concentrations as well as for different exposure times. Prior to the treatment with CRMs, PBMCs were seeded in 6-well cell culture dishes (Sarstedt AG & Co., Nuembrecht, Germany) containing 3 ml PBMC medium.  $3 \times 10^6$  PBMCs were used for each concentration as well as for each exposure time. In addition,  $3 \times 10^6$  PBMCs were left untreated as control sample for each exposure time. Dissolving resveratrol in DMSO required a solvent control consisting of  $3 \times 10^6$  PBMCs and 40  $\mu$ l of DMSO (the volume used for the highest concentration of resveratrol) for each exposure time. Since spermidine could be resolved in water, this was not necessary for this CRM. Following **Table 11** displays the concentrations and exposure times of the CRMs.

**Table 11:** Concentration and exposure times of CRMs

CRM	exposure time [h]	concentration [ $\mu$ M]
Resveratrol	24	0
		75
		100
		DMSO only
	48	0
		75
		100
		DMSO only
	72	0
		75
		100
		DMSO only
Spermidine	24	0
		10
		20
		30
	48	0
		10
		20
		30
	72	0
		10
		20
		30

PBMCs were kept in an incubator at 37 °C and 5 % CO<sub>2</sub> with renewal of cell culture medium and corresponding concentrations of the CRM every 24 h. For this purpose, PBMCs were centrifuged at 250 x g for 10 min at room temperature and suspended in new PBMC medium and the correct concentration of the CRM. After the incubation times displayed in **Table 11**, PBMCs were collected by centrifugation (250 x g, 10 min and room temperature) and RNA was isolated directly as described in 2.2.9. All experiments described in this section were carried out as two independent approaches. Statistical analyses for the CRM treatments were conducted as two-way ANOVA with Dunnet's multiple comparisons test.

### 2.2.9 RNA extraction and synthesis of cDNA

Total RNA was isolated from 10<sup>6</sup> PBMCs for the study of the influence of CR on mRNA expression and from 3x10<sup>6</sup> PBMCs for the CRM studies. High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used in both cases. cDNA was amplified using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) and random hexamer primers (CR specimens) or random hexamer and anchored-oligo(dT)<sub>18</sub> primers for CRM samples. The amplification of cDNA was carried out with the maximum volume of RNA possible, which were 11 µl for the amplification with random hexamer primers and 10 µl for the amplification with random hexamer and anchored-oligo(dT)<sub>18</sub> primers. Both kits were used as proposed by the manufacturer.

### 2.2.10 Precipitation of cDNA

Prior to qPCR analyses, ethanol precipitation of cDNA was performed as follows. In the first step, 2 µl of 3 M sodium acetate and 60 µl pre-chilled (-86 °C) 99.8 % ethanol were added to each 0.2 ml PCR tube (Sarstedt AG & Co., Nuembrecht, Germany) containing cDNA. The solution was incubated at -86 °C for 30 min and subsequently centrifuged at 15,000 x g for 15 min at 0 °C. The cDNA pellet should then be easily recognizable. In the following, the supernatant was removed, 100 µl of 75 % ethanol were added and the tubes were centrifuged at 15,000 x g for 5 min at 0 °C. The supernatant was again removed and the washing and centrifugation were repeated. After the last removal of the supernatant, pellets were dried under the laminar flow clean bench. The dried pellets were then dissolved in 100 µl PCR-grade water (Roche Diagnostics GmbH, Mannheim, Germany). cDNAs were then stored at -86 °C until qPCR was performed.

### 2.2.11 Primer design

Primers for the aging-associated genes *SIRT1*, *XPA*, *SIRT3*, *FOXO3*, *SOD2*, *AMPK $\alpha$* , *LC3B* and *BECN1* as well as for the housekeeping genes  *$\beta$ -actin*, *G3PDH* and *GUSB* were designed by using mRNA sequences from NCBI-database (<https://www.ncbi.nlm.nih.gov/gene>). By alignment of the individual splice variants, a common sequence was determined and suitable primers were selected. For primer design the program Primer3 plus web interface was used. Primers for the housekeeping gene *RPLP0* were used as designed by Gebhard *et al.* (Gebhard *et al.*, 2014). All primers were purchased via biomers.net GmbH (biomers.net GmbH, Ulm, Germany). The table containing primer sequences can be found in chapter 2.1, **Table 7**.

### 2.2.12 qPCR analyses

Gene expression analyses were conducted on a LightCycler® 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany). PCR reactions contained 1  $\mu$ L (0.5 pmol/ $\mu$ L) of each primer (biomers.net GmbH, Ulm, Germany), 10  $\mu$ L SYBR Green I Master and 8  $\mu$ L of cDNA. For no template controls 8  $\mu$ L PCR-grade water (Roche Diagnostics GmbH, Mannheim, Germany) were used instead of cDNA. qPCR of each sample was carried out in triplicates. 45 cycles of the following PCR program were performed: 5 min pre-incubation (95 °C), followed by 95 °C for 10 sec, 62 °C for 10 sec and 72 °C for 10 sec. Fluorescence measurements were conducted at the end of the elongation phase. Melting curve analysis after 45 cycles completed the PCR program.

Target genes for the CR samples were *SIRT1*, *XPA*, *SIRT3*, *SOD2*, *FOXO3*, *AMPK $\alpha$* , *LC3B* and *BECN1*. For normalization of the target genes the housekeeping genes *RPLP0*, *G3PDH*,  *$\beta$ -Actin* and *GUSB* were used. Genes of interest for the CRM samples were *SIRT1*, *SIRT3*, *FOXO3* and *SOD2*. These genes were normalized to the housekeeping genes *RPLP0*, *G3PDH* and  *$\beta$ -Actin*. In both cases, the  $2^{-\Delta\Delta CP}$  method was used to calculate n-fold mRNA expression. In order to take a closer look at the basal mRNA expression only compared to the housekeeping genes,  $\Delta CP$  values of *SIRT1* and *XPA* mRNA expression were calculated and plotted separately. This procedure allows for the detection of changes in the basal level of mRNA expression. Sequences of the primers used for gene expression analyses are shown in **Table 7**.

### 2.2.13 Treatment of human fibroblasts with spermidine and subsequent mHCRA

In order to research the influence of the CRM spermidine on DNA repair capacity of human fibroblasts a 24 h spermidine treatment of the cells was conducted. Afterwards mHCRA was performed by electroporation of the treated fibroblasts. To assure that a sufficient number of fibroblasts was available for three independent experiments, fibroblasts of the two donors Ast72 and K25 (see **Table 8**) were expanded in 20 ml fibroblast medium in T175 Cell+ cell culture flasks (Sarstedt AG & Co., Nuembrecht, Germany) until an adequate number of cells per donor could be cryopreserved. Sub-cultivation took place at a confluency of approximately 70 % by detaching of the cells from the cell culture flasks using 3 ml 1x trypsin/EDTA (1:10 dilution of 10 x stock; Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany) and collected by adding 17 ml stopping solution (10 % FBS in 1x PBS).  $5 \times 10^6$  fibroblasts each were suspended in 1 ml cryo medium (90 % FBS and 10 % DMSO), transferred into CryoPure tubes (Sarstedt AG & Co., Nuembrecht, Germany) and stored at  $-86^\circ\text{C}$  until further use.

Three days prior to the spermidine treatment,  $5 \times 10^6$  fibroblasts were thawed and seeded into three T175 Cell+ cell culture flasks (Sarstedt AG & Co., Nuembrecht, Germany) containing 20 ml fibroblast medium. On the day of the spermidine treatment  $3 \times 10^6$  fibroblasts were seeded into a 150 cm<sup>2</sup> cell culture dish (Sarstedt AG & Co., Nuembrecht, Germany) and 20 ml of fibroblast medium containing the different spermidine concentrations. Two dishes were treated with 10  $\mu\text{M}$  spermidine, two with 20  $\mu\text{M}$  spermidine and two dishes remained untreated to serve as control. All approaches were kept at 37 C and 5 % CO<sub>2</sub> for 24 h in an incubator. After 24 h the fibroblasts were washed with 20 ml 1 x PBS, detached from the cell culture dishes using 3 ml 1 x trypsin/EDTA (1:10 dilution of 10 x stock; Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany) and collected by adding 17 ml stopping solution (10 % FBS in 1x PBS). After centrifugation (500 x g, 3 min) and removal of the supernatant, each cell pellet was resuspended in 600  $\mu\text{l}$  Ham's F12 medium (Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany) in order to form a triplicate. mHCRA requires the transfection of cells with UVC-irradiated pDsRed-Express-N1 (15  $\mu\text{g}$ ) and pEGFP-N1 (5  $\mu\text{g}$ ) for the determination of DNA repair capacity and the transfection of cells with non-irradiated pDsRed-Express-N1 (15  $\mu\text{g}$ ) and pEGFP-N1 (5  $\mu\text{g}$ ) in a second test approach for calculation of the correction factor F (correction



of DNA repair capacity with regard to transfection efficacy). Therefore one triplicate of a spermidine treated cells was transfected with the irradiated pDsRed-Express-N1 and pEGFP-N1 and the other triplicate was transfected with non-irradiated pDsRed-Express-N1 and pEGFP-N1. After adding the corresponding amount of plasmid DNA to the cell suspension, the approach was distributed in three 0.4 cm electroporation cuvettes (Molecular BioProducts, Inc., purchased via Thermo Fisher Scientific GmbH, Dreieich, Germany), so that each cuvette contained  $10^6$  fibroblasts. Following the electroporation (320 kV and 500  $\mu$ F), each approach was seeded into a 6-well cell culture dish (Sarstedt AG & Co., Nuembrecht, Germany) containing 3 ml fibroblast medium and incubated at 37 °C and 5 % CO<sub>2</sub> for 24 h in an incubator. 24 h after the electroporation the cells were detached from the cell culture dishes using 0.5 ml 1 x trypsin/EDTA (1:10 dilution of 10 x stock; Gibco® by Life Technologies, purchased via Fisher Scientific GmbH, Schwerte, Germany) and collected by adding 1 ml stopping solution. Subsequently, FACS analysis was performed using BD FACSVerser™ flow cytometer and BD FACSuite™ software (both Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Calculation of DNA repair capacity was conducted as described in chapter 2.2.5.

#### **2.2.14 Ethical standards**

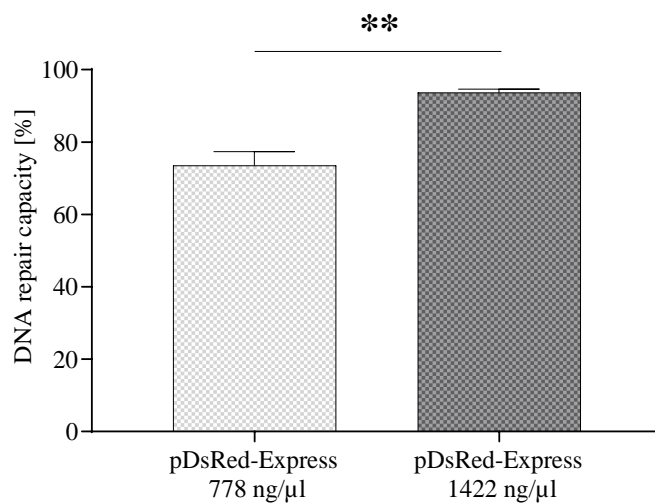
All experiments conducted during the preparation of this thesis were performed in accordance with the declaration of Helsinki and approved by the ethics committee of the Landesärztekammer Baden-Württemberg. This includes experiments conducted using human PBMCs and human fibroblasts. All individuals, who voluntarily provided a skin sample for the isolation of fibroblasts, voluntarily provided blood for the experiments or participated in a CR were informed in advance and gave their written consent to the use of the skin sample or before the drawing of the blood samples. All information on the subjects taking part in F.X. Mayr therapy or consenting to the use of their skin sample were pseudonymized.

### 3 Results

#### 3.1 Establishment of mHCRA for an application with human PBMCs

Transfection of human PBMCs was firstly established within the scope of M. Seid's Bachelor thesis (Seid, 2013). Further optimization was conducted within the scope of this thesis, as described in chapter 2.2.4. Highest transfection efficiencies were achieved by using  $2 \times 10^6$  PBMCs in a total volume of 250  $\mu\text{l}$ , 20  $\mu\text{g}$  pEGFP-N1, a total concentration of 40 % EB buffer, RPMI w/o phenol red, 400 V and 500  $\mu\text{F}$ . By applying these settings, a transfection efficiency of 28.8 % up to 40.5 % could be obtained. mHCRA performs best when using  $2 \times 10^6$  PBMCs in a total volume of 250  $\mu\text{l}$ , 40 % EB buffer, RPMI w/o phenol red, 400 V and 500  $\mu\text{F}$ . Furthermore 10  $\mu\text{g}$  pEGFP-N1 and 30  $\mu\text{g}$  pDsRed-Express-N1 (either irradiated or non-irradiated) are required. By applying these settings, average transfection efficiencies of 21.5 % (SEM = 0.8 %) for the transfections with irradiated pDsRed-Express-N1 and pEGFP-N1 and average transfection efficiencies of 35.3 % (SEM = 1.4 %) for the transfections with non-irradiated pDsRed-Express-N1 and pEGFP-N1 were obtained. The final protocol for mHCRA was published in the peer-reviewed protocol journal Bio-protocol (Matt and Bergemann, 2019).

In order to research the influence of plasmid DNA concentration on DNA repair capacity of NER, mHCRA was conducted using human PBMCs and two different concentrations of pDsRed-Express-N1. Plasmid solutions of a concentration of 1422 ng/ $\mu\text{l}$  (the original concentration of the isolated plasmid DNA) as well as 778 ng/ $\mu\text{l}$  (a 1:2 dilution of the originally isolated plasmid DNA solution) were used. Results are displayed in **Figure 6**. Transfection with the 778 ng/ $\mu\text{l}$  pDsRed-Express-N1 resulted in a DNA repair capacity of 73.5 % ( $\pm 2.22$  %), whereas conducting mHCRA using the 1422 ng/ $\mu\text{l}$  plasmid DNA solution resulted in a DNA repair capacity of 93.72 % ( $\pm 0.50$  %). Comparing the two results, a significantly higher DNA repair capacity results from transfection of human PBMCs with the 1422 ng/ $\mu\text{l}$  plasmid DNA solution compared to the 778 ng/ $\mu\text{l}$  pDsRed-Express-N1 solution (\*\* $p = 0.0091$ ; unpaired t-test with Welch's correction;  $n = 3$ ). Experiments were conducted within the scope of M. Weber's bachelor thesis (Weber, 2015).

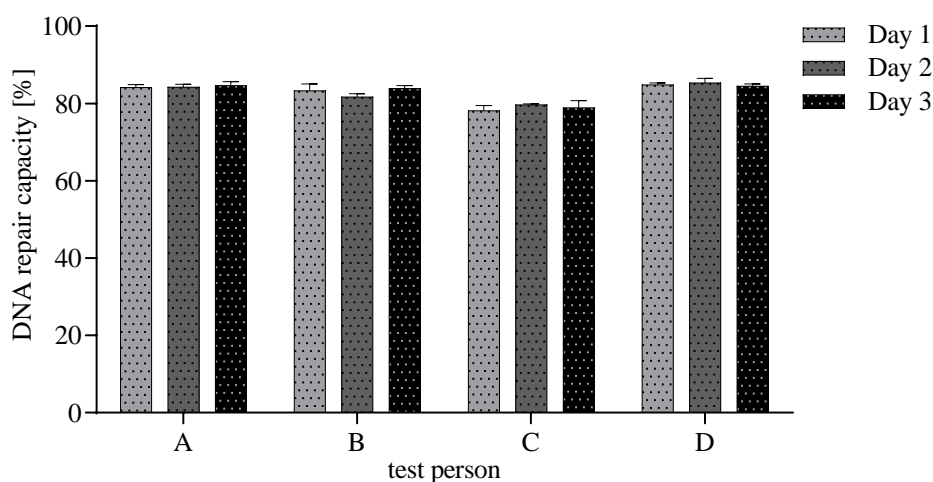


**Figure 6: Concentration of plasmid DNA influences DNA repair capacity.**

Depending on the concentration of the plasmid solution, DNA repair capacity varies significantly (\*\* $p = 0.0091$ ; unpaired t-test with Welch's correction;  $n = 3$ ). Results were obtained in context of M. Weber's bachelor thesis (Weber, 2015).

### 3.2 Reproducibility of mHCRA

Before conducting DNA repair capacity measurements within the scope of a study involving test persons taking part in any kind of intervention, mHCRA as it has been established was tested for its reproducibility. For this purpose, NER capacity of four test persons was determined by conducting mHCRA on three consecutive days as triplicates. All transfections were carried out using the same plasmid mixture to guarantee comparability. The graphic depiction of these experiments is shown in **Figure 7**. Parts of these reproducibility studies were published in Matt *et al.* 2016 (Matt *et al.*, 2016). None of the test persons showed significant differences regarding DNA repair capacity on the three days. Among the four test persons, person A showed the most constant DNA repair capacity. Furthermore, DNA repair capacities are slightly different in the four individuals, with test person C displaying the lowest and test person D displaying the highest DNA repair capacity.



**Figure 7: Reproducibility of mHCRA – four test persons over three consecutive days.**

DNA repair capacity does not change significantly throughout three consecutive days in any of the four test persons. The level of DNA repair capacity, however, varies from one test person to another, with test person C displaying the lowest and test person D displaying the highest NER capacity. Data shown represent the mean of triplicates. Statistical analyses were conducted as two-way ANOVA with Tukey's multiple comparisons test.

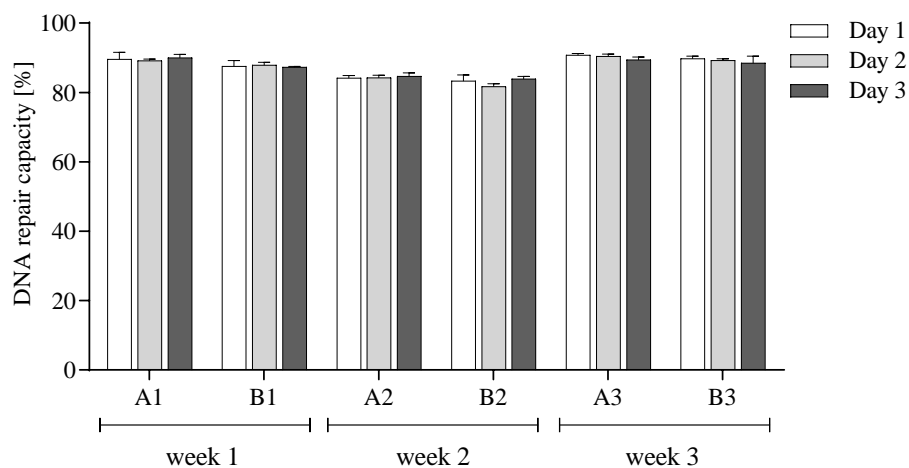
Percentages of DNA repair capacity measurements as well as statistical analyses of this reproducibility study are shown in **Table 12**. Statistical analyses were carried out as two-way ANOVA with Tukey's multiple comparisons test. *p* values confirm that there are no significant differences regarding DNA repair capacities between the three days of reproducibility tests in any of the test persons.

**Table 12:** Three days reproducibility study involving four test persons.

		DNA repair capacity [%]	SEM [%]	<i>p</i> values		
				Day 1 - Day 2	Day 2 - Day 3	Day 1 - Day 3
Test person A	Day 1	84.19	0.67			
	Day 2	84.26	0.70	0.9995	0.9775	0.9706
	Day 3	84.69	0.94			
Test person B	Day 1	83.38	1.70			
	Day 2	81.72	0.80	0.7266	0.5632	0.9572
	Day 3	83.98	0.67			
Test person C	Day 1	78.17	1.28			
	Day 2	79.69	0.21	0.7641	0.9321	0.9345
	Day 3	78.92	1.78			
Test person D	Day 1	84.86	0.41			
	Day 2	85.40	1.13	0.9650	0.9136	0.9873
	Day 3	84.53	0.53			

mHCRA was tested for reproducibility two more times within a period of ten months, using PBMCs isolated from anticoagulated blood of test person A and B. In summary, three day reproducibility was carried out three time for test person A and B. Results of these reproducibility studies are presented in **Figure 8** and were published in Matt *et al.* 2016 (Matt *et al.*, 2016). For every three-day reproducibility study, a different batch of isolated plasmid DNA and resulting plasmid mixture was used.

DNA repair capacity measurements showed a high reproducibility for both donors, however, NER capacity varied slightly more in test person B compared to test person A. Furthermore, test person B displayed lower DNA repair capacity values than test person A throughout all reproducibility studies.



**Figure 8: Reproducibility of mHCRA – two test persons over three consecutive days, conducted three times.**

Both test persons show a high reproducibility regarding DNA repair capacity measurements. No significant differences can be seen for both individuals. However, percentages of DNA repair vary slightly more for test person B than for test person A. Furthermore, test person A displays higher DNA repair capacity levels than test person B. Results shown represent the means of triplicates with SEM. Statistical analyses were carried out as two-way ANOVA with Tukey's multiple comparisons test.

For the second reproducibility study, statistical analyses were conducted as two-way ANOVA with Tukey's multiple comparisons test. Resulting percentages of DNA repair capacity measurements as well as statistical analyses of this study are shown in **Table 13**. Displayed  $p$  values show that there are no significant changes in NER capacity between the three days in the period of ten months. Furthermore, mHCRA measurements using PBMCs of test person A show more constant DNA repair capacity values throughout the three consecutive days than mHCRA measurements using PBMCs of test person B.

**Table 13:** Three days reproducibility study of mHCRA.

PBMCs of two test persons were investigated regarding reproducibility of mHCRA on three consecutive days three times throughout ten months.

		DNA repair capacity [%]	SEM [%]	<i>p</i> values			
				Day 1 - Day 2	Day 2 - Day 3	Day 1 - Day 3	
Test person A	Study 1	Day 1	89.65	1.95			
		Day 2	89.18	0.47	0.8820	0.6372	0.8975
		Day 3	90.08	0.87			
	Study 2	Day 1	84.19	0.67			
		Day 2	84.26	0.70	0.9977	0.8974	0.8678
		Day 3	84.69	0.94			
	Study 3	Day 1	90.80	0.48			
		Day 2	90.02	0.36	0.9424	0.5740	0.3955
		Day 3	89.47	0.78			
Test person B	Study 1	Day 1	87.63	1.59			
		Day 2	87.98	0.75	0.9339	0.7994	0.9545
		Day 3	87.35	0.16			
	Study 2	Day 1	83.38	1.70			
		Day 2	81.72	0.80	0.2511	0.0984	0.8132
		Day 3	83.98	0.67			
	Study 3	Day 1	89.79	0.71			
		Day 2	89.27	0.44	0.8550	0.7214	0.4164
		Day 3	88.50	2.00			

### 3.3 DNA repair capacity and calorie restriction – first study

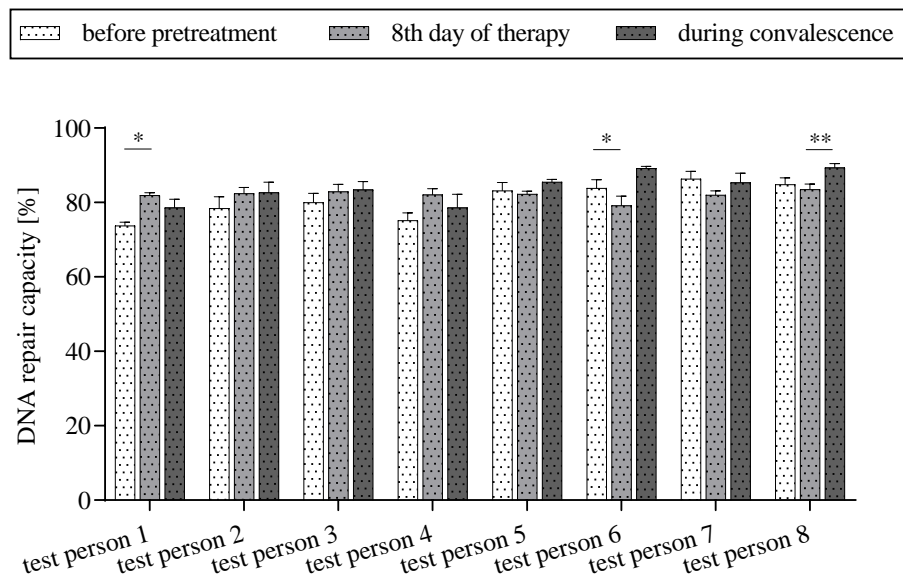
Since there is a strong presumption that CR influences DNA repair positively, but few studies involving humans exist, mHCRA was used to measure DNA repair capacity of different test persons within the scope of this thesis. As described before, mHCRA is a suitable and reliable method to assess NER capacity in humans. The advantage of this assay is, that the ability of a cell to repair DNA damages is measured by the functional reactivation of the previously damaged reporter gene. The induction of the damage on the plasmid DNA is carried out outside the cell and thus the damaging event takes place spatially separated from the DNA repair. Individuals voluntarily took part in a F.X. Mayr therapy, which represents a kind of CR for humans due to the average loss of body weight of 5 kg. A detailed description of F.X. Mayr therapy can be found in chapter 2.2.7. Within the scope of the first study, DNA repair capacity of eight test persons was determined three times throughout the six-week procedure of F.X. Mayr therapy. The first time of measurement was before the beginning of pretreatment, the second on the eighth day of fasting and the last one during convalescence. Results of this study were published in Matt *et al.* 2016 (Matt *et al.*, 2016).

#### 3.3.1 Influence of calorie restriction on DNA repair capacity

The previously established mHCRA was applied to research the influence of CR on human DNA repair capacity. As shown in **Figure 9**, the eight individual test persons display differing values of NER capacity before pretreatment, on the eighth day of therapy as well as during convalescence. Three test persons show a significant alteration of DNA repair capacity during the calorie restrictive period. DNA repair capacity of test person 1 increases significantly on the eighth day of fasting (\*  $p = 0.0278$ ). However, DNA repair capacity of test person 6 decreases significantly on the eighth day (\*  $p = 0.0212$ ), whereas DNA repair capacity of test person 8 increases significantly during convalescence compared to the eighth day of fasting (\*\* $p = 0.0047$ ). All other subjects show no significant alteration of DNA repair capacity throughout CR. Test persons 2 and 3 demonstrate a continuous moderate increase in DNA repair capacity, whereas test persons 5 and 7 show a slight decline on the eighth day of therapy, followed by an increase of NER capacity during convalescence. Test person 4, however, displays an increase in DNA repair capacity on the eighth day and a subsequent decrease during convalescence. Furthermore, only DNA repair capacity of test person 7 is lowest during convalescence, all other test persons' DNA repair capacities are higher in the



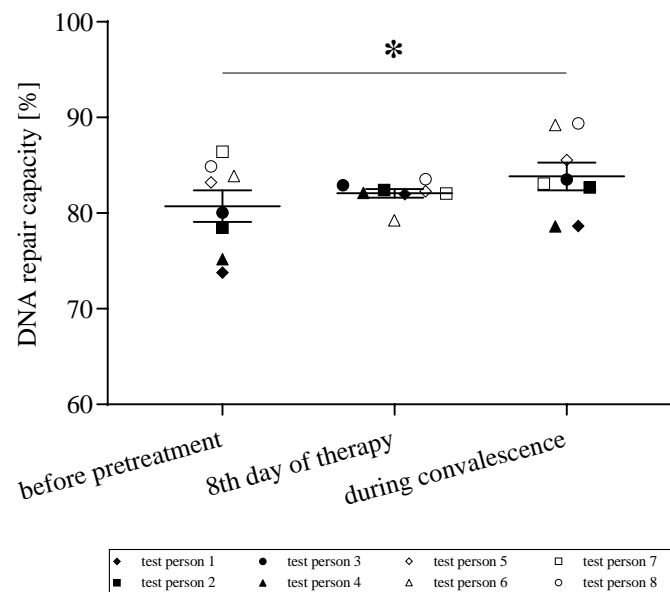
end of the study than before pretreatment. Statistical analyses were conducted as one-way ANOVA with subsequent Tukey's multiple comparisons test.



**Figure 9: DNA repair capacity of human PBMCs changes during calorie restriction.**

Eight test persons underwent a period of CR in form of F.X. Mayr therapy, resulting in different alterations of DNA repair capacity. Three of the eight test persons display significant changes in NER capacity. DNA repair capacity of test person 1 increases significantly on the eighth day of therapy (\*  $p = 0.0278$ ), DNA repair capacity of test person 6 decreases significantly on the eighth day of fasting (\*  $p = 0.0212$ ) compared to the measurement before pretreatment. Finally, DNA repair capacity of test person 8 increases significantly from the eighth day of fasting to the assessment during convalescence (\*\*  $p = 0.0047$ ). Presented data are means of triplicates and corresponding SEM. Statistical analyses were carried out as one-way ANOVA with Tukey's multiple comparisons test.

Since the test persons react differently to CR, grouped analysis was performed in order to point out the influence of CR on the study cohort. Results of this statistical analysis, which was carried out as repeated measure one-way ANOVA with Dunnett's multiple comparisons test, are displayed in **Figure 10**. On the average, individuals show a slight but not significant increase in DNA repair capacity from the first time of assessment to second on the eighth day of therapy. During convalescence, DNA repair is increased significantly compared to the time of assessment before pretreatment (\*  $p = 0.0272$ ).



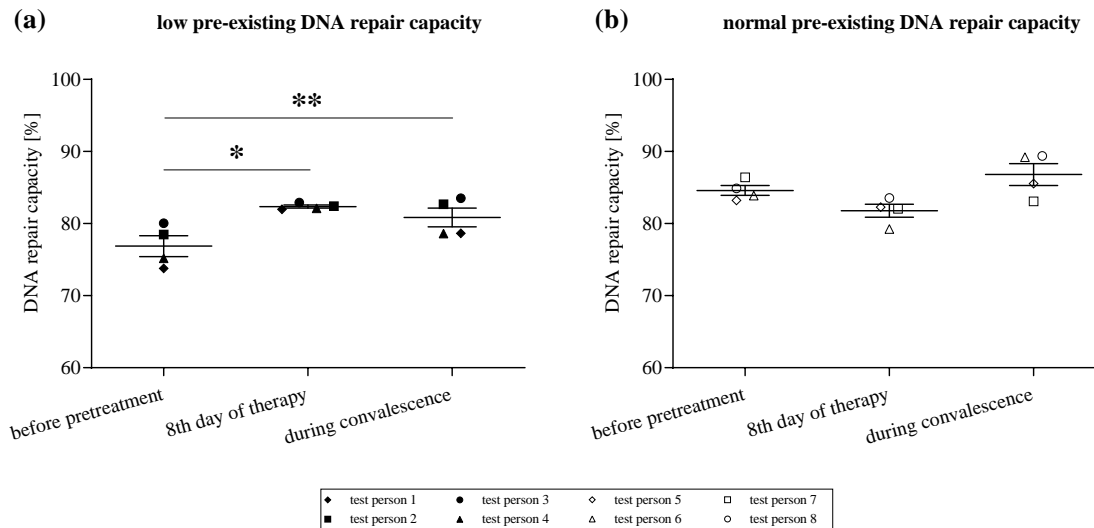
**Figure 10: Increase of DNA repair capacity during calorie restriction.**

On the eighth day of therapy a slight increase in DNA repair capacity can be seen on the average. During convalescence DNA repair capacity increases significantly (\*  $p = 0.0272$ ) compared to the measurement before pretreatment. Statistical analyses were carried out as repeated measure one-way ANOVA with Dunnett's multiple comparisons test.

When looking at the individual levels of DNA repair capacity it is visible that some test persons show a lower NER capacity than other ones in the beginning of the study. Due to that observation, the test persons included in this study were grouped into the lower 50 % and the higher 50 % depending on their pre-existing DNA repair capacity before pretreatment, as described in 2.2.7. The group consisting of the lower 50 % of individuals will hereinafter be referred to as “low pre-existing DNA repair capacity” group, whereas the higher 50 % of individuals will be referred to as “normal pre-existing DNA repair capacity” group. Results are shown in **Figure 11**.

DNA repair capacity of test persons, who display a low DNA repair capacity in the beginning of the study increases significantly on the eighth day of therapy compared to the NER capacity before pretreatment (\*  $p = 0.0355$ ). During convalescence this increase persists, resulting in a significantly higher DNA repair capacity compared to the measurement before pretreatment (\*\*  $p = 0.0022$ ), as shown in **Figure 11 a**. In the group displaying a normal DNA repair capacity in the beginning of the study, a slight decrease of DNA repair capacity can be seen on the

eighth day of therapy followed by a slight increase during convalescence, as presented in **Figure 11 b**. None of the changes in DNA repair capacity are significant in this group. Statistical analyses were conducted as repeated measure one-way ANOVA with Dunnett's multiple comparisons test.



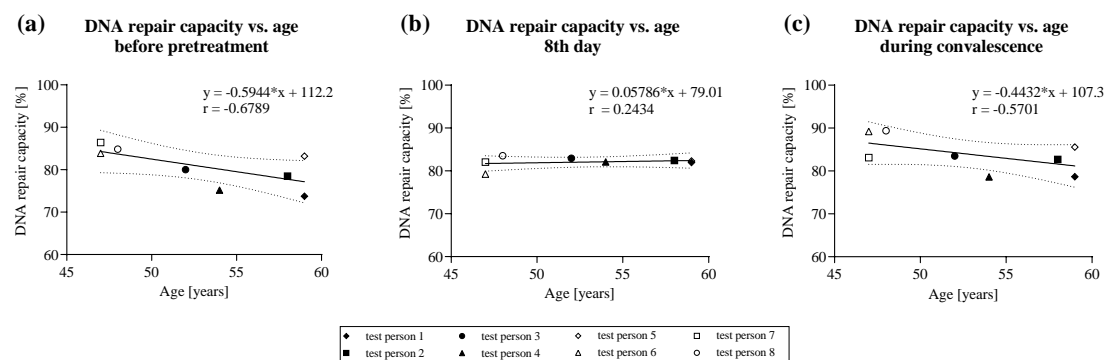
**Figure 11: Differences in DNA repair capacity during calorie restriction in individuals with low vs. normal pre-existing DNA repair capacity.**

(a) Individuals, who display a low DNA repair capacity before pretreatment show a significant increase in NER capacity on the eighth day of therapy ( $* p = 0.0355$ ), as well as during convalescence ( $** p = 0.0022$ ). (b) Test persons belonging to the group displaying a normal pre-existing DNA repair capacity show a slight decrease in NER capacity on the eighth day, followed by a slight increase during convalescence. In either case, the alterations in this group are not significant. Presented data are means of triplicates and corresponding SEM. Statistical analyses were carried out as repeated measure one-way ANOVA with Dunnett's multiple comparisons test.

### 3.3.2 Age and calorie restriction

Within the scope of the first study, individuals, who voluntarily participated in a CR were aged between 47 and 59 years. Since it is assumed that DNA repair capacity declines with increasing age, NER capacity measured via mHCRA and age of the test persons are correlated in the following. Linear regression analyses were performed for researching the correlation between DNA repair capacity and the test person's age. Additionally, Pearson's correlation coefficient was calculated. Interpretation of correlation coefficients was carried out using Mukaka's proposition (Mukaka, 2012). Analyses focusing on the link between age and DNA repair capacity are displayed in **Figure 12**.

Before the beginning of the CR period a negative correlation of DNA repair and age can be observed, as displayed in **Figure 12 a**. Although this correlation is not significant, the Pearson's correlation coefficient ( $r = -0.6789$ ) hints at a moderate negative correlation according to Mukaka (Mukaka, 2012). On the eighth day of therapy, Pearson's correlation coefficient increases to  $r = 0.2434$ , which can be interpreted as a negligible correlation (see **Figure 12 b**). Compared to the measurement before pretreatment DNA repair capacity of elder individuals increases on the eighth day of therapy. As shown in **Figure 12 c**, Pearson's correlation coefficient decreases again during convalescence ( $r = -0.5701$ ), indicating a moderate negative correlation of DNA repair capacity and age of the test persons. Furthermore, a donor variability can be stated regarding NER capacity at different years of age. These findings were published as supplemental material in a combined analysis with the second study in Matt *et al.* 2016 (Matt *et al.*, 2016).



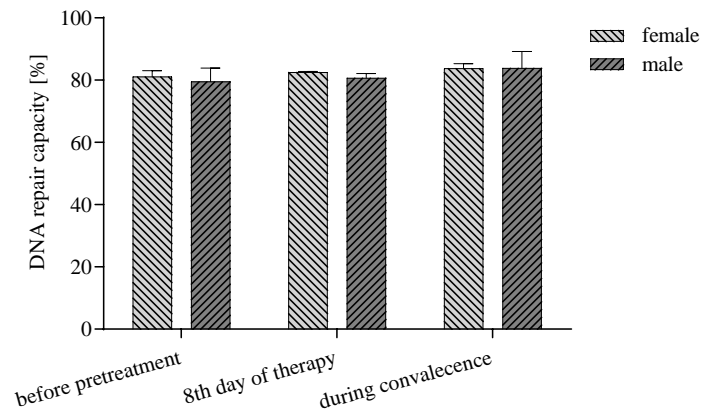
**Figure 12: Correlation of DNA repair capacity and age.**

(a) Before pretreatment a moderate negative correlation of DNA repair capacity and age of the test persons can be observed (Pearson's correlation coefficient  $r = -0.6789$ ). (b) On the second time of assessment, Pearson's correlation coefficient increases to  $r = 0.2434$ , indicating a negligible correlation of NER capacity and age. (c) During convalescence Pearson's correlation coefficient decreases ( $r = -0.5701$ ), which hints at a moderate negative correlation of age and DNA repair capacity.

### 3.3.3 Gender and calorie restriction

After assessing the correlation of DNA repair capacity and age of the test persons, it was investigated whether there is a difference in DNA repair capacity of female and male test persons throughout the study. As mentioned in chapter 2.2.7, the study cohort consists of six female and two male test persons. Results of this analysis are displayed in **Figure 13**. There is no significant difference in DNA repair capacity on all three times of assessment, although male test persons display slightly reduced DNA repair capacities at the first two

measurements compared to the female test persons. However, during convalescence there is no difference in DNA repair capacities of male and female individuals. Statistical analyses were conducted as two-way ANOVA with Sidak's multiple comparisons test.



**Figure 13: DNA repair capacity during calorie restriction – gender differences.**

There is no significant difference between female ( $n = 6$ ) and male ( $n = 2$ ) test persons throughout the study. Male individuals show a slightly lower NER capacity than females before pretreatment and on the eighth day of therapy, whereas DNA repair capacity is the same during convalescence. Statistical evaluation was carried out as two-way ANOVA with Sidak's multiple comparisons test.

### 3.4 DNA repair capacity and calorie restriction – second study

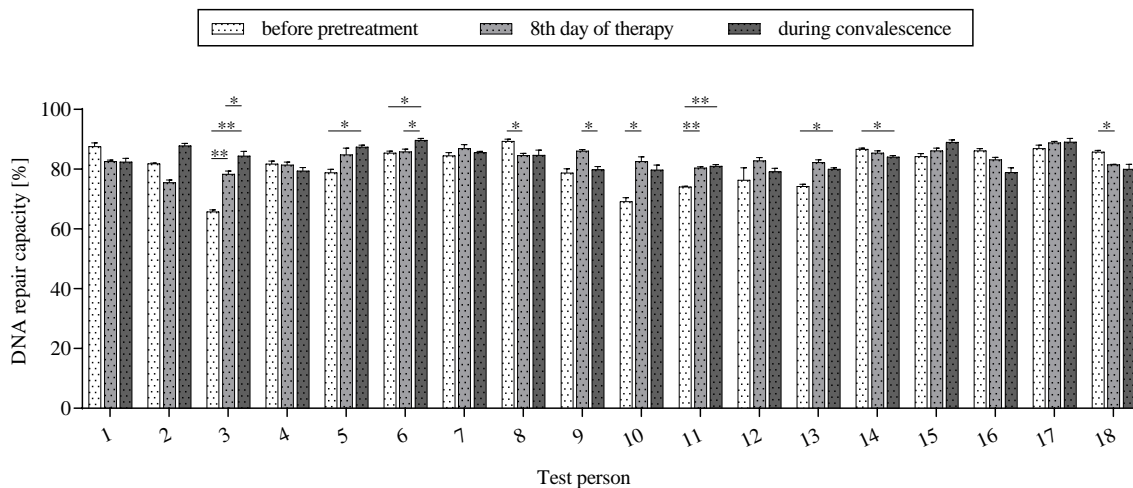
The first study on the influence of CR on DNA repair capacity of human test persons led to promising results, as describe in chapter 3.3. Since only eight test persons could be included in the first study at that time, a second study was conducted one year after the first one. This time 18 individuals were taking part in a CR in form of F.X. Mayr therapy voluntarily. As in the first study, test persons lost an average of 5 kg of their initial body weight. The first time of measurement was once more before the beginning of pretreatment, the second on the eighth day of fasting and the last one during convalescence. In addition to the measurement of NER capacity via mHCRA, gene expression analyses were conducted. Specifically the aging-associated genes *SIRT1*, *XPA*, *SIRT3*, *FOXO3*, *SOD2*, *AMPK $\alpha$* , *LC3B* and *BECN1* were analyzed regarding their expression during CR. Results of mHCRA measurements were published in Matt *et al.* 2016, whereas Matt *et al.* 2020 contains the results of mRNA expression analyses (Matt *et al.*, 2016; Matt *et al.*, 2020).

#### 3.4.1 Influence of calorie restriction on DNA repair capacity

As in the first study, mHCRA was carried out for the second study to measure possible alterations of DNA repair capacity before, during and after a CR in form of F.X. Mayr therapy. It is clearly visible in **Figure 14** that the 18 test persons display varying degrees of altered DNA repair in the time period studied. Statistical analyses for these measurements were carried out as one-way ANOVA with Tukey's multiple comparisons test. Since NER capacity of test person 2 could only be measured in duplicates, no significances can be given here using one-way ANOVA.

Three test persons (test persons 3, 10 and 11) show a significant increase in DNA repair capacity on the eighth day compared to the measurement before pretreatment, whereas two test persons (test persons 8 and 18) display a significant reduction. In turn, twelve test persons (test persons 1, 4, 5, 6, 7, 9, 12, 13, 14, 15, 16 and 17) show no statistically significant change during this period. From the measurement before pretreatment to the one during convalescence, five individuals (test persons 3, 5, 6, 11 and 13) display a significant increase in DNA repair capacity and only test person 14 displays a significant reduction of DNA repair capacity. Eleven test persons (test persons 1, 4, 7, 8, 9, 10, 12, 15, 16, 17 and 18) show no alteration. From the eighth day of therapy to the measurement during convalescence, two test

persons' (test persons 3 and 6) DNA repair capacity increases significantly, whereas test person 9 displays a significantly reduced NER capacity. However, 14 test persons (test persons 1, 4, 5, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17 and 18) show no significant changes in DNA repair capacity from the eighth day to the measurement during convalescence.



**Figure 14: DNA repair capacity of human PBMCs changes during calorie restriction.**

Individuals display varying values of DNA repair capacity throughout the investigation period. Compared to the measurement before pretreatment, NER capacity of three test persons increases significantly on the eighth day of therapy, two test persons show a significant reduction and twelve test persons show no alteration of DNA repair capacity. During convalescence, DNA repair capacity increases significantly in five individuals, compared to the measurement before pretreatment. One individual displays a significantly decreased DNA repair capacity at that time and eleven individuals show no change in NER capacity. Compared to the eighth day, two test persons show a significantly increased DNA repair capacity during convalescence, one test person shows a significant reduction and 14 test persons show no alteration. Presented data are means of triplicates (duplicates for test person 2) and corresponding SEM. Statistical analyses were conducted as one-way ANOVA with Tukey's multiple comparisons test.

Due to the number of significant values, **Table 14** displays according  $p$  values as well as the arrow-marked direction of the change of DNA repair capacity for a better overview. It is striking that in seven test persons (test persons 3, 5, 6, 11, 15, 16 and 17) DNA repair capacity is highest in the end of the study. These test persons display a steady increase in NER capacity throughout the study, whereby only in test persons 15 and 17 no significant increases can be detected. However, six test persons (test persons 1, 4, 8, 14, 16 and 18) display only a decreasing DNA repair capacity. All of these individuals show a lower DNA repair capacity in the end of the study, compared to the measurement before pretreatment, but significant decreases are only obvious for test persons 8, 14 and 18. Furthermore, DNA repair capacity of test person 4 decreases only marginally throughout the study. In test persons 7, 9, 10, 12 and

13 the highest NER capacity is displayed on day eight, but during convalescence DNA repair capacity is still higher than before pretreatment. As mentioned in the beginning of this chapter, no statistical analysis could be conducted for test person 2. Nevertheless, it is important to note that DNA repair capacity of test person 2 first decreases on the eighth day and then increases during convalescence to a higher level than in the beginning of the study.

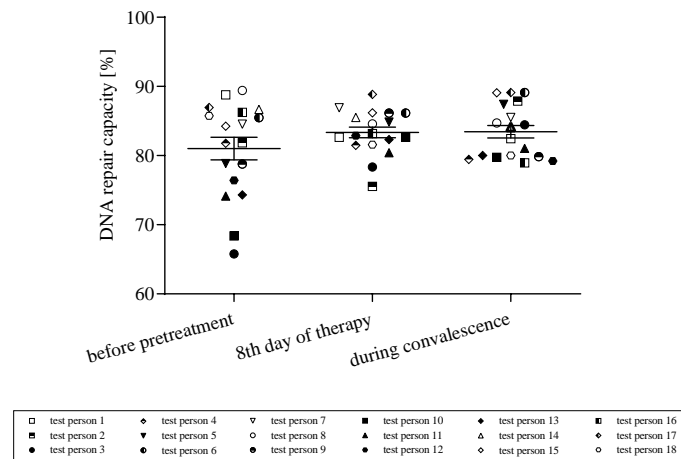
**Table 14:** Presentation of the test persons' individual *p* values (one-way ANOVA with Tukey's multiple comparisons test) of DNA repair capacity alterations.

test person	before pretreatment → 8th day of therapy	before pretreatment → during convalescence	8th day of therapy → during convalescence
1	n. s.	n. s.	n. s.
2	-	-	-
3	** 0.0038 ↑	** 0.0046 ↑	* 0.0101 ↑
4	n. s.	n. s.	n. s.
5	n. s.	* 0.0395 ↑	n. s.
6	n. s.	* 0.0172 ↑	* 0.0132 ↑
7	n. s.	n. s.	n. s.
8	* 0.0216 ↓	n. s.	n. s.
9	n. s.	n. s.	* 0.0277 ↓
10	* 0.0473 ↑	n. s.	n. s.
11	** 0.0025 ↑	** 0.0052 ↑	n. s.
12	n. s.	n. s.	n. s.
13	n. s.	* 0.0290 ↑	n. s.
14	n. s.	* 0.0433 ↓	n. s.
15	n. s.	n. s.	n. s.
16	n. s.	n. s.	n. s.
17	n. s.	n. s.	n. s.
18	* 0.0317 ↓	n. s.	n. s.

As already pointed out for the first study on the influence of CR on human DNA repair capacity (see chapter 3.3.1), individual test persons also respond differently to CR in form of F.X. Mayr therapy in context of the second study. Therefore, grouped analyses were also performed for the second study in order to analyze the overall influence of CR on the study cohort. Grouped analysis of the second study is displayed in **Figure 15**. Unlike in the first study, no significant increase in DNA repair capacity can be stated for the second study. Nevertheless, a continuous increase in DNA repair capacity is observable for the group of 18 test persons involved in this study. In addition, it is striking that DNA repair capacity level of



the study cohort approximate one another during convalescence, resulting in the highest average values with the lowest divergence in the end of the study.



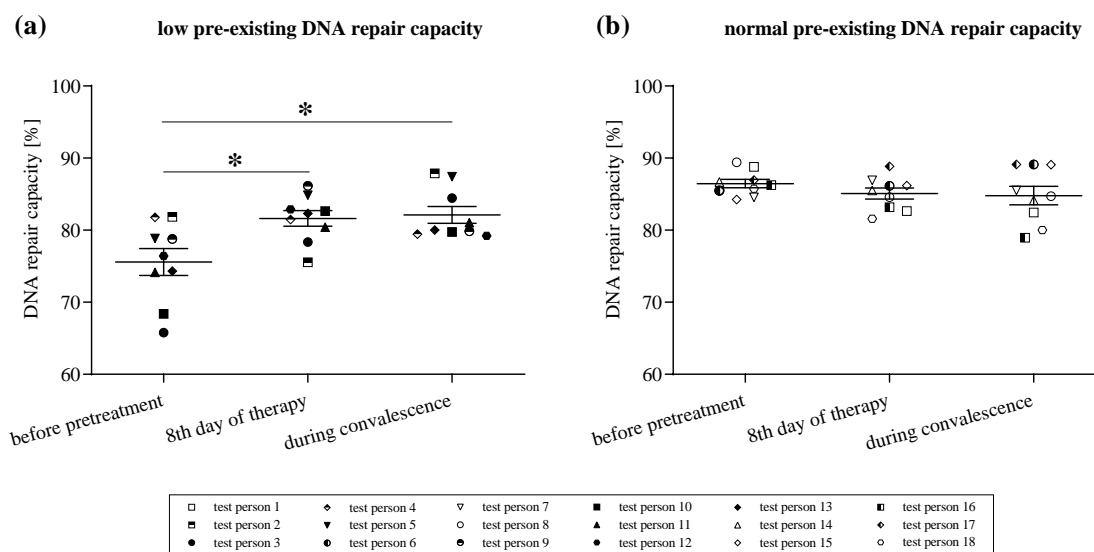
**Figure 15: Alteration of DNA repair capacity during calorie restriction.**

A steady but not significant increase can be observed for this study cohort over the study period. The highest DNA repair capacity values can be observed during convalescence. Additionally, average values of DNA repair capacity approximate one another over the study period. Statistical analyses were carried out as repeated measure one-way ANOVA with Dunnett's multiple comparisons test.

DNA repair capacity of the individual study participants varies during the course of the second study just like it did during the first study. Here it is particularly striking that the highest variability in NER capacity level is displayed before pretreatment. To be able to look more closely at this circumstance, individuals included in the second study were also grouped into the lower 50 % and the higher 50 % depending on their pre-existing DNA repair capacity before pretreatment, as described in 2.2.7. The groups will be referred to as “low pre-existing DNA repair capacity” group (lower 50 % of test persons) and “normal pre-existing DNA repair capacity” group (higher 50 % of test persons). The results of this grouped analysis is displayed in **Figure 16**.

In the “low pre-existing DNA repair capacity” group, NER capacity increases significantly on the eighth day of therapy compared to the measurement before pretreatment (\*  $p = 0.0457$ ). In this group an even slightly higher significant increase can be stated during convalescence, again compared to the first assessment before pretreatment (\*  $p = 0.0299$ ). For the “low pre-

existing DNA repair capacity” group the significant changes in NER capacity are displayed in **Figure 16 a**. Concerning the “normal pre-existing DNA repair capacity” group, a slight but not significant decrease in DNA repair capacity can be seen on the eighth day compared to the measurement before pretreatment. The same can be stated for the measurement during convalescence, again compared to the one before pretreatment. **Figure 16 b** depicts these findings. Statistical analyses for both groups were carried out as repeated measure one-way ANOVA with Dunnett’s multiple comparisons test.



**Figure 16: Differences in DNA repair capacity during calorie restriction in individuals with low vs. normal pre-existing DNA repair capacity.**

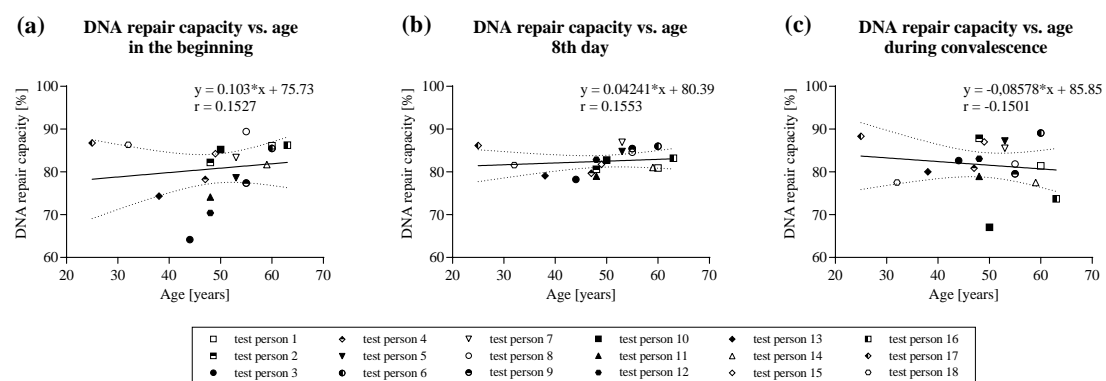
(a) Individuals with a low DNA repair capacity before pretreatment show a significant increase in NER capacity on the eighth day of therapy (\*  $p = 0.0457$ ), as well as during convalescence (\*  $p = 0.0299$ ). (b) Test persons assigned to the “normal pre-existing DNA repair capacity” group show a slight decrease in DNA repair capacity on the eighth day as well as during convalescence. In either case, the alteration in this group is not significant. Statistical analyses were carried out as repeated measure one-way ANOVA with Dunnett’s multiple comparisons test.

### 3.4.2 Age and calorie restriction

Participants of the second study on the influence of F.X. Mayr therapy on DNA repair were aged between 25 and 63 years and thus covering a greater age range than participants of the first study. As conducted for the first study, DNA repair capacity measured via mHCRA is correlated with the age of the volunteers in the following. In order to research the correlation between DNA repair capacity and the test person’s age, linear regression analyses were performed and Pearson’s correlation coefficient was calculated. As within the scope of the first study, interpretation of correlation coefficients of the second study was carried out using

Mukaka's proposition (Mukaka, 2012). **Figure 17** shows the results of linear regression analyses and corresponding correlation coefficients.

In the beginning of F.X Mayr therapy, a negligible correlation (Pearson's correlation coefficient  $r = 0.1527$ ) of NER capacity and age of the study participants is observable (see **Figure 17 a**). **Figure 17 b** displays that on the eighth day of the calorie restrictive period a negligible correlation ( $r = 0.1553$ ) persists for the 18 test persons. During convalescence, there is also a negligible correlation observable ( $r = -0.1501$ ), as shown in **Figure 17 c**. Just like in the first study, a donor variability can be stated regarding DNA repair capacity at different years of age. These findings were published as supplemental material in a combined analysis with the first study in Matt *et al.* 2016 (Matt *et al.*, 2016).

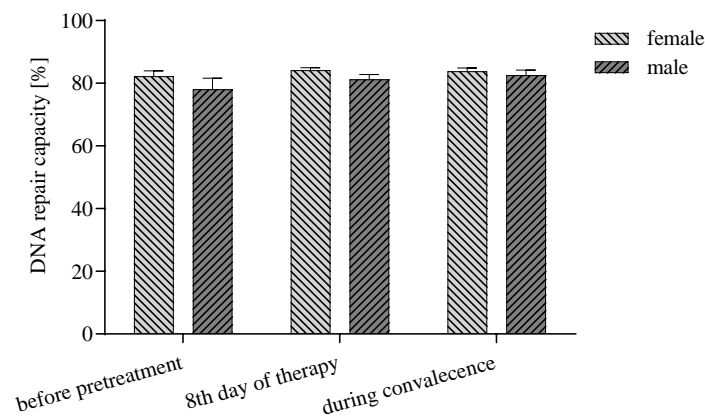


**Figure 17: Correlation of DNA repair capacity and age.**

(a) Before pretreatment, a negligible correlation ( $r = 0.1527$ ) of DNA repair capacity and age of the test persons is visible. (b) The negligible correlation from the beginning of the study persists on the eighth day of CR ( $r = 0.1553$ ). (c) During convalescence a negligible correlation is also evident ( $r = -0.1501$ ).

### 3.4.3 Gender and calorie restriction

As mentioned in chapter 2.2.7, 13 female test persons and five male test persons participated in the second study. Just like within the scope of the first study, a possible difference in DNA repair capacity between female and male test persons was researched for the second study. Results of these analyses are shown in **Figure 18**. At no time of measurement any statistically significant differences in the NER capacity of female and male individuals are visible. However, male test persons display a slightly reduced DNA repair capacity at all three time points. Statistical analyses were carried out as two-way ANOVA with Sidak's multiple comparisons test.



**Figure 18: DNA repair capacity during calorie restriction – gender differences.**

No significant differences regarding DNA repair capacity between female ( $n = 13$ ) and male ( $n = 5$ ) test persons are visible throughout the study. Male individuals show a slightly lower DNA repair capacity than females during all three measurements. Statistical evaluation was conducted as two-way ANOVA with Sidak's multiple comparisons test.

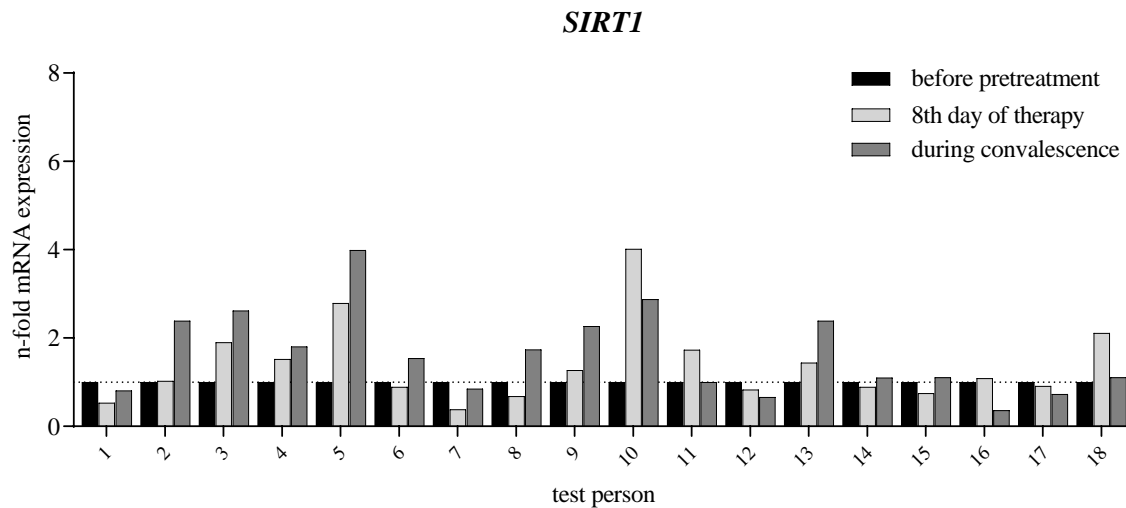
### 3.5 mRNA expression of aging-associated genes – second study

Within the scope of the second study on CR in humans, mRNA was isolated from test persons' PBMCs and cDNA was synthesized in order to analyze the influence of CR on the expression of aging-associated genes. The focus was the detection of gene expression alterations of *SIRT1*, *XPA*, *SIRT3*, *SOD2*, *FOXO3*, *AMPK $\alpha$* , *LC3B* and *BECN1* before, during and after the CR period of F.X. Mayr therapy. mRNA expression was determined on the three time points mentioned above, so that exactly the same time points were used as in the determination of DNA repair capacity previously. Gene expression analyzes were performed for all 18 test persons included into the second study. As with the mHCRA, individuals were divided into two groups according to their pre-existing DNA repair capacity for a closer look at the results of the mRNA expression studies. The detailed procedure of mRNA extraction, cDNA synthesis and qPCR is explained in chapters 2.2.9, 2.2.10 and 2.2.12. Due to the offsetting of the triplicates of CP values during qPCR evaluation, no statistical analysis of the individual subjects is possible with only one value per time point. This applies for all aging-associated genes presented in the following. However, grouped analyses were conducted as one-way ANOVA with subsequent Tukey's multiple comparisons test. Results of mRNA expression analyses of *SIRT1*, *SIRT3*, *XPA*, *SOD2* and *FOXO3* were published in "Nutrition and Health" (Matt *et al.*, 2020).

#### 3.5.1 Alteration of *SIRT1* mRNA expression during calorie restriction

As mentioned earlier, *SIRT1* is one of the most commonly studied genes in terms of CR. It is located mainly in the nucleus but also in the cytoplasm and influences aging positively in various species. The influence of F.X. Mayr therapy on mRNA expression in human test persons is shown in **Figure 19** for all 18 individuals. It is clearly visible that the mRNA expression profile of *SIRT1* varies widely in different subjects. Nine subjects (test persons 2, 3, 4, 5, 6, 8, 9, 10 and 13) display a higher mRNA expression of *SIRT1* during convalescence than in the beginning. Seven of these individuals (test person 2, 3, 4, 5, 8, 9 and 13) show the highest mRNA expression during convalescence. mRNA expression of *SIRT1* is highest on the eighth day in three individuals (test persons 10, 11 and 18). However, these test persons still display a higher mRNA expression during convalescence compared to the one in the beginning of the study. Finally, seven individuals display either no change in mRNA expression or a decrease

thereof. Some of which display the lowest expression on the eighth day (test persons 2, 7, 14 and 15) and some during convalescence (test persons 12, 16 and 17).

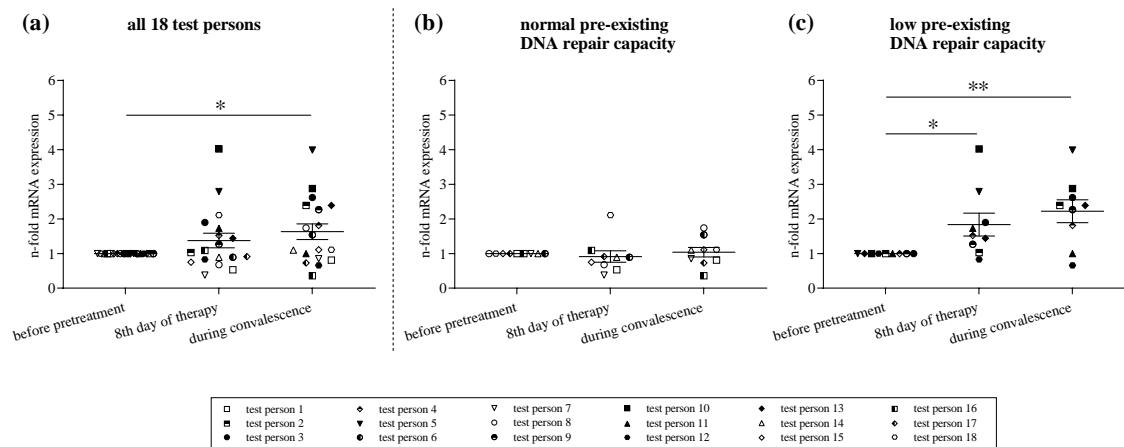


**Figure 19: mRNA expression profiles of *SIRT1* during calorie restriction.**

Test persons 2, 3, 4, 5, 6, 8, 9, 10 and 13 show a higher *SIRT1* mRNA expression during convalescence compared to the beginning of the study and in test persons 2, 3, 4, 5, 8, 9 and 13 mRNA expression is even highest on the last time of measurement. Test persons 10, 11 and 18 first display an increase in mRNA expression on the eighth day and a subsequent decrease to an expression level that is still higher than in the beginning. Test persons 2, 7, 14 and 15 show the lowest *SIRT1* mRNA expression on the eighth day, whereas test persons 12, 16 and 17 display the lowest expression during convalescence.

Since it has been shown that *SIRT1* mRNA expression is closely linked to DNA repair, the 18 test persons were again grouped into the lower 50 % and the higher 50 % depending on their pre-existing DNA repair capacity before pretreatment. This allows a grouped analysis of the study cohort by conducting one-way ANOVA with subsequent Tukey's multiple comparisons test to identify possible significant influences of CR on *SIRT1* mRNA expression. Results of the grouped analysis of all 18 test persons as well as analyses of the "normal" and the "low" pre-existing DNA repair groups are plotted in **Figure 20** and were published as Matt *et al.* 2020 (Matt *et al.*, 2020). When regarding all 18 test persons (**Figure 20 a**), a significant increase in *SIRT1* mRNA expression is obvious during convalescence compared to the measurement before pretreatment (\*  $p = 0.0121$ ). The "normal pre-existing DNA repair" group displays no significant alterations of *SIRT1* mRNA expression on either time point, as shown in **Figure 20 b**. On the other hand, the "low pre-existing DNA repair" group presents a significantly increased *SIRT1* mRNA expression on the eighth day (\*  $p = 0.0468$ ) as well as during convalescence (\*\*  $p = 0.0040$ ), in each case in comparison to the first measurement before

pretreatment (**Figure 20 c**). When looking at **Figure 20 a-c**, it is noticeable, that the significant increases seem to exclusively result from the individuals displaying a low pre-existing DNA repair in the beginning.

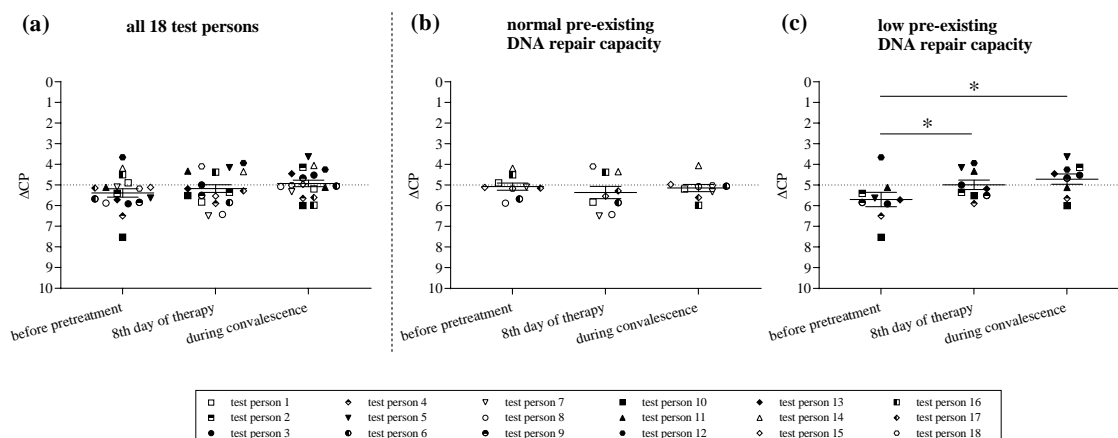


**Figure 20: Grouped analyses of *SIRT1* mRNA expression.**

(a) A significant increase in *SIRT1* mRNA expression is visible during convalescence compared to the measurement before pretreatment (\*  $p = 0.0121$ ). No further significant changes occurred for all 18 test persons. (b) The group consisting of individuals who display a normal DNA repair capacity in the beginning of the study does not show any significant alterations of *SIRT1* mRNA expression. (c) *SIRT1* mRNA expression increases significantly on the eighth day (\*  $p = 0.0468$ ) and during convalescence (\*\*  $p = 0.0040$ ) in test persons with a low pre-existing DNA repair capacity. Statistical analyses were conducted as one-way ANOVA with subsequent Tukey's multiple comparisons test.

In order to look at the basal level of *SIRT1* mRNA expression before normalization to the first time of measurement, which cannot be seen when regarding the n-fold mRNA expression,  $\Delta$ CP values are presented in **Figure 21**. Here, a  $\Delta$ CP value represents the normalization of mRNA expression of the gene of interest to the housekeeping genes. Thereby higher  $\Delta$ CP values point at a lower relative expression of *SIRT1*, whereas lower  $\Delta$ CP values hint at a higher relative expression of the gene of interest. Furthermore, a decrease of a  $\Delta$ CP value of 1 means a doubling of mRNA expression. For a better understanding, axes are inverted. Statistical analyses were conducted as one-way ANOVA with subsequent Tukey's multiple comparisons test. When looking at all 18 test persons,  $\Delta$ CP values do not change significantly throughout the study period (see **Figure 21 a**). In test persons displaying a normal DNA repair capacity in the beginning of the study  $\Delta$ CP values also do not change significantly, although values are slightly lower compared to the presentation of all test persons (see **Figure 21 b**). **Figure 21 c** shows  $\Delta$ CP values of *SIRT1* mRNA expression for the "low pre-existing DNA repair" group. In this group,  $\Delta$ CP values before pretreatment are higher than  $\Delta$ CP values of the "normal pre-

existing DNA repair” group before pretreatment and consequently also higher than in all 18 test persons. Furthermore, a significant decrease of  $\Delta CP$  values occurs on the eighth day of therapy ( $* p = 0.0370$ ) as well as during convalescence ( $* p = 0.0154$ ), hinting at an increase in *SIRT1* mRNA expression. Additionally,  $\Delta CP$  values of the “low” group approach and finally exceed  $\Delta CP$  values of the “normal” group during the study period, suggesting an adaption of mRNA expression in individuals with low pre-existing DNA repair capacity.



**Figure 21:  $\Delta CP$  values of *SIRT1* mRNA expression.**

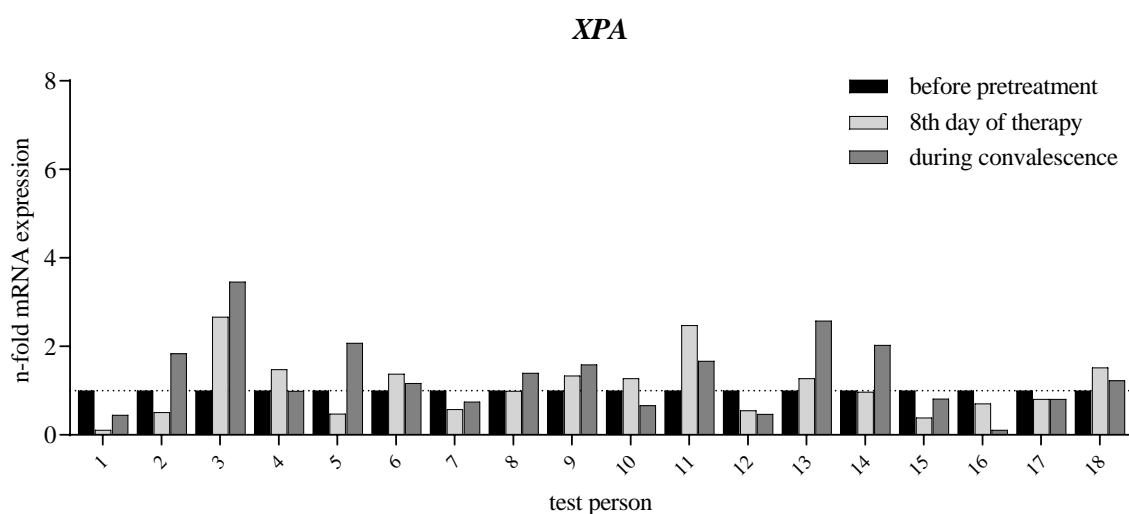
(a)  $\Delta CP$  values of *SIRT1* mRNA expression do not alter significantly in all 18 test persons. (b)  $\Delta CP$  values of *SIRT1* mRNA expression also change not significantly in test persons displaying a normal DNA repair capacity in the beginning. (c)  $\Delta CP$  values increase significantly in the “low pre-existing DNA repair” group on the eighth day ( $* p = 0.0370$ ) as well as during convalescence ( $* p = 0.0154$ ). Statistical analyses were carried out as one-way ANOVA with Tukey’s multiple comparisons test.

### 3.5.2 Alteration of *XPA* mRNA expression during calorie restriction

*XPA* is a core factor of nucleotide excision repair. Furthermore, *XPA* is closely linked to *SIRT1* by the ability of *SIRT1* to modulate the acetylation status of *XPA* and thus regulating nucleotide excision repair (Fan and Luo, 2010). Since *SIRT1* can be influenced by CR and since *XPA* is closely linked to *SIRT1*, mRNA expression of *XPA* was also researched regarding alterations due to CR. **Figure 22** shows the results of mRNA expression analyses for all 18 test persons. As with the expression profiles of *SIRT1*, the 18 individuals display varying *XPA* mRNA expression patterns. Nine of the subjects (test persons 2, 3, 5, 8, 9, 11, 13, 14 and 18) included into the second study display a higher *XPA* mRNA expression during convalescence compared to the time point before pretreatment. Furthermore, seven of these individuals (test persons 2, 3, 5, 8, 9, 13 and 14) show the highest *XPA* expression during convalescence. In five test persons (test persons 4, 6, 10, 11 and 18), however, *XPA* expression is highest on the



eighth day of F. X .Mayr therapy. Test persons 4, 6, 11 and 18 display a lower *XPA* mRNA expression during convalescence after an increase on the eighth day. However, *XPA* expression is still higher or the same during convalescence than before pretreatment. Six individuals (test persons 1, 7, 12, 15, 16 and 17) exhibit either no alteration of or a decrease of mRNA expression, in some of which *XPA* expression is lowest on the eighth day (test persons 1, 7 and 15) and in some during convalescence (test persons 12 and 16). Solely *XPA* mRNA expression of test person 17 is the same on the eighth day and during convalescence.

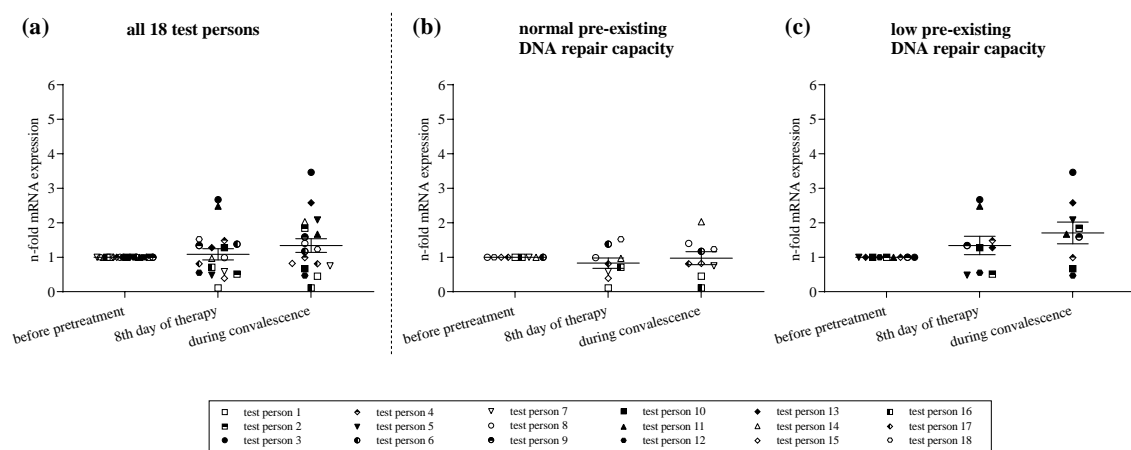


**Figure 22: mRNA expression profiles of *XPA* during calorie restriction.**

Test persons 2, 3, 5, 8, 9, 11, 13, 14 and 18 demonstrate a higher *XPA* mRNA expression during convalescence than before pretreatment and in test persons 2, 3, 5, 8, 9, 13 and 14 *XPA* expression is even highest on the last time of measurement. Test persons 4, 6, 10, 11 and 18 show the highest expression on the eighth day, followed by a decrease during convalescence, but *XPA* mRNA expression is still higher or the same in the end of the study in test persons 4, 6, 11 and 18. Test persons 1, 7, 12, 15, 16 and 17 show no alteration or a decrease of *XPA* expression on either time point, whereby test persons 1, 7 and 15 display the lowest expression on the eighth day and test persons 12 and 16 display the lowest expression during convalescence. *XPA* mRNA expression is the same on the eighth day and during convalescence in test person 17.

In the process of NER, *XPA* is responsible for damage recognition and verification, amongst other factors (Reardon and Sancar, 2003). In order to obtain further information about the influence of CR on *XPA* mRNA expression, previously mentioned grouping according to the pre-existing DNA repair capacity was once again implemented. In the following, grouped analyses of the study cohort were conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test. Results of these analyses were published in Matt *et al.* 2020 (Matt *et al.*, 2020) and are plotted in **Figure 23**. Looking at the whole study cohort in **Figure 23 a**, it is

apparent that an increase in *XPA* mRNA expression occurs with increasing time of CR, although this increase is statistically not significant. However, individuals displaying a normal pre-existing DNA repair capacity show no alteration of *XPA* expression, as shown in **Figure 23 b**. On the contrary, the “low pre-existing DNA repair” group displays a not significant increase in *XPA* mRNA expression on the eighth day as well as during convalescence, as displayed in **Figure 23 c**. Regarding **Figure 23 a-c** more precisely, the increase in *XPA* expression of all 18 test persons seems to be attributed solely to individuals with low pre-existing DNA repair capacity, since the “normal pre-existing DNA repair” group shows no change in mRNA expression. Interestingly, this has already been the case for *SIRT1* mRNA expression (see chapter 3.5.1).

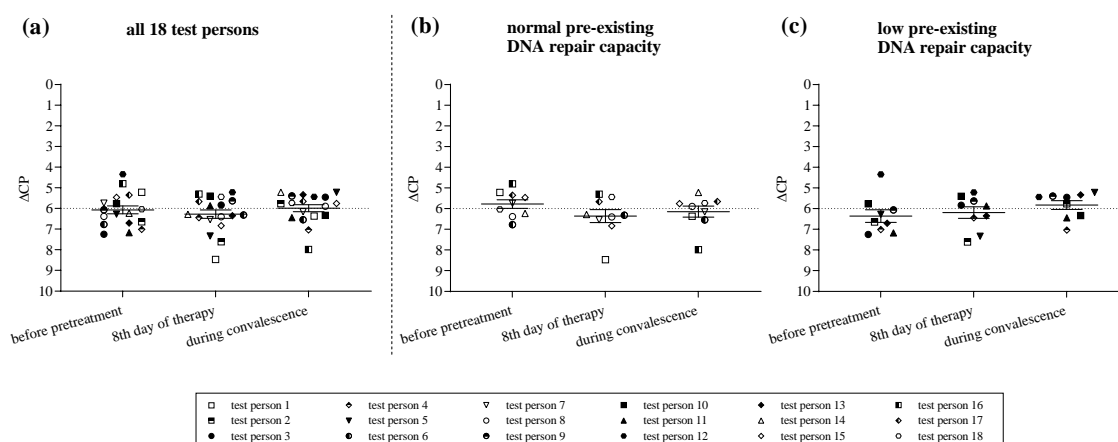


**Figure 23: Grouped analyses of *XPA* mRNA expression.**

(a) An increase in *XPA* mRNA expression of all 18 test persons is observable on the eighth day and during convalescence compared to the measurement before pretreatment. (b) The group consisting of individuals displaying a normal DNA repair capacity in the beginning of the study does not show any significant alterations of *XPA* mRNA expression. (c) Test persons with a low pre-existing DNA repair capacity show a not significant increase in *XPA* mRNA expression on the eighth day and during convalescence. Statistical analyses were carried out as one-way ANOVA with Tukey’s multiple comparisons test.

Since *XPA* mRNA expression alters similarly to *SIRT1* mRNA expression, a presentation of  $\Delta$ CP values was also prepared for *XPA*, as shown in **Figure 24**. As previously mentioned, higher  $\Delta$ CP values point at a lower relative mRNA expression, whereas lower  $\Delta$ CP values hint at a higher relative expression of the gene of interest. For a better understanding, axes are inverted. Statistical analyses were carried out as one-way ANOVA with subsequent Tukey’s multiple comparisons test. **Figure 24 a** displays  $\Delta$ CP values of all 18 test persons. These analyses were also published in Matt *et al.* 2020 (Matt *et al.*, 2020). It is clearly visible that the

values do not change during the study period. When looking at the individuals with normal pre-existing DNA repair capacity, as shown in **Figure 24 b**,  $\Delta$ CP values before pretreatment are slightly higher compared to the presentation of all 18 test persons. Furthermore, no considerable alterations of  $\Delta$ CP values occur, although  $\Delta$ CP values decrease marginally. The “low pre-existing DNA repair capacity” group displays slightly lower  $\Delta$ CP values in the beginning, as displayed in **Figure 24 c**. In the course of the study,  $\Delta$ CP values of this group increase slightly. In the end of the study,  $\Delta$ CP values are on approximately the same level as  $\Delta$ CP values of the “normal” group in the beginning.



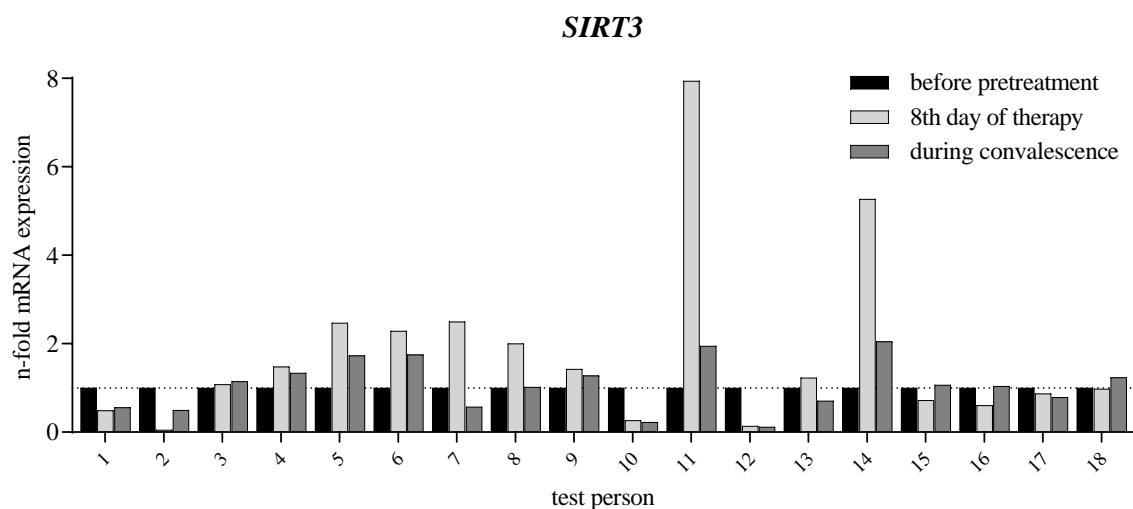
**Figure 24:  $\Delta$ CP values of XPA mRNA expression.**

(a)  $\Delta$ CP values of XPA mRNA expression do not change when regarding all 18 test persons. (b)  $\Delta$ CP values of XPA mRNA expression also do not change in test persons of the “normal pre-existing DNA repair capacity” group. (c) In the beginning,  $\Delta$ CP values of the “low pre-existing DNA repair” group are lower than  $\Delta$ CP values of the “normal pre-existing DNA repair” group.  $\Delta$ CP values increase slightly during the study, so that in the end,  $\Delta$ CP values of the “low” group are at the same level as  $\Delta$ CP values of the “normal” group. Statistical analyses were carried out as one-way ANOVA with Tukey’s multiple comparisons test.

### 3.5.3 Alteration of SIRT3 mRNA expression during calorie restriction

As stated in chapter 1.7.1, sirtuins are known to be influenced positively by CR, to contribute to healthier aging and to prolong lifespan. Besides the aforementioned sirtuin 1, the mitochondria-located sirtuin 3 is closely linked to aging for example by preserving mitochondrial integrity (Kim *et al.*, 2010). Therefore, SIRT3 mRNA expression was also researched during the CR period of F.X. Mayr therapy. Results of gene expression analyses are displayed in **Figure 25**. As with SIRT1 and XPA mRNA expression, the 18 test persons show differing expression patterns of SIRT3 throughout the study. Seven test persons (test persons 4, 5, 6, 9, 11, 14 and 18) display a higher SIRT3 mRNA expression during

convalescence compared to the beginning of the study. Only test person 18 shows the highest expression on the last point of measurement. Furthermore, in nine individuals (test persons 4, 5, 6, 7, 8, 9, 11, 13 and 14) *SIRT3* expression is highest on the eighth day. Six of these persons (test persons 4, 5, 6, 9, 11 and 14) first display an increase in mRNA expression on the eighth day and a decrease during convalescence to a level, which is still higher than in the beginning of the study. After the increase on the eighth day, *SIRT3* mRNA expression level decreases to the lowest overall level in the end in test persons 7 and 13, whereas in test person 8, *SIRT3* expression in the end is similar to the one at the beginning of the study. Eight subjects (test persons 1, 2, 3, 10, 12, 15, 16 and 17) show no reaction to CR or a decrease of *SIRT3* mRNA expression. Some of these individuals (test persons 1, 2, 15 and 16) display the lowest expression on the eighth day, others (test persons 3, 10, 12 and 17) during convalescence.

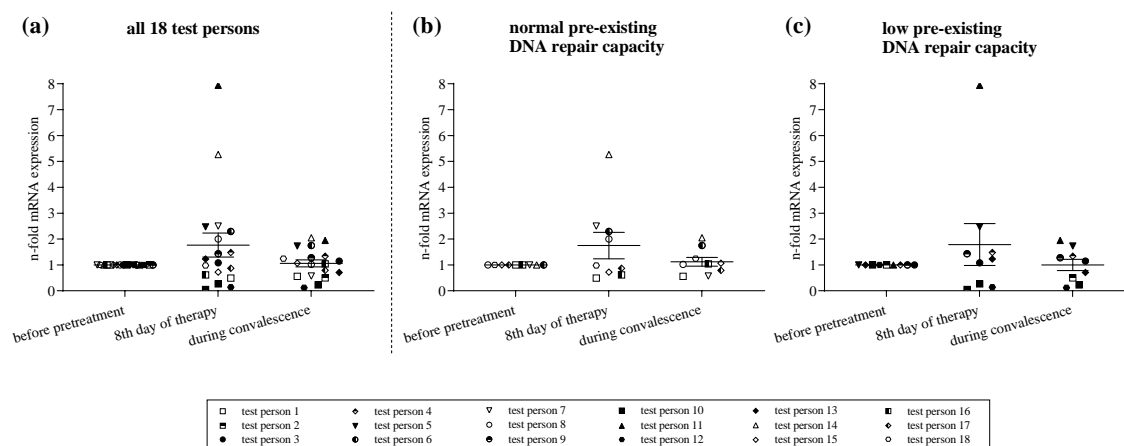


**Figure 25: mRNA expression profiles of *SIRT3* during calorie restriction.**

Test persons 4, 5, 6, 9, 11, 14 and 18 exhibit a higher *SIRT3* mRNA expression during convalescence than in the beginning and in test person 18 *SIRT3* expression is even highest on the last time of measurement. Test persons 4, 5, 6, 7, 8, 9, 11, 13 and 14 display the highest expression on the eighth day, however, in test persons 4, 5, 6, 9, 11 and 14 *SIRT3* expression decreases again during convalescence, but remains higher than in the beginning of the study, whereas test persons 7 and 13 display lower expression levels than in the beginning. *SIRT3* expression returns to the initial level during convalescence in test person 8. Test persons 1, 2, 3, 10, 12, 15, 16 and 17 show no alteration or a decrease of *SIRT3* expression on either time point, whereby test persons 1, 2, 15 and 16 display the lowest expression on the eighth day and test persons 3, 10, 12 and 17 show the lowest expression during convalescence.

As conducted for the *SIRT1* and *XPA* mRNA expression, the 18 individuals were subdivided into test persons displaying a normal and test persons displaying a low initial DNA repair capacity. Resulting grouped analyses were conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test are exhibited in **Figure 26**. Furthermore, the results were

published in Matt *et al.* 2020 (Matt *et al.*, 2020). An increasing trend of *SIRT3* mRNA expression can be observed on the eighth day of therapy, when looking at the whole study cohort (see **Figure 26 a**). Furthermore, the high mRNA expression level of test persons 11 and 14 on the eighth day is striking. During convalescence, however, *SIRT3* expression level decreases again to its original level. Individuals displaying a normal DNA repair capacity before pretreatment show the same pattern in terms of an increase of *SIRT3* mRNA expression on the eighth day and a subsequent decrease during convalescence, as plotted in **Figure 26 b**. Test person 14 shows the highest *SIRT3* mRNA expression in this group, which contributes to the average increase on the eighth day. As with the consideration of all subjects as well as the "normal" group, the same course of *SIRT3* mRNA expression can be determined for the "low" group as well (see **Figure 26 c**). On the eighth day the average *SIRT3* expression level increases, before it decreases again during convalescence. However, the exceptionally high mRNA expression in test person 11 probably leads to the seemingly increasing *SIRT3* expression on the eighth day. Within the "low" group, test person 11 is worth mentioning by showing by far the highest *SIRT3* mRNA expression. None of the alterations mentioned before are statistically significant.

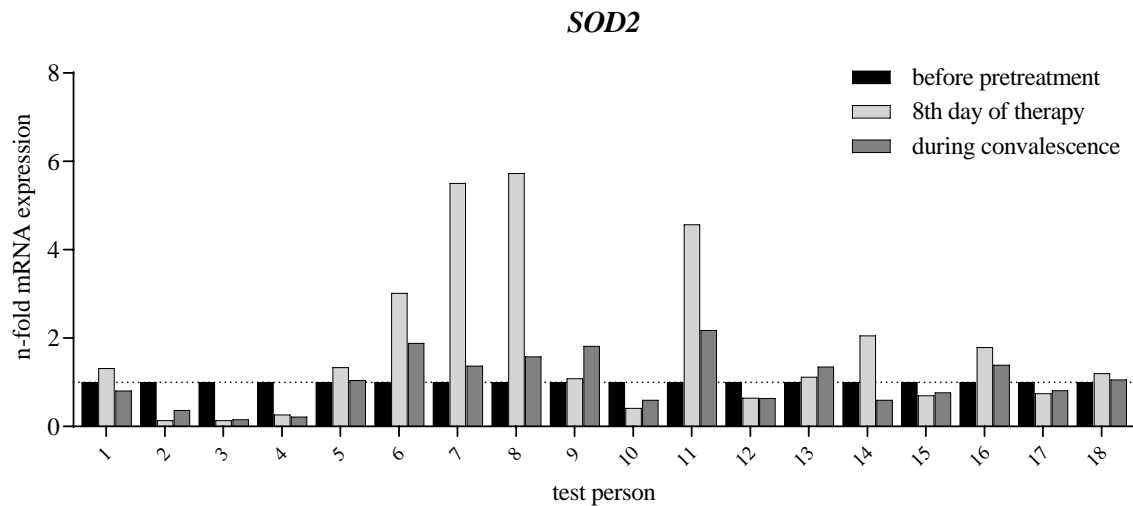


**Figure 26: Grouped analyses of *SIRT3* mRNA expression.**

(a) An increase of the average *SIRT3* mRNA expression of all 18 test persons is evident on the eighth day of therapy, followed by a decrease of the average expression level. Test persons 11 and 14 are outstanding by displaying a rather high *SIRT3* expression. (b) The "normal" group also shows an increase on the eighth day and a subsequent decrease of *SIRT3* mRNA expression, whereby test person 14 displays the highest mRNA expression on the eighth day. (c) Test persons with a low pre-existing DNA repair capacity also show an increase in *SIRT3* mRNA expression on the eighth day, followed by a decrease during convalescence. Here, test person 11 shows by far the highest *SIRT3* expression on the eighth day, which probably leads to the average increase in *SIRT3* mRNA expression. None of the changes of *SIRT3* mRNA expression are statistically significant. Statistical analyses were conducted as one-way ANOVA with subsequent Tukey's multiple comparisons test.

### 3.5.4 Alteration of *SOD2* mRNA expression during calorie restriction

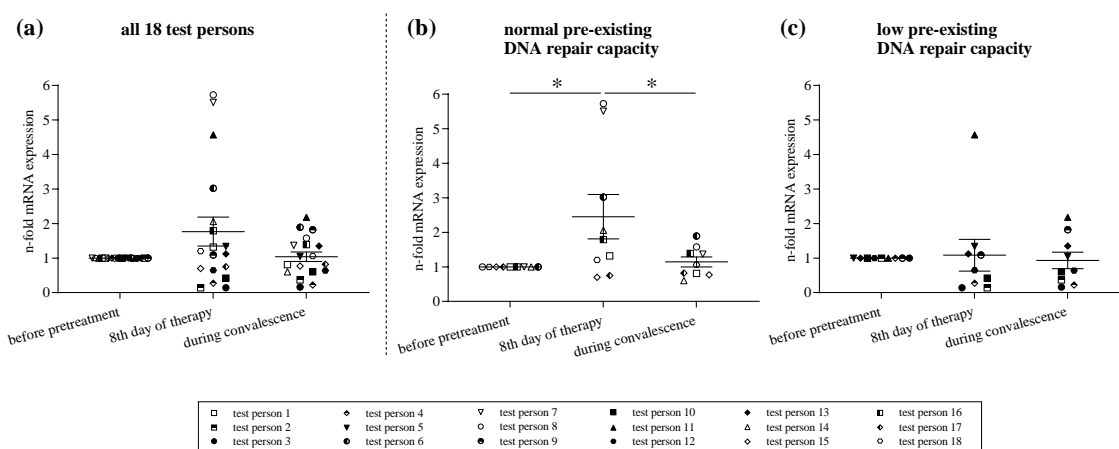
As an aging-associated gene, *SOD2* was also studied regarding an influence of CR. Due to its function as antioxidant, *SOD2* takes part in protecting cells from oxidative damage and thus contributes to the preservation of a healthy organism. mRNA expression analyses of the F.X. Mayr study cohort are displayed in **Figure 27** as individual values per test person. As it has been shown for *SIRT1*, *XPA* and *SIRT3* mRNA expression, the 18 individuals also display different expression patterns of *SOD2* throughout the study. Seven subjects (test persons 6, 7, 8, 9, 11, 13 and 16) exhibit a higher *SOD2* mRNA expression during convalescence than in the beginning of the study. However, test persons 9 and 13 display the highest expression during convalescence. *SOD2* mRNA expression is highest on the eighth day in eight individuals (test persons 1, 5, 6, 7, 8, 11, 14 and 16), whereby five of them (test persons 6, 7, 8, 11 and 16) show an increase in *SOD2* expression on the eighth day and a subsequent decrease. However, in these individuals *SOD2* expression is still higher during convalescence compared to the beginning of the study. Eight subjects (test persons 2, 3, 4, 10, 12, 15, 17 and 18) display no alteration or a decrease of *SOD2* mRNA expression throughout the study period. Five of them (test persons 2, 3, 10, 15 and 17) show the lowest *SOD2* expression on the eighth day, whereas test persons 4 and 12 display the lowest expression during convalescence. Solely *SOD2* expression of test person 18 decreases to its initial level during convalescence.



**Figure 27: mRNA expression profiles of *SOD2* during calorie restriction.**

Test persons 6, 7, 8, 9, 11, 13 and 16 display a higher *SOD2* mRNA expression during convalescence than in the beginning. In test persons 9 and 13 *SOD2* expression is highest during convalescence. Test persons 1, 5, 6, 7, 8, 11, 14 and 16 show the highest expression on the eighth day, but in test persons 6, 7, 8, 11 and 16 *SOD2* expression decreases during convalescence, but remains higher than in the beginning. Test persons 2, 3, 4, 10, 12, 15, 17 and 18 show no alteration or a decrease of *SOD2* expression on either time point. Test persons 2, 3, 10, 15 and 17 display the lowest expression on the eighth day, whereas test persons 4 and 12 show the lowest expression during convalescence. *SOD2* mRNA expression of test person 18 decreases to its initial level after a slight increase on the eighth day.

Hereinafter, the study cohort was subdivided into individuals displaying a normal pre-existing DNA repair capacity and those displaying a low DNA repair capacity in the beginning of the study. Grouped analyses of all 18 test persons were conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test. Results of the analyses were published in Matt *et al.* 2020 (Matt *et al.*, 2020) and are plotted in **Figure 28**. When regarding all 18 test persons, a not significant increase in *SOD2* mRNA expression is visible on the eighth day, followed by a decrease during convalescence (see **Figure 28 a**). Individuals assigned to the "normal pre-existing DNA repair capacity" group show a significantly elevated average *SOD2* expression on the eighth day ( $* p = 0.0265$ ) as well as a significant decrease during convalescence ( $* p = 0.0466$ ), as displayed in **Figure 28 b**. In this group, test persons 7 and 8 stand out by displaying the highest *SOD2* mRNA expression by far on the eighth day. **Figure 28 c** shows the grouped analysis of *SOD2* mRNA expression for the "low" group. On the average, no alteration of *SOD2* expression is visible, but mRNA expression in test person 11 is considerably higher than the average on the eighth day.



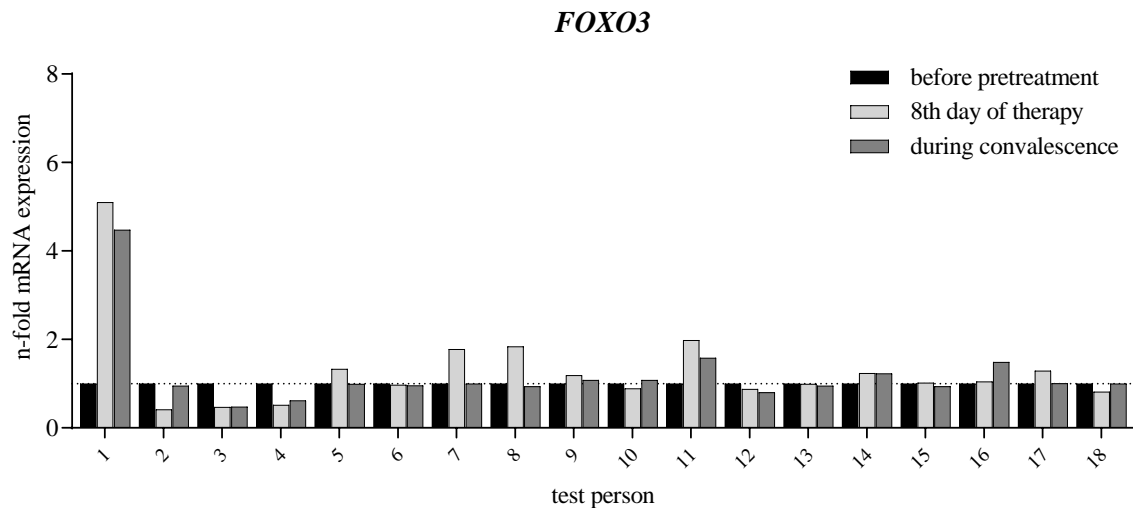
**Figure 28: Grouped analyses of *SOD2* mRNA expression.**

(a) *SOD2* mRNA expression increases on the eighth day of therapy, followed by a decrease of the average expression level during convalescence. (b) The "normal" group shows a significant increase of *SOD2* mRNA expression on the eighth day ( $* p = 0.0265$ ) and a subsequent significant decrease ( $* p = 0.0466$ ). (c) Test persons with a low pre-existing DNA repair capacity show no alteration of *SOD2* mRNA expression throughout the study. *SOD2* mRNA expression of test person 11 is noticeably higher than the average expression level on the eighth day of therapy. Statistical analyses were carried out as one-way ANOVA with subsequent Tukey's multiple comparisons test.



### 3.5.5 Alteration of *FOXO3* mRNA expression during calorie restriction

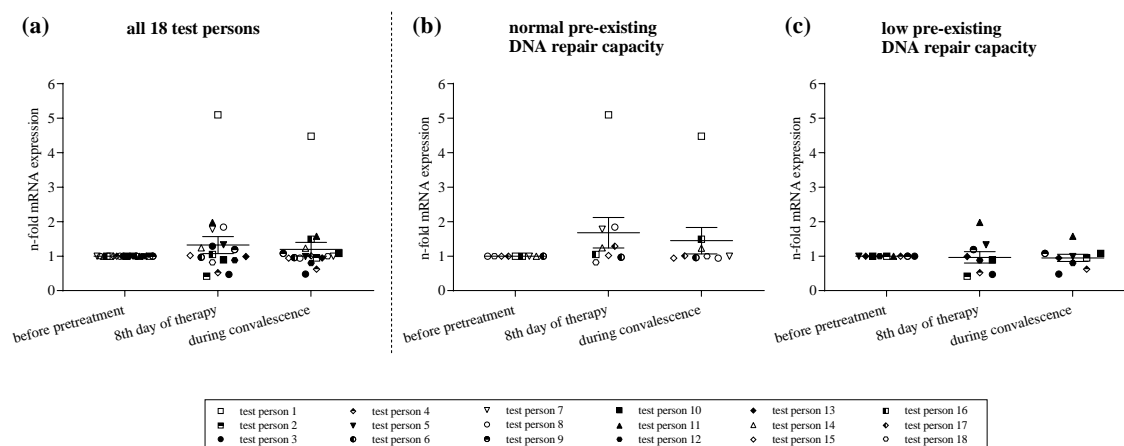
*FOXO3* is associated with longevity in humans (Flachsbart *et al.*, 2009). Moreover, it is able to trigger autophagy (Mammucari *et al.*, 2007). In turn, CR leads to an increase in autophagic activity, whereas a decreased autophagy is linked to aging processes (Uddin *et al.*, 2012). Therefore, the influence of F.X. Mayr therapy as a CR intervention on *FOXO3* mRNA expression was researched within the scope of this work. Individual *FOXO3* mRNA expression profiles for all 18 test persons are plotted in **Figure 29**. In general, there are no major changes in *FOXO3* expression during the study, with the exception of test person 1. Four test persons (test persons 1, 11, 14 and 16) display a higher mRNA expression during convalescence than in the beginning. Solely test person 16 shows the highest *FOXO3* expression during convalescence. *FOXO3* mRNA expression is highest on the eighth day in six individuals (test persons 1, 5, 7, 8, 11 and 17). Test persons 1 and 11 display a decreasing *FOXO3* expression during convalescence, but at that time mRNA expression is still higher than in the beginning. Ten of the 18 subjects (test persons 2, 3, 4, 6, 9, 10, 12, 13, 15 and 18) show no alteration or a decrease of *FOXO3* mRNA expression at either time point. Four of these individuals (test persons 2, 4, 10 and 18) display the lowest expression on the eighth day, whereas test person 12 displays the lowest expression during convalescence. Five subjects (test persons 6, 9, 10, 13 and 15) show no alteration at all. In test persons 3 and 4 *FOXO3* mRNA expression decreases on the eighth day and remains at that level during convalescence. *FOXO3* expression pattern is striking in test person 1, who displays the most pronounced increase in mRNA expression on the eighth day as well as during convalescence.



**Figure 29: mRNA expression profiles of *FOXO3* during calorie restriction.**

Test persons 1, 11, 14 and 16 display a higher *FOXO3* mRNA expression during convalescence than before pretreatment and in test person 16 *FOXO3* expression is highest during convalescence. Test persons 1, 5, 7, 8, 11 and 17 show the highest expression on the eighth day, followed by a decrease during convalescence. *FOXO3* expression is still higher in the end of the study in test persons 1 and 11. Test persons 2, 3, 4, 6, 9, 10, 12, 13, 15 and 18 show no alteration or a decrease of mRNA expression on either time point, whereby test persons 2, 4, 10 and 18 display the lowest expression on the eighth day and test person 12 displays the lowest *FOXO3* expression during convalescence. Test persons 3 and 4 show a decreased *FOXO3* mRNA expression on the eighth day and during convalescence.

As with the results of gene expression alterations upon CR, which are described in the previous chapters 3.5.1 - 3.5.4, a subdivision of the study cohort into two groups according to their initial DNA repair capacity was conducted for *FOXO3* mRNA expression. Grouped analyses of the 18 individuals as well as of the “normal” and the “low pre-existing DNA repair capacity” groups were conducted via one-way ANOVA with subsequent Tukey’s multiple comparisons test. Results were published in Matt *et al.* 2020 (Matt *et al.*, 2020) and are furthermore shown in **Figure 30**. No significant alterations of *FOXO3* mRNA expression can be stated for all 18 test persons, as displayed in **Figure 30 a**. Within the persons exhibiting a normal pre-existing DNA repair capacity *FOXO3* mRNA expression is visible, but not significant (see **Figure 30 b**). Moreover, the average *FOXO3* expression remains on that level during convalescence. As plotted in **Figure 30 c**, no alteration of *FOXO3* mRNA expression can be recorded for the “low pre-existing DNA repair capacity” group on either time point. Looking at the **Figure 30 a-c**, test person 1 is worth mentioning due to the exceptionally high *FOXO3* mRNA expression on the eighth day as well as during convalescence. This presumably leads to the increased average *FOXO3* mRNA expression shown in **Figure 30 a and b**.

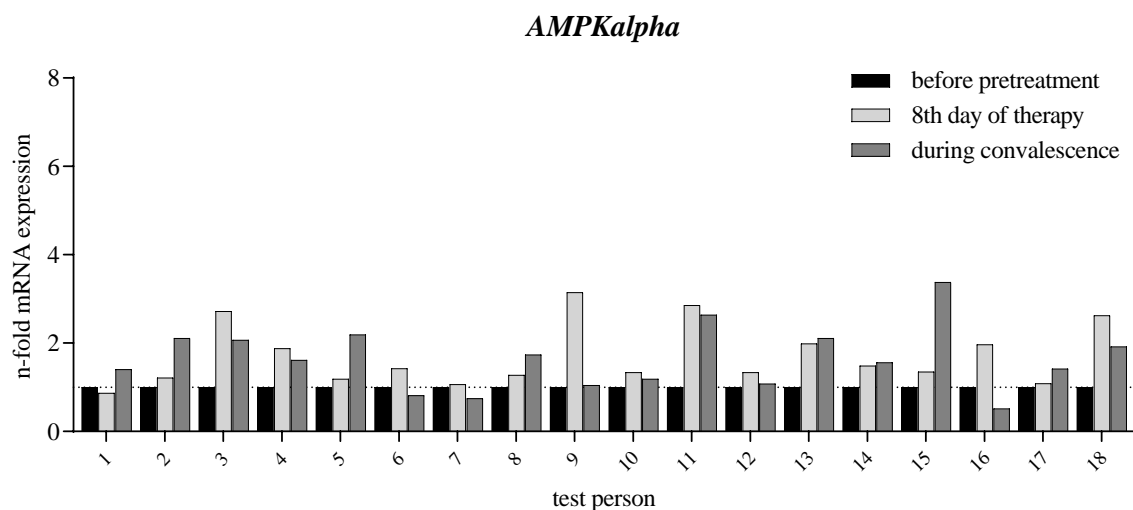


**Figure 30: Grouped analyses of *FOXO3* mRNA expression.**

(a) *FOXO3* mRNA expression does not change significantly when analyzing all 18 test persons. (b) Within the “normal” group, a slight increase in *FOXO3* expression is visible on the eighth day and during convalescence. (c) No alterations of *FOXO3* mRNA expression occur in the “low pre-existing DNA repair capacity” group. Regarding all three figures, test person 1 is notable for the exceptionally high expressions on the eighth day and during convalescence. Statistical analyses were conducted as one-way ANOVA with Tukey’s multiple comparisons test.

### 3.5.6 Alteration of *AMPK $\alpha$* mRNA expression during calorie restriction

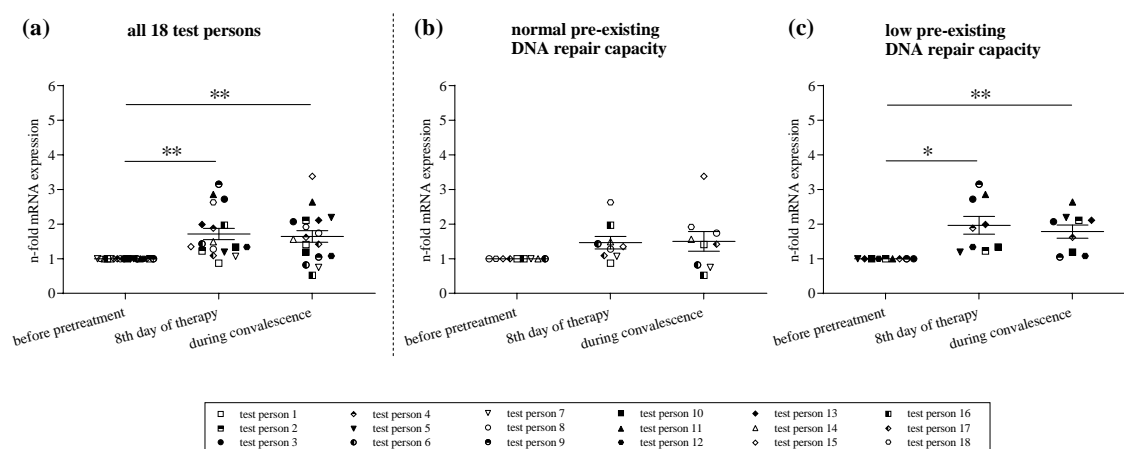
*AMPK* can be activated by nutrient scarcity or the CRM resveratrol (Mihaylova and Shaw, 2011). As an important nutrient sensor, an influence of CR on *AMPK $\alpha$*  mRNA expression is possible and was therefore researched within this thesis. The influence of F.X. Mayr therapy on human mRNA expression is shown in **Figure 31**. It is clearly visible that mRNA expression profile of *AMPK $\alpha$*  varies in different subjects. Twelve test persons (test persons 1, 2, 3, 4, 5, 8, 11, 13, 14, 15, 17 and 18) display a higher *AMPK $\alpha$*  mRNA expression during convalescence compared to the beginning of the study. Seven of the individuals (test persons 1, 2, 5, 8, 13, 14 and 15) show the highest expression on the last point of measurement. *AMPK $\alpha$*  expression is highest on the eighth day of therapy in nine individuals (test persons 3, 4, 6, 9, 10, 11, 12, 16 and 18), whereby six of them (test persons 3, 4, 10, 11, 16 and 18) show an increase in *AMPK $\alpha$*  expression on the eighth day and a subsequent decrease. However, apart from test person 6, *AMPK $\alpha$*  expression in these individuals is still higher during convalescence compared to the beginning of the study. Only one individual (test person 7) displays no change in mRNA expression on the eighth day and a slight decrease during convalescence.



**Figure 31: mRNA expression profiles of *AMPK $\alpha$*  during calorie restriction.**

Test persons 1, 2, 3, 4, 5, 8, 11, 13, 14, 15, 17 and 18 exhibit a higher *AMPK $\alpha$*  mRNA expression during convalescence than in the beginning. In seven of these test persons (test persons 1, 2, 5, 8, 13, 14 and 15) *AMPK $\alpha$*  expression is even highest on the last time of measurement. Test persons 3, 4, 6, 9, 10, 11, 12, 16 and 18 display the highest expression on the eighth day, however, in test persons 3, 4, 10, 11, 16 and 18 *AMPK $\alpha$*  expression decreases again during convalescence. Apart from test person 16 mRNA expression remains higher than in the beginning of the study. *AMPK $\alpha$*  mRNA expression of test person 7 does not change on the eighth day and decreases slightly during convalescence.

As conducted for the five aging-associated genes mentioned before, results of *AMPK $\alpha$*  gene expression analyses of the 18 individuals were subdivided into test persons displaying a normal and test persons displaying a low initial DNA repair capacity. In the following, grouped analyses of the study cohort were conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test. Results are displayed in **Figure 32**. When regarding all 18 test persons (**Figure 32 a**), a significant increase in *AMPK $\alpha$*  mRNA expression is obvious on the eighth day of therapy (\*\*  $p = 0.0011$ ) and during convalescence (\*\*  $p = 0.0038$ ), in each case compared to the measurement before pretreatment. The "normal pre-existing DNA repair" group shows no significant alterations of *AMPK $\alpha$*  mRNA expression on either time point, as shown in **Figure 32 b**. However, the "low pre-existing DNA repair" group presents a significantly increased *AMPK $\alpha$*  mRNA expression on the eighth day (\*  $p = 0.0134$ ) as well as during convalescence (\*\*  $p = 0.0083$ ), in each case in comparison to the first measurement before pretreatment (**Figure 32 c**). As with *SIRT1*, the significant increases in mRNA expression is only obvious in the "low pre-existing DNA repair" group.

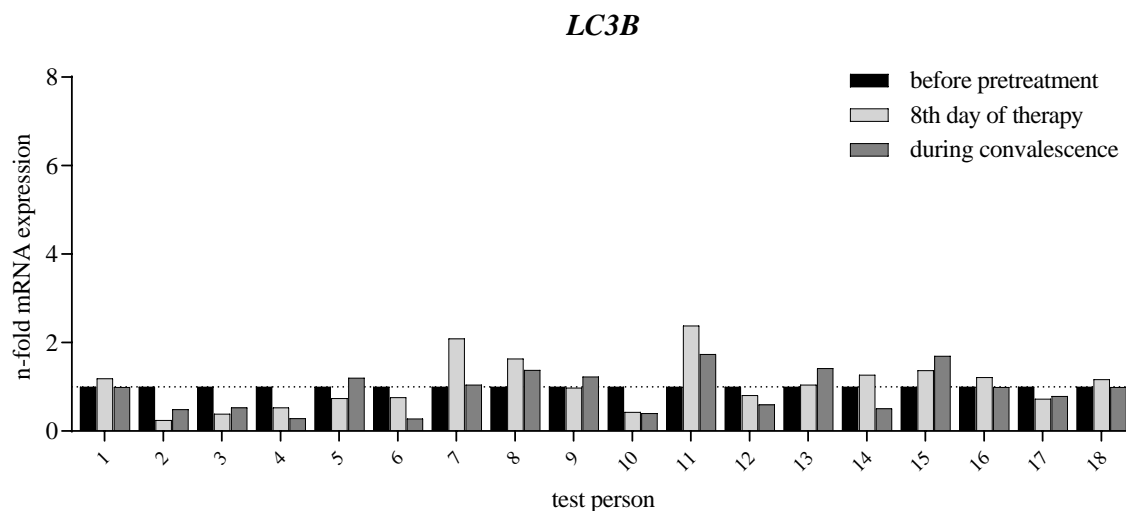


**Figure 32: Grouped analyses of *AMPK $\alpha$*  mRNA expression.**

(a) A significant increase in *AMPK $\alpha$*  mRNA expression of all 18 test persons is observable on the eighth day (\*\*  $p = 0.0011$ ) and during convalescence (\*\*  $p = 0.0038$ ) compared to the measurement before pretreatment. (b) The group consisting of individuals displaying a normal DNA repair capacity in the beginning of the study does not show any significant alterations of *AMPK $\alpha$*  mRNA expression. (c) Test persons with a low pre-existing DNA repair capacity show significant increases in *AMPK $\alpha$*  mRNA expression on the eighth day (\*  $p = 0.0134$ ) and during convalescence (\*\*  $p = 0.0083$ ). Statistical analyses were carried out as one-way ANOVA with Tukey's multiple comparisons test.

### 3.5.7 Alteration of *LC3B* mRNA expression during calorie restriction

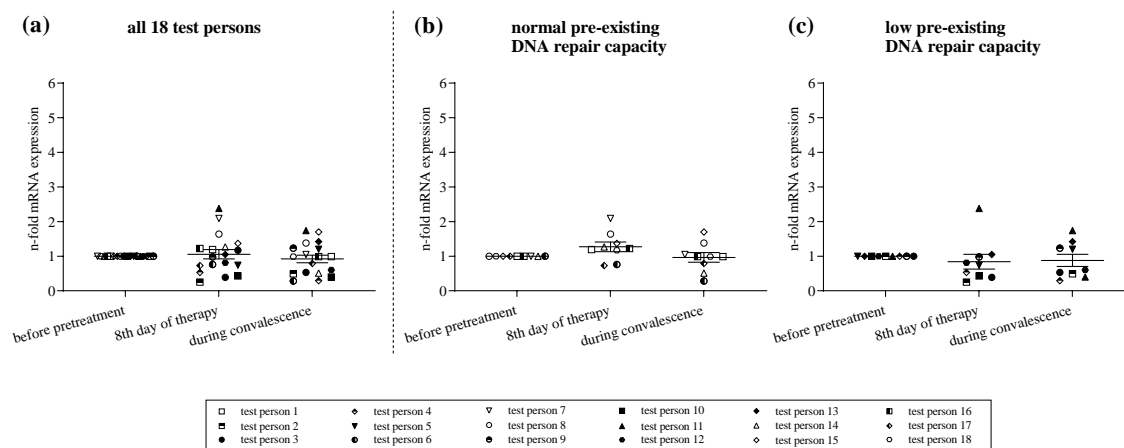
Long-term CR leads to a significantly increased *LC3* gene and protein expression (Yang *et al.*, 2016). Therefore the question arose, whether F.X. Mayr therapy also influences *LC3B* mRNA expression. Results of mRNA expression analyses of the F.X. Mayr study cohort are displayed in **Figure 33** as individual values per test person. Few individuals display alterations of *LC3B* mRNA expression. Four subjects (test persons 8, 11, 13 and 15) show higher *LC3B* expressions during convalescence than in the beginning. Test person 15 displays the highest expression during convalescence. In three individuals (test persons 7, 8 and 11), *LC3B* expression is highest on the eighth day, whereby test persons 8 and 11 show an increase on the eighth day and a subsequent decrease. However, in these individuals *LC3B* expression is still higher during convalescence than in the beginning. Twelve subjects (test persons 1, 2, 3, 4, 5, 6, 9, 10, 12, 16, 17 and 18) display no alteration or a decrease of *LC3B* expression throughout the study. Three of them (test persons 2, 3 and 5) show the lowest *LC3B* expression on the eighth day, whereas test persons 4, 6 and 12 display the lowest expression during convalescence. Test persons 10 and 17 display similarly low expression levels on both time points.



**Figure 33: mRNA expression profiles of *LC3B* during calorie restriction.**

Test persons 8, 11, 13, and 15 show a higher *LC3B* mRNA expression during convalescence than before pretreatment and in test person 15 the expression is highest during convalescence. Test persons 7, 8 and 11 display the highest expression on the eighth day, followed by a decline during convalescence, but *LC3B* mRNA expression is still higher or the same in the end of the study compared to the beginning. Test persons 1, 2, 3, 4, 5, 6, 9, 10, 12, 16, 17 and 18 show no alteration or a decrease of *LC3B* expression on either time point, whereby test persons 2, 3 and 5 display the lowest expression on the eighth day and test persons 4, 6 and 12 display the lowest expression during convalescence. Test persons 10 and 17 show nearly the same low *LC3B* expression on the eighth day and during convalescence.

As conducted previously, the 18 individuals were subdivided into test persons displaying a normal and test persons displaying a low initial DNA repair capacity. Grouped analyses of the 18 individuals as well as the normal and the low pre-existing DNA repair capacity groups were conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test. Results are shown in **Figure 34**. No significant alterations of *LC3B* mRNA expression can be stated for all 18 test persons (**Figure 34 a**). The "normal pre-existing DNA repair" group also shows no significant changes in *LC3B* expression, although a marginal increase is visible (see **Figure 34 b**), followed by a slight, but not significant decrease back to the pre-existing level before pretreatment. As displayed in **Figure 34 c**, no alteration of *LC3B* mRNA expression can be recorded for the "low pre-existing DNA repair capacity" group on either time point.



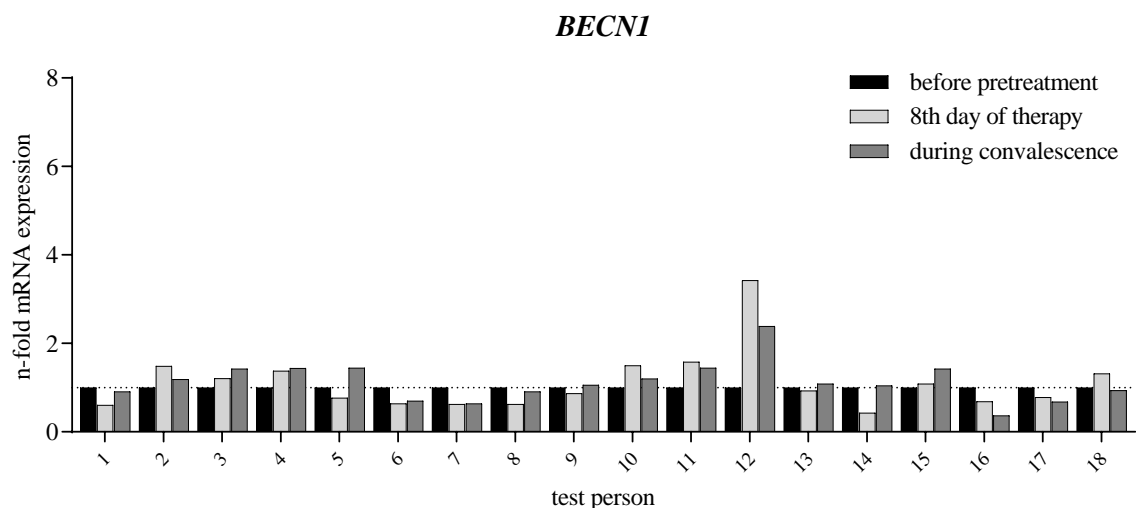
**Figure 34: Grouped analyses of *LC3B* mRNA expression.**

(a) *LC3B* mRNA expression does not change significantly when conducting grouped analyses of all 18 test persons. (b) Within the "normal" group, a marginal, but not significant increase in *LC3B* expression is visible on the eighth day, before expression returns to the initial level during convalescence. (c) No alterations of *LC3B* mRNA expression occur in the "low pre-existing DNA repair capacity" group. Statistical analyses were conducted as one-way ANOVA with Tukey's multiple comparisons test.

### 3.5.8 Alteration of *BECN1* mRNA expression during calorie restriction

The association of Beclin-1 and CR is not characterized very well. One of the few studies states that nutrient deprivation for 48 h leads to significantly increased mRNA level of *BECN1* in human epithelial cells (Pan *et al.*, 2019). In order to contribute to a better understanding of the role *BENC1* plays in the effects of CR, mRNA expression analyses of the 18 volunteers undergoing F.X. Mayr therapy were conducted. Individual values of *BECN1* mRNA expression are displayed in **Figure 35**. Overall, slight changes in *BECN1* expression are observable throughout the study period, with the exception of test person 12. Six test persons

(test persons 3, 4, 5, 11, 12 and 15) display a higher mRNA expression during convalescence than in the beginning of the study. Of these six individuals, four test persons' (test persons 3, 4, 5 and 15) *BECN1* expression is highest during convalescence. mRNA expression is highest on the eighth day in five individuals (test persons 2, 10, 11, 12 and 18), with test persons 11 and 12 displaying still higher expression levels during convalescence compared to the beginning of the study. Nine of the 18 subjects (test persons 1, 6, 7, 8, 9, 13, 14, 16 and 17) show no alteration or a decrease of *BECN1* mRNA expression at either time point. Three of these individuals (test persons 1, 8 and 14) display the lowest expression on the eighth day, whereas test persons 16 and 17 display the lowest expression during convalescence. Two subjects (test persons 9 and 13) show no notable alterations of *BECN1* expression at all. In test persons 7 and 8 *BECN1* mRNA expression decreases on the eighth day and remains at that level during convalescence.

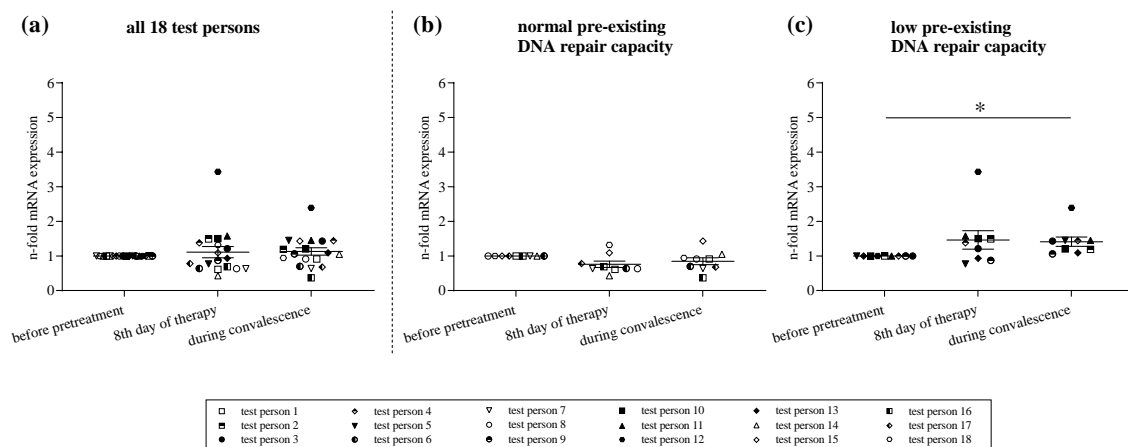


**Figure 35: mRNA expression profiles of *BECN1* during calorie restriction.**

Test persons 3, 4, 5, 11, 12 and 15 demonstrate a higher *BECN1* mRNA expression during convalescence than before pretreatment. In test persons 3, 4, 5 and 15 *BECN1* expression is highest during convalescence. Test persons 2, 10, 11, 12 and 18 show the highest expression on the eighth day, followed by a decrease on the last point of measurement. *BECN1* mRNA expression is still higher in the end of the study in test persons 11 and 12. Test persons 1, 6, 7, 8, 9, 13, 14, 16 and 17 show no alteration or a decrease of *BECN1* expression on either time point, whereby test persons 1, 8 and 14 display the lowest expression on the eighth day and test persons 16 and 17 display the lowest expression during convalescence. Test persons 9 and 13 show a nearly constant *BECN1* mRNA expression throughout the study, whereas *BECN1* expression in test persons 7 and 8 decreases on the eighth day and remains at this level during convalescence.



The study cohort was once more subdivided into individuals displaying a normal pre-existing DNA repair capacity and those displaying a low DNA repair capacity in the beginning of the study. Grouped analyses of all 18 test persons were again conducted via one-way ANOVA with subsequent Tukey's multiple comparisons test. Results of the analyses are plotted in **Figure 36**. When regarding all 18 test persons, no alteration of *BECN1* mRNA expression is visible on the eighth day as well as during convalescence (see **Figure 36 a**). Individuals displaying a normal pre-existing DNA repair capacity show a marginal decrease of *BECN1* expression on the eighth day as well as during convalescence (see **Figure 36 b**). **Figure 36 c** shows the grouped analysis of *BECN1* mRNA expression for the "low pre-existing DNA repair capacity" group. A not significant increase in *BECN1* expression is visible on the eighth day. However, during convalescence, a significant increase is obvious compared to the measurement before pretreatment (\*  $p = 0.0361$ ).



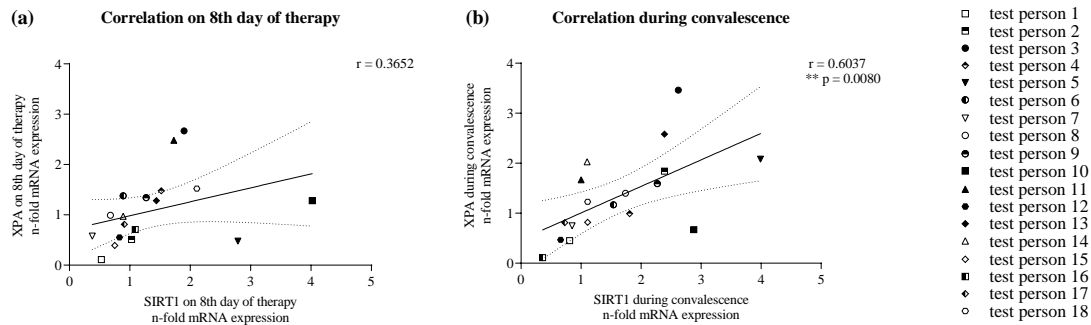
**Figure 36: Grouped analyses of *BECN1* mRNA expression.**

(a) *BECN1* mRNA expression does not change significantly when conducting grouped analyses of all 18 test persons. (b) Within the "normal" group, a marginal, but not significant decrease in *BECN1* expression is visible on the eighth day, as well as during convalescence. (c) A slight, not significant increase in mRNA expression occurs in the "low pre-existing DNA repair capacity" group on the eighth day of therapy. During convalescence, a significant increase in *BECN1* mRNA expression compared to the beginning of the study is visible in this group (\*  $p = 0.0361$ ). Statistical analyses were conducted as one-way ANOVA with Tukey's multiple comparisons test.

### 3.6 Correlations of mRNA expression patterns

In order to examine the underlying mechanism, through which CR influences aging-associated genes more closely, linear regression analyses were performed and Pearson's correlation coefficient was determined. The eighth day and the measurement of mRNA expression during convalescence are hereby considered separately. Interpretation of the extent of correlations were again conducted as suggested by Mukaka (Mukaka, 2012). Since it is known that SIRT1 can deacetylate XPA (Fan and Luo, 2010), it was interesting to look for a correlation of *SIRT1* and *XPA* mRNA expression. As mentioned before, SIRT1 and AMPK are known to interact closely, therefore a possible correlation of mRNA expression of these two aging-associated genes was performed. AMPK, in turn, phosphorylates Beclin-1 and is hence involved in autophagy, as mentioned in chapter 1.7.6. Therefore, a possible correlation of the expression of these two genes was researched. Moreover, SIRT1 also interacts with LC3, as stated in chapter 1.7.7. CR triggers LC3 as well as SIRT1 gene and protein expression, therefore a correlation of the two can be assumed. As stated before, SIRT1 also deacetylates Beclin-1, thus an analysis of the correlation of mRNA expressions was performed. Furthermore, SIRT3 can activate SOD2 (Qiu *et al.*, 2010), resulting in a reduction of oxidative stress. Within the scope of this work, mRNA expression profile of these two aging-associated genes were correlated. Moreover, FOXO3 induces *SOD2* mRNA expression (Kops *et al.*, 2002) and thus regulates ROS signaling. Consequently, a possible correlation of *FOXO3* and *SOD2* mRNA expression was also considered. Lastly, SIRT3 is able to deacetylate FOXO3 (Tseng *et al.*, 2013), which finally results in an increased protection from oxidative stress. In order to clarify a possible correlation on mRNA level, these two genes were also correlated.

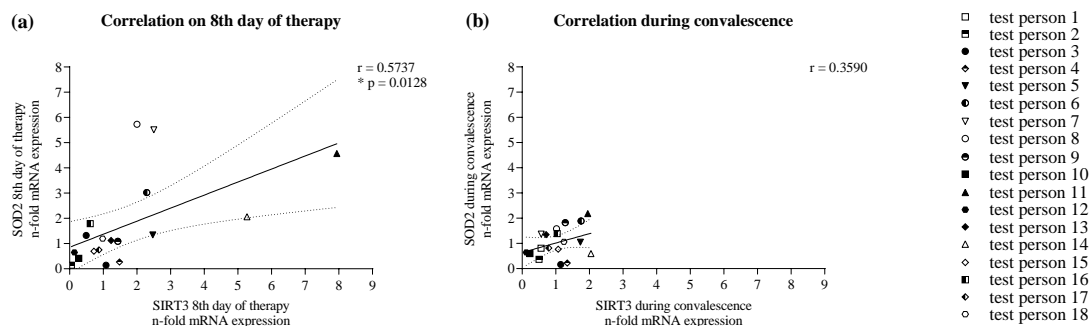
Linear regression analyses of *SIRT1* and *XPA* mRNA expression were published in Matt *et al.* 2020 (Matt *et al.*, 2020). Resulting correlations of both mRNA expressions are displayed in **Figure 37**. On the eighth day of therapy, a low positive ( $r = 0.3652$ ) correlation of *SIRT1* and *XPA* expression is visible (see **Figure 37 a**). During convalescence, however, a moderate positive, significant correlation (\*\*  $p = 0.0080$ ;  $r = 0.6037$ ) is evident, as shown in **Figure 37 b**.



**Figure 37: Correlation of *SIRT1* and *XPA* mRNA expression.**

(a) A low positive correlation ( $r = 0.3652$ ) of *SIRT1* and *XPA* mRNA expression can be stated for the eighth day of therapy. (b) A moderate significant positive correlation ( $** p = 0.0080$ ;  $r = 0.6037$ ) of *SIRT1* and *XPA* mRNA expression is apparent during convalescence.

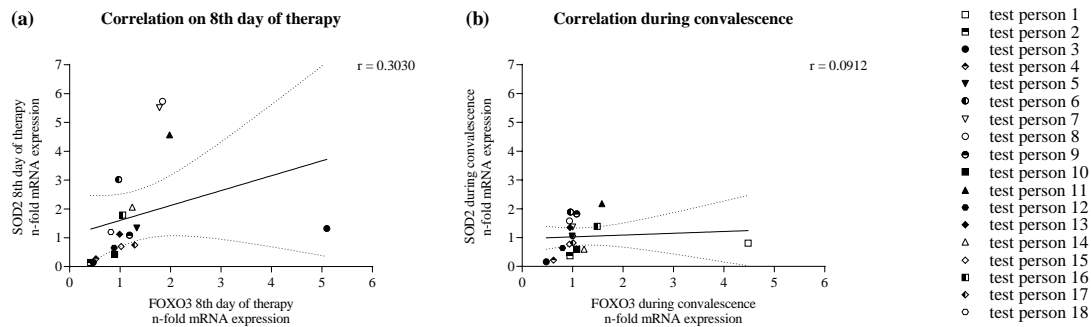
Linear regression analyses as well as correlations of *SIRT3* and *SOD2* mRNA expression are displayed in **Figure 38**. *SIRT3* and *SOD2* mRNA expression show a moderate positive, but significant correlation ( $* p = 0.0128$ ;  $r = 0.5737$ ) on the eighth day of therapy (see **Figure 38 a**), whereas only a low positive correlation ( $r = 0.3590$ ) persists during convalescence (see **Figure 38 b**).



**Figure 38: Correlation of *SIRT3* and *SOD2* mRNA expression.**

(a) A moderate significant positive correlation ( $* p = 0.0128$ ;  $r = 0.5737$ ) of *SIRT3* and *SOD2* mRNA expression is visible on the eighth day of therapy. (b) During convalescence a low positive correlation can be stated for *SIRT3* and *SOD2* mRNA expression.

**Figure 39** displays linear regression analyses and resulting correlations of *FOXO3* and *SOD2* mRNA expression. For either time point, a low positive correlation (eighth day of therapy) or a negligible correlation (during convalescence) of *FOXO3* and *SOD2* mRNA expression can be recorded ( $r = 0.3030$  for the eighth day and  $r = 0.0912$  during convalescence). Results are displayed in **Figure 39 a** and **Figure 39 b**.



**Figure 39: Correlation of *FOXO3* and *SOD2* mRNA expression.**

(a) A low positive correlation of *FOXO3* and *SOD2* mRNA expression is visible on the eighth day of therapy ( $r = 0.3030$ ). (b) During convalescence, a negligible correlation can be stated for *FOXO3* and *SOD2* expression ( $r = -0.0912$ ).

Since only negligible or low correlations were calculated for the remaining aging-associated genes, further linear regression analyses are displayed in the appendix of this work. More precisely, correlations of mRNA expression patterns of *SIRT3* and *FOXO3*, *SIRT1* and *AMPK $\alpha$* , *SIRT1* and *LC3B*, *SIRT1* and *BECN1* as well as *AMPK $\alpha$*  and *BECN1* were calculated and depicted in chapter 7.1.2.

### 3.7 Calorie restriction mimetics

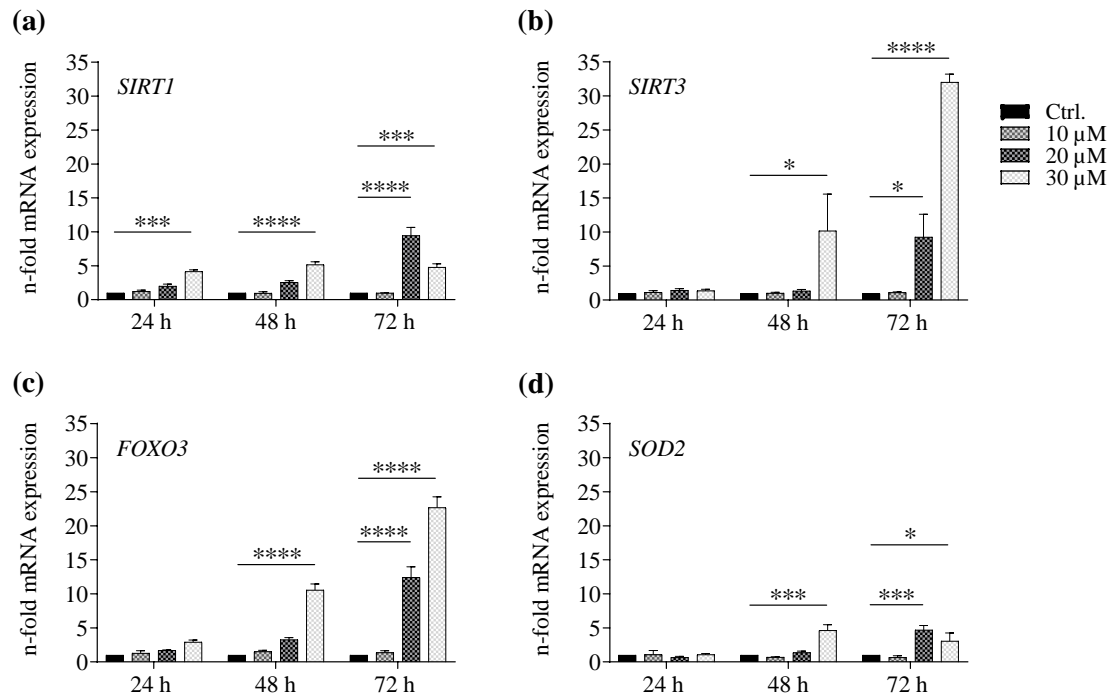
Following a calorie reductive diet in everyday life entails a lot of restrictions, which only few people are willing to accept for longer periods. At the same time, people want to live longer and age healthier. A promising way to achieve this is the use of CRMs, which promise to lead to the same beneficial effects as CR without its restrictions. Within the scope of B. Hochecker's master thesis, two potential CRMs were analyzed with regard to their effects on aging-associated genes (Hochecker, 2018). In brief, freshly isolated human PBMCs were treated *ex vivo* with different concentrations of resveratrol or spermidine and mRNA expression of *SIRT1*, *SIRT3*, *SOD2* and *FOXO3* was analyzed. The exact procedures for CRM treatments can be read in chapter 2.2.8. RNA isolation, cDNA synthesis and qPCR were conducted as presented in chapters 2.2.9, 2.2.10 and 2.2.12. The primers used are listed in chapter 2.1, **Table 7**. For statistical analyses, two-way ANOVA with Dunnett's multiple comparisons test was applied. Results were published in Nutrition and Health (Matt *et al.*, 2020).

#### 3.7.1 *Ex vivo* treatments of human PBMCs with spermidine

Spermidine has become increasingly the focus of aging research, especially as inducer of autophagy (Eisenberg *et al.*, 2009). Furthermore, spermidine shows a prolonging of lifespan in model organisms as well as human immune cells (Eisenberg *et al.*, 2009). To help elucidate the mechanism with which spermidine acts as a CRM, human PBMCs have been treated *ex vivo* with varying concentrations and varying amounts of spermidine. Furthermore, by conducting this approach, a clinical trial was not required. Applied concentrations of spermidine were 10  $\mu$ M, 20  $\mu$ M and 30  $\mu$ M for 24 h, 48 h and 72 h. Results of *ex vivo* treatments of human PBMCs with spermidine are displayed in **Figure 40**.

**Figure 40 a** displays that *SIRT1* mRNA expression increases significantly after a 24 h treatment with 30  $\mu$ M spermidine (\*\* $p = 0.0005$ ), whereas neither 10  $\mu$ M nor 20  $\mu$ M spermidine leads to significant alterations on *SIRT1* mRNA expression. After 48 h and 30  $\mu$ M spermidine, there is also a significant increase in *SIRT1* expression (\*\*\*\* $p < 0.0001$ ). No significant changes of *SIRT1* mRNA expression can be stated for 10  $\mu$ M and 20  $\mu$ M spermidine at 48 h. After a 48 h treatment a significant increase in *SIRT1* expression is visible for 20  $\mu$ M as well as 30  $\mu$ M spermidine (in both cases \*\*\*\* $p < 0.0001$ ). Solely a treatment with 10  $\mu$ M spermidine for 72 h has no effect on *SIRT1* mRNA expression. As shown in **Figure 40 b**, *SIRT3* mRNA expression

does not change significantly after a 24 h treatment with any concentration of spermidine. After a 48 h incubation of PBMCs with 10  $\mu$ M or 20  $\mu$ M spermidine no alteration of *SIRT3* mRNA expression is visible. However, a treatment with 30  $\mu$ M spermidine for 48 h results in a significant increase in *SIRT3* expression (\*  $p = 0.0121$ ). Furthermore, an incubation of PBMCs with 20  $\mu$ M and 30  $\mu$ M spermidine for 72 h results in a significant elevation of *SIRT3* expression levels (\*  $p = 0.0226$  for 20  $\mu$ M and \*\*\*\*  $p < 0.0001$  for 30  $\mu$ M). A 72 h treatment with 10  $\mu$ M spermidine does not change *SIRT3* mRNA expression significantly. As already stated for *SIRT3* mRNA expression, *FOXO3* mRNA expression does not change significantly after a 24 h treatment with the applied concentrations of spermidine (see **Figure 40 c**). A 48 h incubation with 30  $\mu$ M spermidine leads to a significant increase in *FOXO3* expression (\*\*\*\*  $p < 0.0001$ ), whereas 10  $\mu$ M and 20  $\mu$ M do not influence *FOXO3* mRNA expression significantly. An incubation of PBMCs with 10  $\mu$ M spermidine for 72 h has no effect on *FOXO3* expression levels. However, a 72 h treatment with 20  $\mu$ M as well as 30  $\mu$ M spermidine leads to a significant increase in *FOXO3* mRNA expression (in both cases \*\*\*\*  $p < 0.0001$ ). A 24 h treatment with 10  $\mu$ M, 20  $\mu$ M or 30  $\mu$ M spermidine does not affect *SOD2* mRNA expression, as displayed in **Figure 40 d**. After 48 h of 30  $\mu$ M spermidine treatment, a significant increase in *SOD2* mRNA expression is visible (\*\*  $p = 0.0006$ ), whereas 10  $\mu$ M and 20  $\mu$ M do not influence *SOD2* expression. A significant induction of *SOD2* mRNA expression is observable after a 72 h incubation of PBMCs with 20  $\mu$ M and 30  $\mu$ M spermidine (\*\*  $p = 0.0006$  for 20  $\mu$ M and \*  $p = 0.0311$  for 30  $\mu$ M). However, a treatment with 10  $\mu$ M spermidine for 72 h does not affect *SOD2* mRNA expression significantly.



**Figure 40: mRNA expression of aging-associated genes in human PBMCs after spermidine treatment.**

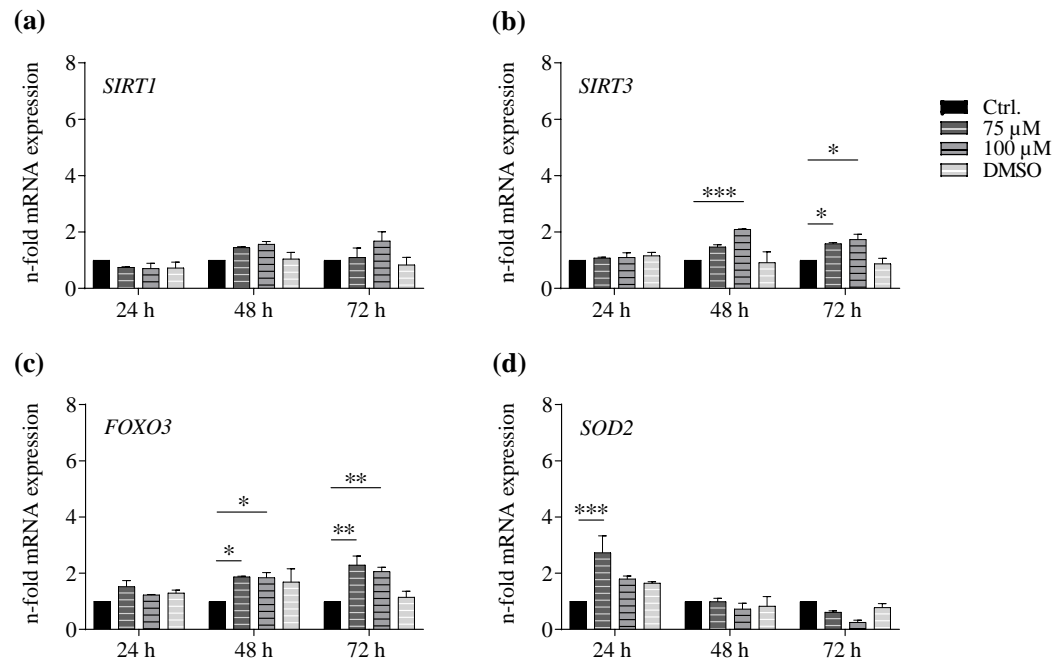
(a) *Ex vivo* treatment of PBMCs with spermidine induces *SIRT1* mRNA expression significantly after 24 h and 30 μM (\*\* $p = 0.0005$ ), 48 h and 30 μM (\*\*\*\*  $p < 0.0001$ ), 72 h and 20 μM (\*\*\*\*  $p < 0.0001$ ) as well as 30 μM (\*\* $p = 0.0001$ ). (b) *SIRT3* mRNA expression increases significantly after 48 h treatment with 30 μM spermidine (\*  $p = 0.0121$ ), 72 h and 20 μM (\*  $p = 0.0226$ ) as well as 30 μM (\*\*\*\*  $p < 0.0001$ ). A 24 h treatment with the mentioned concentrations of spermidine has no effect on *SIRT3* mRNA expression. (c) *FOXO3* mRNA expression level are elevated significantly after a 48 h treatment with 30 μM spermidine (\*\*\*\*  $p < 0.0001$ ), 72 h and 20 μM (\*\*\*\*  $p < 0.0001$ ) as well as 30 μM spermidine (\*\*\*\*  $p < 0.0001$ ). No significant changes of *FOXO3* expression can be stated for the 24 h treatments. (d) *SOD2* mRNA expression does not change significantly after a 24 h treatment with the mentioned spermidine concentrations. A 48 h treatment with 30 μM spermidine induces *SOD2* mRNA expression significantly (\*\* $p = 0.0006$ ). After 72 h of incubation with 20 μM and 30 μM *SOD2* expression level are significantly increased (\*\* $p = 0.0006$  for 20 μM and \*  $p = 0.311$  for 30 μM). Results represent the mean from two independently conducted experiments and corresponding SEM. Statistical analyses were conducted as two-way ANOVA with Dunnet's multiple comparisons test. Data were obtained in context of B. Hochecker's master thesis (Hochecker, 2018).

### 3.7.2 *Ex vivo* treatments of human PBMCs with resveratrol

One of the first substances obtaining the designation CRM was resveratrol. In model organisms, resveratrol displays life-prolonging properties that are very similar to the effects of CR (Howitz *et al.*, 2003; Wood *et al.*, 2004). In order to further elucidate the effect resveratrol has on mRNA expression of human cells, but without having to conduct a clinical trial, *ex vivo* treatments of freshly isolated human PBMCs were conducted. Resveratrol was applied at dosages of 0  $\mu$ M, 75  $\mu$ M, 100  $\mu$ M and a solvent control containing DMSO for 24 h, 48 h and 72 h respectively. Results of *ex vivo* resveratrol treatments are displayed in **Figure 41**.

None of the applied concentrations of resveratrol leads to significant alterations of *SIRT1* mRNA expression after the abovementioned incubation times (see **Figure 41 a**). As shown in **Figure 41 b**, a 24 h treatment with 75  $\mu$ M or 100  $\mu$ M resveratrol does also not affect *SIRT3* mRNA expression. However, *SIRT3* mRNA expression is increased significantly after a 48 h treatment with 100  $\mu$ M resveratrol (\*\* $p = 0.0006$ ). An incubation of PBMCs with 75  $\mu$ M or 100  $\mu$ M resveratrol for 72 h leads to a significant induction of *SIRT3* expression (\* $p = 0.0404$  for 75  $\mu$ M and \* $p = 0.0112$  for 100  $\mu$ M). As aforementioned for *SIRT1* mRNA expression, *FOXO3* mRNA expression does not change significantly after a 24 h treatment with any concentration of resveratrol (see **Figure 41 c**). A 48 h treatment with 75  $\mu$ M resveratrol increases *FOXO3* mRNA expression significantly (\* $p = 0.0243$ ), as well as a 48 h treatment with 100  $\mu$ M resveratrol (\* $p = 0.0285$ ). 72 h of incubation with 75  $\mu$ M as well as 100  $\mu$ M resveratrol induces *FOXO3* expression significantly (\*\* $p = 0.0017$  for 75  $\mu$ M and \*\* $p = 0.0072$  for 100  $\mu$ M). **Figure 41 d** displays the effects of resveratrol treatments on *SOD2* mRNA expression, which only increases significantly after a 24 h treatment with 75  $\mu$ M resveratrol (\*\* $p = 0.0003$ ), whereas the 100  $\mu$ M treatment has no significant effect. Furthermore, incubation times of 48 h and 72 h do not lead to significant alteration of *SOD2* mRNA expression regardless the concentration of resveratrol applied. No significant influence of the solvent control DMSO on the expression of the examined aging-associated genes is evident at any time of incubation.





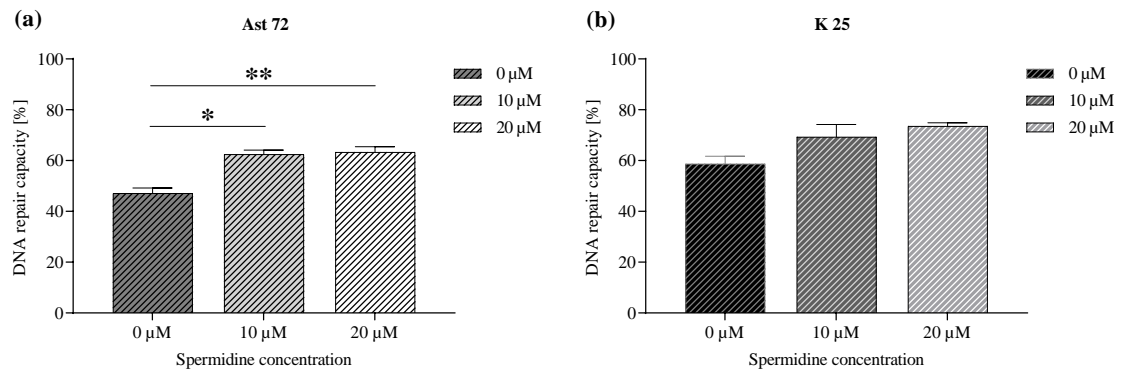
**Figure 41: mRNA expression of aging-associated genes in human PBMCs after resveratrol treatment.**

(a) No alteration of *SIRT1* mRNA expression level is visible for the applied resveratrol concentrations and incubation times. (b) *SIRT3* mRNA expression is increasing significantly after 48 h and a treatment with 100 μM resveratrol (\*\* $p = 0.0006$ ). After 72 h a treatment with 75 μM resveratrol leads to a significant increase in *SIRT3* expression (\*  $p = 0.0404$ ), as well as the treatment with 100 μM resveratrol (\*  $p = 0.112$ ). (c) A treatment with 75 μM or 100 μM resveratrol changes *FOXO3* mRNA expression significantly after 48 h (\*  $p = 0.0243$  for 75 μM and \*  $p = 0.0285$  for 100 μM) and after 72 h (\*\*  $p = 0.0017$  for 75 μM and \*\*  $p = 0.0072$  for 100 μM). A 24 h treatment with the mentioned concentrations of resveratrol does not affect *FOXO3* expression levels significantly. (d) *SOD2* mRNA expression is elevated after a 24 h treatment with 75 μM resveratrol (\*\* $p = 0.0003$ ), whereas the 100 μM treatment has no effect after 24 h. Incubation times of 48 h and 72 h with the abovementioned concentrations of resveratrol also have no effect on *SOD2* mRNA expression. None of the incubation times with the solvent control DMSO affects mRNA expression of the aging-associated genes mentioned. Results represent the mean from two independently conducted experiments and corresponding SEM. Statistical analyses were carried out as two-way ANOVA with Dunnet's multiple comparisons test. Data were obtained in context of B. Hochecker's master thesis (Hochecker, 2018).

### 3.8 Influence of spermidine on DNA repair capacity of human fibroblasts

Since the autophagy-inducing CRM spermidine has become increasingly the focus of aging research, the influence of this CRM on DNA repair capacity was also tested within the scope of this thesis. The practical experiments were carried out in three independent experiments as part of the bachelor thesis by M. Scherer (Scherer, 2020). In brief, primary fibroblasts of two male donors (8 years and 63 years of age) were treated with 0  $\mu\text{M}$ , 10  $\mu\text{M}$  and 20  $\mu\text{M}$  spermidine for 24 h in a cell culture incubator. Subsequently, mHCRA was performed by conducting electroporation of the fibroblasts. The exact protocol is described in chapter 2.2.13. Statistical analyses were conducted as two-way ANOVA with subsequent Tukey's multiple comparisons test.

Results of DNA repair capacity measurements after spermidine treatment of human fibroblasts are depicted in **Figure 42**. As presented in **Figure 42 a**, DNA repair capacity was significantly increased in fibroblasts of the younger donor (Ast 72) after a 24 h incubation with 10  $\mu\text{M}$  spermidine (\*  $p = 0.0100$ ). A treatment with 20  $\mu\text{M}$  spermidine for 24 h resulted in even a slightly higher DNA repair capacity (\*\*  $p = 0.0082$ ). A similar statement can be made for fibroblasts isolated from the older donor (K 25), as shown in **Figure 42 b**. In this case, DNA repair capacity increased clearly, but not significantly after the 10  $\mu\text{M}$  spermidine treatment for 24 h. A treatment with 20  $\mu\text{M}$  spermidine also resulted in a clear, but not significant increase in DNA repair capacity. Compared to the younger donor, the older donor showed a higher DNA repair capacity at all spermidine concentrations and also a higher initial DNA repair capacity (0  $\mu\text{M}$  spermidine). On the other hand, DNA repair capacity could be stimulated by spermidine treatment to a greater extent in fibroblasts isolated from the younger donor in comparison to the older donor. For both donors, the 10  $\mu\text{M}$  spermidine treatment led to a clear increase in DNA repair capacity, whereas the 20  $\mu\text{M}$  treatment resulted only in a slight further increase.



**Figure 42: DNA repair capacity in human fibroblasts after a 24 h spermidine treatment.**

(a) A 10 μM and 20 μM spermidine treatment for 24 h increases DNA repair capacity of human fibroblasts isolated from a younger donor significantly (\*  $p = 0.0100$  for 10 μM spermidine and \*\*  $p = 0.0082$  for 20 μM spermidine). (b) DNA repair capacity of fibroblasts isolated from an older donor also increases after a 24 h treatment with 10 μM and 20 μM spermidine, although not significantly. Results represent the mean and corresponding SEM from three independent experiments. Statistical analyses were carried out as two-way ANOVA with Tukey's multiple comparisons test. Data were obtained in context of M. Scherer's bachelor thesis (Scherer, 2020).

## 4 Discussion

Aging is a multifactorial complex process, which is not completely understood. However, it is certain that with increasing age, the incidence of aging-associated diseases increases. In order to better understand the mechanisms the aging process is accompanied with, intensive research is carried out using model organisms, cell cultures or human test persons. Besides, different theories try to explain aging. A description of all theories would go beyond this thesis, therefore four different types of aging processes are briefly provided here.

The “free radical theory of aging” and the refined “mitochondrial theory of aging” both suggest that ROS are responsible for the age-related decline of cellular processes, whereas the latter describe mitochondria as the main source of ROS (Harman, 1956; Alexeyev, 2009). The human immune system is also subject to aging. Immunoaging, which is also called immunosenescence, describes the inability of the immune system to execute a functional antibody- and cellular response against infections and vaccines due to the natural aging process. This dysfunction leads to an increased risk for infections and pathologies like cancer, since the daily assault of pathogens cannot be effectively countered. (Pawelec, 1999) Hallmarks of immunosenescence are a decrease of the amount of peripheral naïve T and B cells, a decreased capability to react to new antigens, an aggregation of memory T cells and a persistent low-level inflammation called inflamm-aging. However, oxidative stress contributes to maintaining this low-level inflammation, which, in turn, leads to oxidative stress. (Aiello *et al.*, 2019) Consequently one cause of immuno-aging can be countered by reducing oxidative stress. The “DNA damage theory of aging” regards the accumulation of damages to nuclear DNA as cause of the aging process (Best, 2009). To a low proportion, DNA mutations occur spontaneously, but defective DNA repair mechanisms mainly give rise to the formation of mutations out of these DNA damages. This is also the first step of cancer formation. On the other hand, the persistence of DNA damages can inhibit transcription and replication, thus impede normal cellular functions and lead to senescence or apoptosis. (Ou and Schumacher, 2018) Senescence and apoptosis, but also somatic DNA mutations are included in the hallmarks of aging (López-Otín *et al.*, 2013). With regard to an age-dependent accumulation of DNA mutations in humans, there are studies, which show an exponential increase in the mutation frequency with increasing age (Jaiswal *et al.*, 2014). Unlike DNA mutations, DNA damages can, and usually will, be repaired successfully. Since a large portion

of DNA mutations occur due to defective DNA repair systems, a way to reduce DNA mutations is to boost DNA repair mechanisms. Another theory of aging was presented by Pearl, who suggests that metabolic rate and mammalian longevity are inversely related (Pearl, 1928). CR has been shown to extend the lifespan and additionally the healthspan of various model organisms (Most *et al.*, 2017). Furthermore, a large-scale study researched the influence of CR on humans. They found, for example, that insulin sensitivity improves and that cardiometabolic risk factors like cholesterol and blood pressure were reduced as a consequence of CR. (Kraus *et al.*, 2019) Other study designs also researched the influence of CR on human health parameters, but the precise underlying molecular mechanisms remain mostly unknown.

Crucial for a healthy aging is a functional DNA repair in order to prevent or at least delay the manifestation of DNA damages as mutations and subsequently delay the onset of aging-associated diseases like cancer or the aging process itself. Another approach to counteract aging are the so-called CRMs, which are believed to offer the advantages of a CR without the disadvantages.

The present work intends to contribute to the elucidation of the molecular mechanisms of the beneficial effects of CR and CRMs on DNA repair and the expression of genes, which might counteract aging. For this purpose, the influence of CR on DNA repair and the expression of aging-associated genes were examined. In addition, the CRMs resveratrol and spermidine were investigated in terms of their potential to induce the expression of aging-associated genes. Lastly, first experiments were conducted researching the influence of spermidine on DNA repair capacity.

#### 4.1 Establishment of mHCRA

The optimal settings for an electroporation of any cell type have to be determined empirically (Kumar *et al.*, 2019). Therefore, the adaption of mHCRA for an application with human PBMCs, which was conducted within the scope of this thesis, comprised different optimization steps. This included finding a suitable transfection medium, adjusting voltages and capacitance as well as testing the amount of plasmid DNA. Taken together, the optimization steps carried out resulted in transfection efficiencies of 28.8 % - 40.5 % for pEGFP-N1 only,

21.5 % (SEM 0.8 %) for a co-transfection of PBMCs with irradiated pDsRed-Express-N1 and pEGFP-N1 and 35.3 % (SEM 1.4 %) for the co-transfection of PBMCs with non-irradiated pDsRed-Express-N1 and pEGFP-N1. Furthermore 10  $\mu\text{g}$  pEGFP-N1 and 30  $\mu\text{g}$  pDsRed-Express-N1 (either irradiated or non-irradiated) are required. This means that a total amount of 40  $\mu\text{g}$  of plasmid DNA was used for a successful transfection of human PBMCs. According to Potter and Heller, the recommended optimal amount of plasmid DNA per electroporation is 40  $\mu\text{g}$  (Potter and Heller, 2018). By using 40  $\mu\text{g}$  of plasmid DNA, the protocol established here uses the highest recommended amount of plasmid DNA, which, however, leads to good transfection efficiencies (see chapter 3.1).

Based on observations during mHCRA adaption for an application with human PBMCs, a possible influence of the concentration of plasmid DNA solutions on DNA repair capacity was researched in the first step. A transfection of the PBMCs with higher concentrated pDsRed-Express-N1 (1422 ng/ $\mu\text{l}$ ) resulted in a significantly higher NER capacity compared to the transfection with the lower concentration (778 ng/ $\mu\text{l}$ ) of pDsRed-Express-N1 (**Figure 6**). During the process of UVC irradiation, where the plasmid solution is applied in drops, the plasmids in the solution can lie above each other. The plasmids near the surface of the drops can shelter the plasmids below from UVC and thus irradiation is less effective. Consequently, fewer DNA damages are introduced into the plasmids, which leads to a seemingly increased DNA repair capacity. Drops consisting of smaller volumes might circumvent this, but as some liquid evaporates during irradiation, problems with collecting the drops arise with decreasing volume of the drops. Taking this into account, it is of utmost importance that the same plasmid solution with the same DNA concentration is used for all analyzes of the studies. If that is not possible, at least DNA solutions of the same concentration are to be used. In conclusion, volume and concentration of the plasmid-DNA solution play an important role in preparing plasmid DNA for mHCRA. To ensure comparability, one plasmid solution should therefore ideally be prepared for all analyzes in a study.

## 4.2 mHCRA is a highly reproducible method to determine DNA repair capacity of human PBMCs *ex vivo*

In order to obtain reliable data for *ex vivo* DNA repair capacity measurements, mHCRA was tested for reproducibility. Firstly, NER capacity was determined for four volunteers on three consecutive days as triplicates. The second reproducibility study consisted of two volunteers, whose DNA repair capacity was analyzed in triplicates on three consecutive days. This procedure was repeated three times. As already shown in **Figure 7** and **Figure 8**, conducting mHCRA for analyzing NER capacity leads to highly reproducible results. No significant differences regarding DNA repair capacity were detected for all test persons. Even when using only one reporter plasmid like Mendez *et al.*, mHCRA was demonstrated to be highly reproducible (Mendez *et al.*, 2011). This apparently also applies for the mHRCA established within the course of this thesis.

However, DNA repair capacity varied within the different participants of the reproducible studies. These inter-individual differences are in accordance with the findings of Bykov *et al.*, who found an up to 20-fold variation in DNA repair efficiency between test persons (Bykov *et al.*, 1999). Furthermore, it should be noted that for the first reproducibility study the same mixture of plasmid DNA was used, whereas for the second one three different batches were used. This resulted in slight differences regarding DNA repair capacities of the two volunteers over the three independently conducted studies. However, since the proportion of NER capacity from the two test persons remain the same, this circumstance actually underlines the high reproducibility of mHCRA. Taken together, this leads to the conclusion that the refined mHCRA as it was conducted in the course of this work, is suitable for *ex vivo* studies of human DNA repair ability.

## 4.3 Calorie restriction influences nucleotide excision repair in humans

The preservation of the genome's integrity is of utmost importance for the delay or prevention of aging-associated diseases and the aging process itself, since genomic instability is considered one of the hallmarks of aging (López-Otín *et al.*, 2013). Aging and the occurrence of aging-associated diseases, in turn, can be influenced positively by a reduction of calorie intake, as numerous studies using model organisms have already pointed out (McCay *et al.*, 1935; Jiang *et al.*, 2000; Colman *et al.*, 2014). With regard to studies involving human volunteers,

the focus so far seems to be on improving health parameters rather than on molecular biomarkers such as DNA repair (see chapter 1.1.2). One way to promote healthier aging in humans was suggested by Kagawa *et al.* by linking a lower caloric intake to an increased presence of individuals reaching above-average ages (Kagawa, 1978). At the molecular level, CR can reduce oxidative damages to the DNA of white blood cells (Hofer *et al.*, 2008). CR in form of short-term fasting (24 h pre and 24 h post chemotherapy) is also presumed to mitigate the toxicity of chemotherapeutic drugs to DNA and to support the recovery of human blood cells from DNA damages caused by chemotherapeutics (de Groot *et al.*, 2015). The cause of the protective effects of fasting or CR on normal cells from the effects of chemotherapeutics can be found in the hypothesis that normal cells enter a stress-resistant state at the onset of CR (Raffaghello *et al.*, 2008). In this context, cell proliferation is reduced and metabolization of degradation products of fats, proteins or cell organelles is initiated. In contrast to that, cancer cells disobey anti-proliferative signals as a consequence of a self-sufficient supply with growth signals. Mutations in oncogenes lead to a constitutive activation of proliferative pathways and in combination with dysfunctional tumor suppressor genes, this leads to disregarding the proliferation-inhibiting signals (Hanahan and Weinberg, 2000). As a consequence and in contrast to normal cells, cancer cells cannot enter this non-proliferative and protected condition and are probably more prone to chemotherapeutics.

Since the human organism is constantly exposed to DNA damaging agents and up to 70.000 lesions occur per cell and day (Lindahl and Barnes, 2000), the functionality of DNA repair mechanisms is of crucial importance to guard the integrity of the genome. One of the most important defense mechanism against bulky and helix-distorting DNA damages is NER. As mentioned before, mHCRA represents a reliable method for *ex vivo* studies of human NER (see chapter 3.2, **Figure 7**, **Figure 8** and chapter 4.2). In order to contribute to a better understanding of the impact of CR on human NER, mHCRA was conducted using freshly isolated PBMCs of volunteers taking part in F.X. Mayr therapy. Due to the fact that participants of this intervention lost an average of 5 kg of body weight, F.X. Mayr therapy represents a kind of CR. Two studies were performed within the course of this thesis, whereby eight participants were enlisted in the first study and 18 participants were enlisted in the second study. Regarded individually, the test subjects of both studies showed different reactions to the CR in terms of DNA repair capacity. For some, NER capacity increased, for



others it decreased slightly, and some volunteers showed no change in DNA repair capacity (**Figure 9** and **Figure 14**). This indicates a distinct donor variability. However, these results confirm the findings of others, who reported inter-individual variations of DNA repair ability (Bykov *et al.*, 1999).

Interestingly, a grouped analysis of all participants of the first study revealed a significantly increased DNA repair capacity during convalescence, compared to the measurement before pretreatment (**Figure 10**). Moreover, dividing the group of individuals into the lower 50 % and the higher 50 % depending on their pre-existing DNA repair capacity before pretreatment showed a clear picture. Solely individuals of the “low” group benefited from CR in terms of a significantly increased NER capacity on the eighth day of therapy as well as during convalescence (**Figure 11 a** and **b**). However, since only eight test persons were included in the first study, these data should be regarded with reservations. The results of the second study were remarkable, because the results of the first study could be confirmed independently one year later. Although the grouped analysis of all 18 participants of F.X. Mayr therapy did not show a significant increase in DNA repair capacity, a tendency towards an increase was visible throughout the study period (**Figure 15**). As already mentioned for the first study, the test subjects of the second study were also subdivided into a “low” and “normal” group according to the pre-existing DNA repair capacity before pretreatment. Remarkably, just like in the first study, individuals assigned to the “low” group displayed a significantly increased NER capacity on the eighth day as well as during convalescence (**Figure 16 a**). During the study period DNA repair capacity of individuals assigned to the “normal” group did not change in the second study either (**Figure 16 b**). Again, test persons displaying a lower DNA repair capacity before the CR intervention by F.X. Mayr therapy benefit from this CR in form of an increased NER capacity. Taken together, the results of the two studies strongly support the assumption, that CR can modulate human NER. Although with regard to DNA repair capacity, only individuals with a lower initial DNA repair seem to benefit from the intervention. It is also important to mention that repair capacities above 90 % were neither measured in this thesis nor during the establishment and modification of the assay. Therefore the individuals of the “normal” group displayed DNA repair capacities near the upper limit of detection of this assay, whereas the “low” group was clearly below. Up to now, few studies on the influence of CR on human NER exist. Langie *et al.* showed an improved NER upon a

dietary intervention consisting of antioxidant-rich blueberry and apple juice in individuals displaying certain genetic variants of NER genes (Langie *et al.*, 2010). Furthermore, an alternative protocol of F.X. Mayr therapy also led to an increase in DNA repair capacity of individuals, who displayed a lower DNA repair in the beginning of the study (Schöller-Mann *et al.*, 2020). These findings, as well as the findings of this thesis show that NER can be modulated by different kinds of dietary interventions. Furthermore, they confirm the existence of inter-individual differences regarding the benefit of these interventions in form of an increase in DNA repair capacity for the participants. Apart from the stimulatory effect on NER capacity, CR has also beneficial effects during cancer treatment. Side effects of chemotherapy, for example nausea, can be alleviated by fasting cycles prior to and/or after chemotherapy (Safdie *et al.*, 2009). In various model organisms, Raffaghello *et al.* showed an increased effectiveness of chemotherapeutics after a short-term starvation prior to the treatment. They hypothesized that the dependency of cancer cells on glucose and the low glucose level resulting from the fasting left the cancer cells more susceptible to the chemotherapeutics. (Raffaghello *et al.*, 2008)

The results acquired during this work strongly suggest that CR can contribute to a healthier aging by triggering DNA repair capacity and thus supporting the prevention of cancer. One of the steps in the process of cancer formation is acknowledged to be the increase of DNA mutations (Hanahan and Weinberg, 2011), which arise from DNA damages. By supporting DNA repair ability, CR may contribute to the prevention of the detrimental step, which leads to the formation of DNA damages out of DNA mutations and thus contribute to cancer prevention. Since cancer is known to be an aging-associated disease, CR may consequently contribute to a healthier aging process. CR also has further health-beneficial attributes, as described in chapter 1.1.2. Furthermore, a decrease of inflammation and IGF-1 blood level or an increase of insulin sensitivity are well-documented examples of the positive effects of CR on health parameters (Longo and Fontana, 2010; Ribarič, 2012). Hofer *et al.* reported a decrease of oxidative damages to DNA and RNA of blood cells as a consequence of CR (Hofer *et al.*, 2008). In turn, oxidative stress influences gene expression of NER-associated genes and NER capacity negatively (Langie *et al.*, 2007). Taken these findings together with the results of this thesis, the proposed mechanism of action could be as follows. Individuals displaying a lower DNA repair capacity in the beginning could suffer from a higher initial level of oxidative

stress, which caused this lower DNA repair ability. However, CR leads to an increase in NER capacity, which could be a consequence of the reduced level of oxidative stress. This hypothesis is supported by the results of mRNA expression analyses presented here. An increased *SOD2*, *SIRT3* and *FOXO3* mRNA expression probably leads to an increased availability of the corresponding proteins. This subsequently leads to a reduction of oxidative stress, thus supports an increase in NER capacity to a normal level.

#### 4.4 Correlation of CR with age and gender

Several researches already reported a link or an assumed link between a decline of DNA repair and age. In human leukocytes and lung fibroblasts, BER activity presumably decreases with increasing age (Atamna *et al.*, 2000). Furthermore, DSB repair in human PBMCs is also suspected to decrease with increasing age, whereas SSB repair remains at the same level even at older age (Garm *et al.*, 2013). Given the fact that DNA damages accumulate during aging, it is likely that not only DSB repair but also other DNA repair mechanisms show a decreased functionality with increasing age. In turn, impaired DNA repair processes lead to an increased accumulation of DNA damages, leading to a vicious cycle of damage accumulation and deterioration of repair processes with increasing age. Regarding DNA repair in humans, repair capacity for UV-induced DNA damages decreases in human fibroblasts with increasing age of the donor, with the decrease in DNA repair capacity being 0.6 % per year of life (Moriwaki *et al.*, 1996). The same percentage reduction of DNA repair capacity was demonstrated for human T lymphocytes with a value of 0.61 % per year of age (Wei *et al.*, 1993). However, Annett *et al.* found that *in vitro* aged T cell clones obtained from two healthy donors (26 years and 45 years of age) also show a decline in NER capacity, whereas *in vitro* aged T cell clones of an 80 year old donor, who was defined as healthily aged, showed no such decline (Annett *et al.*, 2004). Within the scope of this thesis, NER capacity was shown to be triggered by CR in individuals with an initially lower DNA repair capacity. In order to check whether the age of the individuals of these studies also correlate with their ability to repair DNA damages, these two parameters were compared. Additionally, DNA repair capacity of individuals were evaluated regarding gender differences. The first study revealed a moderate negative correlation of age and NER capacity before pretreatment (**Figure 12 a**), which confirms a decline of DNA repair ability with increasing age. Additionally, a decrease of DNA repair rates for CPDs and 6-4PPs in human fibroblasts was reported by Goukassian *et al.* (Goukassian *et*

*al.*, 2000). Since CPDs and 6-4PPs are repaired via NER, this means an increasing dysfunction of NER during the aging process. Within the scope of this thesis, mHCRA was used to measure NER capacity of human PBMCs. Since CPDs as well as 6-4PPs are repaired via NER, the findings of the first study conducted during this thesis support the previously mentioned statement that DNA repair declines with increasing age. However, this applies for the condition of the test persons' DNA repair capacity before the intervention by CR. On the eighth day, only a negligible correlation of age and NER capacity was noticeable (**Figure 12 b**), suggesting an improvement of DNA repair ability during the CR period. During convalescence, DNA repair and age of the individuals correlated moderately negatively (**Figure 12 c**), just like in the beginning of the study. However, this moderate negative correlation of age and DNA repair during convalescence hints at an improvement of NER capacity in consequence of CR (**Figure 12 c**). Within the course of the second study, only negligible correlations could be detected for all three measurement times (**Figure 17**). This means that regarding the age-dependent decline of DNA repair, the results of the first study could not be confirmed by the second study. However, it must be noted that in the first study conducted within the terms of this thesis, only eight individuals could be examined and interpretation of the results should therefore be viewed with caution. Some studies on a correlation of age and DNA repair show an age-related decline, however the studies by Annett *et al.* (Annett *et al.*, 2004) as well as the results of the work presented here showed no such clear decline. Garm *et al.* 2012 also found no clear decline in DNA repair with increasing age, but only for SSBs, whereas the repair of DSBs declined with increasing age in human PBMCs (Garm *et al.*, 2013). Depending on the type of DNA damage and the repair pathway, there are obviously differences regarding a correlation of repair capacity and age.

However, the results of Annett *et al.* (Annett *et al.*, 2004) and the results of this thesis showed a donor variability with regard to an age-related reduction in the DNA repair capacity. This donor variability could also be the cause of a missing correlation between age and a decline of NER capacity, which is presented here. Additionally, all test persons involved in the studies on the influence of CR on DNA repair were healthy, physically fit and possibly not yet subject to a decline of DNA repair capacity. Taking this into account and remembering the small number of test persons, it was likely that no correlation of age and DNA repair capacity could be observed.

Since it is known that women and men age differently, the results of NER capacity measurements were also analyzed regarding gender differences. DNA repair, particularly the NER pathway, have already been suggested to be subject to gender differences (Slyskova *et al.*, 2011; Wei *et al.*, 1993). However, with regard to possible gender-specific differences in NER capacity, no difference between female and male individuals could be found in both studies presented in this work (**Figure 13** and **Figure 18**). A study of gender-specific differences in the ability to repair DSBs and SSBs came to similar results. Here, too, no difference in the repair of this DNA damage between female and male test subjects could be found in human PBMCs. (Garm *et al.*, 2013) Since the study cohort involved in the analyses conducted within the scope of the thesis presented here was rather small, the results have to be considered as preliminary before drawing final conclusions. Apparently, some DNA repair pathways show gender-specific differences and others do not.

#### 4.5 Calorie restriction influences mRNA expression of aging-associated genes

In order to contribute to a better understanding of the molecular mechanisms leading to beneficial effects of CR on DNA repair and more generally aging, mRNA analyses of aging-associated genes were conducted for participants of the second F.X. Mayr study. Consequently, aging-associated genes of interest were chosen due to their role in aging and DNA repair. mRNA expression of *SIRT1*, *XPA*, *SIRT3*, *SOD2*, *FOXO3*, *AMPK $\alpha$* , *LC3B* and *BECN1* was therefore analyzed using qPCR technique. Regarding the course of mRNA alterations for the individual volunteers, different expression patterns were evident (**Figure 19**, **Figure 22**, **Figure 25**, **Figure 27**, **Figure 29**, **Figure 31**, **Figure 33** and **Figure 35**). Some individuals show an increase of mRNA expression during the course of the CR intervention, whereas some test persons show decreasing mRNA levels and mRNA expression of some individuals does not change at all. Test person 11, who experienced an increase in all mRNA expressions on day eight as well as during convalescence, is noticeable here. Furthermore, test persons 3 and 5 also show increases in mRNA expressions of several aging-associated genes (*SIRT1*, *XPA* and *AMPK $\alpha$*  in test person 3; *SIRT1*, *XPA*, *SIRT3* and *AMPK $\alpha$*  in test person 5). Interestingly all of these three test persons also displayed significant increases in DNA repair capacity and all three were assigned to the “low pre-existing DNA repair capacity” group. As a result of CR, these individuals benefit from the intervention by acquiring a better

protection against DNA damages in form of an improved DNA repair and probably an improved defense against some causes of aging due to a higher expression of aging-associated genes. The course of the mRNA expression from sampling before pretreatment, on the eighth day and during convalescence also differs in individual subjects. Some aging-associated genes seem to be affected by CR to a higher degree and in a higher number of volunteers, like *SIRT1*, *XPA*, *AMPK $\alpha$*  and *SOD2*. On the other hand, mRNA expression levels of some of the genes only alter in few individuals or they do not change at all (*SIRT3*, *FOXO3*, *LC3B* and *BECN1*). Just like the results of DNA repair capacity measurements, the results of gene expression analyses support the presence of a distinct donor variability regarding the effect of CR on the individual test persons.

Following the analyses of the individual courses of mRNA expression, grouped analyses of all 18 test persons were performed. Subsequently, the same grouping as with DNA repair analyses was applied, thus stratifying the test persons according to their pre-existing DNA repair capacity and resulting in groups of nine test persons each. Significant alterations of mRNA expressions were not visible for *SIRT3*, *FOXO3*, *LC3B* and *BECN1* neither on the eighth day nor during convalescence or from the eighth day to the measurement during convalescence. This applies for the analysis of all 18 test persons and for the “low” and the “normal” groups. *SIRT1* mRNA expression was significantly increased during convalescence, compared to the first analysis before pretreatment, when regarding all 18 test persons. The “low” group also displayed a significant increase on the eighth day and during convalescence, each compared to the measurement before pretreatment, whereas no alterations could be detected for the “normal” group. Strikingly, a similar course of mRNA expression was visible for *XPA*, but these alterations were not significant. In contrast to these findings, Schöller-Mann *et al.* found no significant increase in *SIRT1* mRNA expression during a 12-day CR in form of F.X. Mayr therapy (Schöller-Mann *et al.*, 2020). Presumably *SIRT1* reacts very sensitively to different kind of CR, therefore its mRNA expression might be affected differently by different study designs. Besides, *SIRT1* is an important factor for the regulation of NER, as it modulates the acetylation status of *XPA* (Fan and Luo, 2010), which in turn, plays an essential role in NER (Sugitani *et al.*, 2016). An increased expression of *SIRT1* mRNA may thus lead to increased protein levels of *SIRT1* and subsequently to increased deacetylation of *XPA*. The increase in *XPA* mRNA expression thus proposes an improved NER, which, in turn, is triggered by CR.

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An improvement of DNA repair may consequently contribute to maintaining the genome's integrity and thus contribute to decelerate the aging process.

mRNA expression of *SIRT3* and *FOXO3* did not change significantly during CR, but a distinct donor variability was observable. Even when stratifying the test persons into the “low” and “normal” group, mRNA expression did not alter significantly. However, individual test persons display an increased *SIRT3* or *FOXO3* mRNA expression, although the whole cohort shows no change at all. *SOD2* mRNA expression, in turn, increased significantly on the eighth day in individuals assigned to the group displaying a normal DNA repair in the beginning, but decreased significantly from the eighth day to the measurement during convalescence. Neither analysis of all 18 test persons, nor analysis of the “low” group revealed significant changes of *SOD2* expression. Just like mRNA expressions of *SIRT3* and *FOXO3*, the expression of *SOD2* is subject to donor variability. One main function of *SIRT3* is to regulate the quantity of ROS within cells. In order to achieve this, *SIRT3* deacetylates and thus activates *SOD2*, which subsequently eliminates ROS and protects the cells from oxidative stress (Qiu *et al.*, 2010). Contrarily to the results presented here, *SIRT3* mRNA expression can also be significantly induced by a 12-day CR, thus improving the defense against oxidative stress (Schöller-Mann *et al.*, 2020). F.X. Mayr therapy as researched by Schöller-Mann *et al.* comprised twelve days and the significant increase in *SIRT3* mRNA expression was shown at the end of the study period on day twelve (Schöller-Mann *et al.*, 2020). However, nine individuals included into the F.X. Mayr study presented here showed the highest *SIRT3* expression on the eighth day of therapy, which means 15 days after the beginning of the intervention. The increase in *SIRT3* mRNA expression occurred at a similar time point as in the 12-day CR study presented by Schöller-Mann *et al.*, which leads to the assumption that the time of sampling is also crucial for the verification of positive influences of CR on gene expression. Apart from that, *SIRT3* protein level might be stabilized by the CR intervention investigated in this work. However, this was not the scope of this thesis. With regard to oxidative stress and aging, an increased level of ROS and the resulting damages to nucleic acids and proteins are proposed to contribute to the aging process (Harman, 1956), therefore a reduced amount of ROS in a cell contributes to defying aging. Individuals displaying an increased *SIRT3* or *SOD2* mRNA expression might thus benefit from CR in form of a better protection against oxidative stress and a healthier course of aging. However, it must be noted that in the present work this only

seems to be the case for individuals and not for the entire cohort. A reduction of systemic oxidative stress has already been documented by Redman *et al.* in a two-year CR study on humans (Redman *et al.*, 2018). This supports the hypothesis suggested here that a boost of antioxidant mechanisms such as SOD2 might finally contribute to a slowing of aging.

A clear association of FOXO3 with aging and longevity has already been reported for humans (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). Furthermore, FOXO3 controls ROS signaling by inducing SOD2 (Kops *et al.*, 2002), which increases the protection from oxidative damage. Consequently, the increase in SOD2 mRNA expression might also not be caused directly by CR, but indirectly by the transcription factor FOXO3. Moreover, FOXO3 can be deacetylated by SIRT3 as a consequence of oxidative stress. This leads to an upregulated expression of genes, which are crucial for mitochondrial homeostasis and finally contribute to a better protection of mitochondria from oxidative stress. (Tseng *et al.*, 2013) Additionally, FOXO3 can be deacetylated by SIRT1 as a response to oxidative stress, leading to an increased stress resistance (Brunet *et al.*, 2004). Taking this into account, an induction of SIRT1 mRNA expression by CR, as presented here, can eventually lead to an increased deacetylation of FOXO3, which increases stress resistance and hence counteracts aging.

Although it was described for LC3 mRNA expression to be inducible in humans by CR (Yang *et al.*, 2016), this could not be confirmed for the CR intervention researched within the scope of this thesis. Neither the analysis of all 18 test persons, nor analyses of the subdivided groups according to the pre-existing DNA repair capacity resulted in alterations of LC3B mRNA expression. However, it must be noted that the aforementioned increase in LC3 mRNA expression was observable in test persons, who performed CR three or more years. The individuals researched for this thesis took part in a six-week F.X. Mayr therapy. This difference in duration of the two CR studies and the accompanying differing sampling times could be reasons for the dissenting results regarding LC3 mRNA expression. However, this is a very preliminary interpretation of the collected data. Lastly, the inter-individual variability of mRNA expression, which was described for the other aging-associated genes earlier, is also slightly noticeable for LC3B. This supports the assumption, that not all individuals react the same to CR and that some individuals will benefit more from such an intervention.



Grouped analyses of mRNA expression of the metabolic sensor *AMPK $\alpha$*  revealed a significant induction on the eighth day and during convalescence for all 18 test persons. After subdivision into the two groups “low” and “normal” according to the pre-existing DNA repair capacity, only individuals assigned to the “low” group displayed a significantly increased *AMPK $\alpha$*  expression at both times of measurement. However, a tendency towards an increase was also observable for the “normal” group. The significantly increased *AMPK $\alpha$*  expression of the whole study cohort and in the “low” group is similar to the mRNA expression observed for *SIRT1*. As an important nutrient sensor, it is likely that *AMPK $\alpha$*  expression is affected by CR. Nutrient scarcity, for example, activates AMPK (Mihaylova and Shaw, 2011), but also SIRT1 (Cohen *et al.*, 2004). Activated AMPK leads to an increase in NAD<sup>+</sup>, which stimulates SIRT1 activity. This leads to an altered activity of SIRT1 targets like FOXO3. (Cohen *et al.*, 2004) Furthermore, this activation of SIRT1 also activates AMPK, resulting in a bidirectional activation of SIRT1 and AMPK (Hou *et al.*, 2008). Although on the level of gene expression and not protein expression, the results presented in this work show a similar reaction of *SIRT1* and *AMPK $\alpha$*  to CR in terms of a significant increase in gene expression. On a lower level, *XPA* mRNA expression is also affected similarly to *SIRT1* and *AMPK $\alpha$* . Furthermore, the increase in mRNA expression is significant in individuals displaying a low DNA repair capacity before CR. Taken into account that AMPK and SIRT1 are closely linked, that SIRT1 and XPA are also closely linked and that the increases in mRNA expression of these genes were observed in individuals displaying a low DNA repair capacity in the beginning, the stimulation of mRNA expression might improve DNA repair and hence support a healthy aging process.

It is documented that the expression of Beclin-1 decreases in aging human brain (Shibata *et al.*, 2006). Beclin-1 is an important factor in the process of autophagy, which, when acetylated, inhibits autophagosome maturation and thus an important step in autophagy. On the other hand, SIRT1 can deacetylate Beclin-1 and therefore promote autophagosome maturation. (Sun *et al.*, 2015) Consequently, SIRT1 and Beclin-1 are interacting in order to assure autophagy. Just like demonstrated for *SIRT1* within the scope of this thesis, mRNA expression of *BECN1* is inducible by nutrient deprivation in cell cultures of human cells (Pan *et al.*, 2019). *BECN1* mRNA analyses conducted in this thesis showed a donor variation, just like analyses of the other aging-associated genes. For individuals displaying a low pre-existing DNA repair capacity, grouped analysis displayed a significant increase in *BECN1* mRNA expression

during convalescence compared to the beginning of the study. This increase has also been shown for *SIRT1*, *AMPK $\alpha$*  and, in tendency, for *XPA* mRNA expression, respectively. An elevated *BECN1* mRNA expression in combination with an increased *SIRT1* mRNA expression could thus hint at an improved autophagy due to CR.

#### 4.6 Alterations of mRNA expression of *SIRT1* and *XPA* upon calorie restriction correlate

As displayed in **Figure 4**, the aging-associated genes researched within the scope of this thesis interact and are possibly inducible by CR. In order to take a closer look at the mechanisms by which CR influences these aging associated genes, correlation of two genes each was determined. The most obvious correlation based on the course of mRNA expression during CR was between *SIRT1* and *XPA*. Fan and Luo pointed out that the interaction of *SIRT1* and *XPA* is required for an optimal proceeding of NER (Fan and Luo, 2010). At the level of mRNA expression, a significant correlation of the two genes was obvious during convalescence (see **Figure 37**) and thus verified the hypothesis that the expression of these two genes is affected similarly by CR. *SIRT1* showed a significantly increased mRNA expression in individuals displaying a low pre-existing DNA repair capacity, *XPA* mRNA expression was also slightly elevated in these individuals and the two genes correlate significantly. Additionally, NER capacity was also significantly enhanced in these individuals. Taken together, these results give rise to the assumption, that CR not only induces mRNA expression of the aforementioned genes, but leads to an improved NER capacity, at least partly due to this higher mRNA expressions. *SIRT1* and *XPA* are known to interact closely in order to assure an optimal procedure of NER (Fan and Luo, 2010). Moreover, *XPA* is an essential factor for damage verification of NER (Li *et al.*, 2015) and *SIRT1* is crucial for the efficient repair of UVB-induced DNA damages (Ming *et al.*, 2010). Consequently, a higher mRNA expression of these factors can lead to enhanced DNA repair capacity. This hypothesis is confirmed by the significantly improved NER capacity presented in this thesis. Besides, a shift from growth towards DNA repair caused by CR was reported by Mercken *et al.* in 2013 (Mercken *et al.*, 2013). The results of this work, especially the improved DNA repair as well as the higher mRNA expression of *SIRT1* and *XPA* are in accordance with these findings. However, it should be noted that not all individuals benefit from the CR intervention in terms of an

improved NER. But then again it is questionable, whether individuals already displaying a normal DNA repair capacity could further benefit from an improvement in NER.

A possible correlation has also been proposed for the aging-associated genes *SIRT3* and *SOD2*, because *SIRT3* deacetylates *SOD2* upon CR and thus improves its anti-oxidative properties (Qiu *et al.*, 2010). Moreover, a mild stimulation of mRNA expression by intermittent fasting was reported for both genes (Wegman *et al.*, 2015). Although the results of this thesis did not confirm an induction of mRNA expression for these two genes, a significant correlation of *SIRT3* and *SOD2* mRNA expression was revealed on the eighth day of F.X. Mayr therapy (see **Figure 38**). An enhanced expression of both genes might thus lead to an increased availability of both proteins and finally to an improved defense against oxidative stress. Furthermore, a reduction of oxidative stress supports a slowing of aging, since higher ROS levels as a consequence of mitochondrial dysfunction are suggested to be one cause of aging (Barja, 2014). An increased availability of *SOD2* and *SIRT3* protein as a final result of an increased mRNA expression might thus support a slowing of aging.

Since *FOXO3* has been shown to induce *SOD2* mRNA expression (Kops *et al.*, 2002), a correlation of mRNA expression of both genes was researched but no significant correlation was shown for *FOXO3* and *SOD2* mRNA expression (see **Figure 39**). The same can be stated for *FOXO3* and *SIRT3* mRNA expression, as displayed in **Figure 44**, although both genes have been shown to correlate in that *SIRT3* deacetylates and thus activates *FOXO3* (Tseng *et al.*, 2013). Nevertheless, individuals displaying increases in mRNA expression of these genes likely benefit from a CR intervention, as mentioned in chapter 4.5.

Although *SIRT1* and *AMPK $\alpha$*  are influencing each other bidirectionally (Hou *et al.*, 2008), no correlation of mRNA expression could be shown within the scope of this thesis (see **Figure 45**). *SIRT1* mRNA expression as well as *AMPK $\alpha$*  mRNA expression was significantly induced when analyzing all test persons and when analyzing individuals exhibiting a low pre-existing DNA repair capacity (see **Figure 20** and **Figure 32**). It was assumed that with both genes significantly triggered by CR, a correlation of both genes might be possible. This was not the case for the results of this work.

Yang *et al.* reported an induction of *LC3* mRNA expression by long-term CR (Yang *et al.*, 2016), whereas *SIRT1* deacetylates *LC3* (Huang *et al.*, 2015). Consequently, these two proteins interact. This gave rise to the assumption that mRNA expressions of these two also correlate. Since *SIRT1* mRNA expression was increased significantly, but *LC3B* expression was not affected by CR (see **Figure 19** and **Figure 34**), the verification of the aforementioned assumption was not likely. Indeed, the results of this work did not show a significant correlation for *SIRT1* and *LC3B* mRNA expression, or for *SIRT1* and *BECN1* mRNA expression, as displayed in **Figure 46**, **Figure 39** and **Figure 47**. Despite the lack of a correlation of *SIRT1* and *LC3B* mRNA expression, the significant induction of *SIRT1* mRNA expression is rated positive for the aging process.

Since the gene expression patterns of *AMPK $\alpha$*  and *BECN1* throughout the study period were similar, a correlation of mRNA expression was assumed. AMPK functions as activator of autophagy by phosphorylating Beclin-1 (Kim *et al.*, 2013), hence an interaction of both proteins is proven. Although mRNA expression of both genes were significantly increased during convalescence in individuals showing a low pre-existing DNA repair capacity, no significant correlation could be determined (see **Figure 48**). Despite that, an induction of *AMPK $\alpha$*  and *BECN1* mRNA expression presumably contributes to an improved autophagy. The process of autophagy can be triggered by CR. While autophagy itself was not researched within the course of this thesis, the increase of autophagy-related genes like *AMPK $\alpha$*  and *BECN1* give rise to the assumption, that F.X. Mayr therapy might trigger autophagy. Since a decline of autophagy is associated with aging in rats (Donati *et al.*, 2001) and might also decline in aging humans due to the conserved mechanism of this process, an increase in autophagy supports a healthier aging.

#### **4.7 Ex vivo treatments of human PBMCs with CRMs alter mRNA expression of aging-associated genes**

Although CR has a multitude of positive effects on aging and human wellbeing, it is accompanied with restrictions in everyday life. Due to that, few people are willing to adjust their lifestyle permanently to CR. One way to experience the positive effects of CR and at the same time live without restrictions are possibly CRMs. These substances promise to be health-beneficial without the drawbacks of CR. Up to now, few studies on mRNA expression of

aging-associated genes after a treatment with either resveratrol or spermidine were published. Hence, the results of the work presented here contribute to a better understanding of the underlying molecular mechanisms of CRM treatments on the level of gene expression. Two CRMs were researched within the scope of this thesis, namely spermidine and resveratrol. Both CRMs have been shown to extend lifespan of model organisms (Eisenberg *et al.*, 2009; Howitz *et al.*, 2003; Wood *et al.*, 2004). With regard to mRNA expression after CRM treatment, mRNA expression of *SIRT1* was induced in human monocytes after resveratrol treatment (Tsuchiya *et al.*, 2017). Furthermore, mRNA expression of *SIRT1*, *SIRT3* and *SOD2* was shown to be increased significantly after a resveratrol treatment, suggesting an enhanced anti-oxidative defense (Cosín-Tomás *et al.*, 2019). However, spermidine has recently become the focus of anti-aging research and is considered safe for dietary supplementation in humans (Schwarz *et al.*, 2018). Therefore, few studies on the influence of spermidine on human mRNA expression exist. A spermidine treatment for example restores a dysfunctional autophagy in aged human chondrocytes, whereby the spermidine treatment significantly induced *BECN1* and *LC3* mRNA expression (Sacitharan *et al.*, 2018). Autophagy is known to possess anti-aging properties (Rubinsztein *et al.*, 2011). Hence, the induction of the expression of these two autophagy- and aging-associated genes can contribute to a slowing of aging.

If CRMs are actually able to mimic the effects of CR, a similar induction of mRNA expression of aging-associated genes should be detectable within the course of this work. Therefore, mRNA expression of the aging-associated genes *SIRT1*, *SIRT3*, *FOXO3* and *SOD2* was analyzed in human PBMCs 24 h, 48 h and 72 h after treatment with resveratrol or spermidine, respectively. Before the results of CRM treatments are discussed in detail in the following chapters 4.7.1 and 4.7.2, it can be anticipated that both CRMs induced mRNA expression of the researched aging-associated genes even to a greater extent than the CR in form of F.X. Mayr therapy. Nevertheless, only the influence on the expression of aging-associated genes were researched within this work. This represents an important fraction of beneficial effects, but simultaneously only a part of the bigger picture. The influence of CRMs on a process like NER in PBMCs, for example, is pending. Considering the results of this work in context of CRMs so far, it can be said that CRMs can currently support a CR intervention, but that they cannot replace the holistic approach of a CR. F.X. Mayr therapy, as is was researched in this work, consists of different treatments, e. g. manual abdominal treatments, whereby CR is one

important part of these treatments. However, taking these promising results on CRMs into account, they may have a supportive effect, especially when the expression of aging-associated genes cannot be triggered by CR in an individual.

#### 4.7.1 Spermidine induces mRNA expression of aging-associated genes

Spermidine has only recently become the focus of health and geriatric research. Consequently, only a few studies researched the influence of spermidine on mRNA expression of aging-associated genes up to now. The results of this work underline the CRM properties of spermidine, as they show a significant induction of *SIRT1*, *SIRT3*, *FOXO3* and *SOD2*, as displayed in **Figure 40**. A dose of 30  $\mu\text{M}$  spermidine induced *SIRT1* expression significantly after 24 h, 48 h and 72 h of treatment, whereas 20  $\mu\text{M}$  only had a significant influence after 72 h of treatment. *SIRT3* mRNA expression was significantly elevated by 30  $\mu\text{M}$  spermidine treatment after 48 h and 72 h, as well by a 20  $\mu\text{M}$  treatment after 72 h. The same pattern was obvious for *FOXO3* and *SOD2* mRNA expression after spermidine treatment. However, *SOD2* expression was least affected by this *ex vivo* treatment. The spermidine treatment applied here led to increases in *SIRT3* mRNA expression just like nutrient starvation did in the cell culture study conducted by Chen *et al.*, who also found an increased *SIRT3* mRNA expression. Furthermore, they stated, that this led to a *SOD2* activation and subsequently to an increased protection against oxidative damage. (Chen *et al.*, 2011) This statement can also be applied for the results presented here. Consequently, the hypothesis is that spermidine increases *SIRT3* mRNA expression, which, in the following, leads to an increased availability of *SIRT3* protein. *SIRT3* activates *SOD2* by deacetylation and hence increases the anti-oxidative defense. Additionally, an increased *SOD2* mRNA expression presumably leads to an increased availability of *SOD2* protein, which contributes to an increased resistance against oxidative stress. An increased *SIRT3* and *SOD2* mRNA expression due to intermittent fasting could already be shown by Wegmann *et al.* (Wegman *et al.*, 2015). The fact that the spermidine treatment used here also led to this result shows that spermidine can mimic a CR in this regard.

The influence of spermidine on *SIRT1* mRNA expression has not been researched yet. However, an increase of *SIRT1* protein expression in cardiac tissue and increased *SOD*-activity was shown after spermidine treatment of old rats, resulting in an improved mitochondrial biogenesis (Wang *et al.*, 2020). Since spermidine treatment led to an improved mitochondrial

biogenesis, mitochondrial function might also be enhanced. Additionally, a spermidine treatment leads to a reduction of oxidative stress due to an increased SOD-activity. The increase in *SIRT1* mRNA expression shown here could also support this hypothesis, especially since the *SOD2* mRNA expression was also increased.

So far, the influence of spermidine on FOXO3 has also hardly been researched, although both spermidine and FOXO3 are closely linked to aging. After spermidine treatment, FOXO3 protein expression was increased in artificially aged rat muscle, the AMPK/FOXO3 pathway was activated and deficits in autophagy were presumably improved (Fan *et al.*, 2017). Within the course of this work, a treatment of human PBMCs with spermidine led to significant increases in *FOXO3* mRNA expression. Just like the interpretation of the results of *SIRT1* mRNA expression, these results suggest that this increase in *FOXO3* expression might lead to an improved autophagy. However, this has to be researched and verified separately.

A population-based study linked an increased spermidine intake to a decreased mortality of test persons and provides the hypothesis that a spermidine-rich diet increases the survival of humans (Kiechl *et al.*, 2018). Taken together, the results presented in this work as well as the results of others point to a CR-mimicking property of spermidine by a possible contribution to a reduction of mortality similarly to CR. This might be achieved by reducing oxidative stress. Oxidative stress, in turn, is known to promote the aging process, whereby oxidative phosphorylation in mitochondria is the main source of endogenous ROS (Balaban *et al.*, 2005). *SIRT3*, which is located in mitochondria, and *SOD2* play an important role in reducing oxidative stress. Stimulating the expression of both genes, both by CR and by a CRM such as spermidine, can thus help to ensure the integrity of mitochondria by reducing oxidative stress and thus help to slow down aging.

#### 4.7.2 Resveratrol induces mRNA expression of aging-associated genes

Several research groups have shown that resveratrol can stimulate mRNA and protein expression of aging-associated genes like *SIRT3* or *SOD2* in human cell cultures (Zhou *et al.*, 2014; Mathieu *et al.*, 2016). Within the course of this thesis, resveratrol was applied to human PBMCs for 24 h, 48 h and 72 h (see **Figure 41**). Subsequently, mRNA expression of *SIRT1*, *SIRT3*, *FOXO3* and *SOD2* was determined. A significant induction of *SIRT3* mRNA expression

was observable after a treatment with 100  $\mu$ M resveratrol for 48 h and 72 h. Furthermore, a treatment with 75  $\mu$ M resveratrol for 72 h induced *SIRT3* expression significantly. *FOXO3* mRNA expression was elevated significantly after 48 h and 72 h with 75  $\mu$ M or 100  $\mu$ M resveratrol, whereas *SOD2* mRNA expression was only significantly induced after a 24 h treatment with 75  $\mu$ M resveratrol.

Surprisingly, none of the selected concentrations or incubations times had a significant influence on *SIRT1* mRNA expression, although resveratrol is considered a *SIRT1* stimulator (Borra *et al.*, 2005). Since Borra *et al.* studied the influence of resveratrol on *SIRT1* enzyme activation, the results of the study presented here are not directly comparable, although an increase in mRNA expression might lead to increased protein levels. Regarding resveratrol as a CRM, a dose of 500 mg resveratrol and a CR of 1000 calories per day led to a similar increase in *SIRT1* plasma concentrations, but not to an increase in *SIRT1* mRNA expression (Mansur *et al.*, 2017). These findings correspond only partially with the findings presented here. While an *ex vivo* treatment of PBMCs with resveratrol did not increase *SIRT1* mRNA expression significantly, a CR in form of F.X. Mayr therapy significantly increased *SIRT1* expression. This underlines the assumption that CRMs can support a CR, but that they cannot replace a CR.

Nevertheless, the findings presented in this work emphasize the capability of resveratrol to act as a CRM, since mRNA expression of the aging-associated genes *SIRT3*, *SOD2* and *FOXO3* could be increased significantly. Zhou and colleagues showed an enhanced activity and deacetylation of *SOD2*, a reduced mitochondrial ROS generation due to an enrichment of *SIRT3* in mitochondria and an increase in *SIRT3* gene expression (Zhou *et al.*, 2014). At least the increase in *SIRT3* mRNA expression is consistent with the findings presented here. Apart from that, the effect of resveratrol as a CRM could also take place in form of a reduction of oxidative stress. This is supported by the increase in *SIRT3* and *SOD2* mRNA expression. Resveratrol has been shown to increase *SOD2* protein level under oxidative stress conditions in mice, whereby *FOXO3* is needed for that process (Hori *et al.*, 2013). The results presented here confirm that *SOD2* and *FOXO3* are inducible by resveratrol on the mRNA level. Furthermore, an increase in autophagy might be a result of resveratrol treatment, since *FOXO3* expression was also significantly induced. *FOXO3* has been shown to trigger autophagy in mice (Mammucari *et al.*, 2007). Maybe this is also the case in humans, since the process of



autophagy is highly conserved. By stimulating autophagy-related genes and genes, which are involved in anti-oxidative defenses, a deceleration of the aging process could be achieved.

#### 4.8 Spermidine increases DNA repair capacity in human fibroblasts

With regard to DNA repair mechanisms it has been shown that polyamines, amongst them spermidine, influence homology-directed DNA repair. An increased export of polyamines out of cells as well as an impaired polyamine synthesis leads to a decreased homology-directed DNA repair, whereas an increased cellular polyamine production increases it. (Lee *et al.*, 2019) Yet there seem to be no data on the influence of spermidine on NER capacity. As displayed in chapter 3.7.1, the CRM spermidine induced the expression of aging-associated genes in *ex vivo* treated human PBMCs. In order to research a possible influence of this CRM on DNA repair capacity, experiment were conducted using human fibroblasts of one younger (8 years of age) and one older donor (63 years of age). In both cases 10  $\mu$ M as well as 20  $\mu$ M spermidine increased NER capacity, although a significant increase was only observable in fibroblasts of the younger donor (see **Figure 42**). An inhibition of SIRT1 deacetylase was shown to impair the GGR subpathway of NER by suppressing the transcription of XPC and that SIRT1 is essential for the repair of UVB-induced DNA damages (Ming *et al.*, 2010). Furthermore, SIRT1 is known to deacetylate XPA, the main damage recognition factor of the GGR subpathway of NER (Fan and Luo, 2010). Amongst the aging-associated genes researched here, mRNA expression of *SIRT1* was significantly elevated after spermidine treatment of human PBMCs, probably leading to an increased SIRT1 protein level. Since SIRT1 plays an important role as a regulator of NER (Jarrett *et al.*, 2018) and spermidine increases mRNA expression of *SIRT1*, the hypothesis is that NER is promoted by spermidine via an increase in SIRT1 and subsequently increased transcription of the repair factors XPA and XPC. Since no mRNA expression data of spermidine treatment of fibroblasts were collected within the course of this thesis, this can only be an assumption. However, spermidine increased NER capacity of human fibroblasts like the CR intervention of F.X. Mayr therapy did in human PBMCs. Although a positive influence of spermidine on NER in human PBMCs has to be researched in future, the results presented here show the potential of spermidine as a CRM. Looking into the future, a spermidine treatment might not only support the positive effects of CR on mRNA expression of aging-associated genes, but also on DNA repair capacity of NER. However, more experiments involving more test persons have to be conducted to confirm this assumption.

## 5 Conclusions

Within the scope of this thesis, the influence of CR on NER and the expression of aging-associated genes was researched using PBMCs of human test persons. Furthermore, two promising CRMs, resveratrol and spermidine were studied regarding their ability to trigger mRNA expression of aging-associated genes. Finally, the influence of spermidine on human NER capacity was studied using human fibroblasts.

The results obtained show convincingly that DNA repair capacity of human PBMCs can be determined reliably by conducting mHCRA. By using this functional assay, CR in form of a F.X. Mayr therapy could be shown to stimulate NER capacity. However, this seems not to be the case for all individuals, since a significant increase was only observable in individuals displaying a low pre-existing DNA repair capacity. These results strongly support the hypothesis that CR has indeed an influence on NER in humans. Additionally, the expression of the two aging-associated genes *SIRT1* and *XPA*, which are also closely linked to NER, increased as a consequence of CR. Both genes showed correlating expression patterns, but only *SIRT1* mRNA expression significantly increased. Strikingly the significant increase was solely attributed to the “low pre-existing DNA repair” group, just like the significant increase in DNA repair capacity. NER works probably at its optimum in individuals displaying a normal DNA repair in the beginning and a further increase by CR is not beneficial or not possible. This also demonstrates that individuals of the “low DNA repair capacity” group benefit from CR in form of an increase in NER capacity so that at the end of the study DNA repair capacity roughly corresponds to that of the “normal” group. DNA repair capacity of individuals of the “low” group seemed to be below-average. Due to CR, this deficiency can be balanced and individuals may be protected better against DNA damages and their consequences. The increases in *SIRT1* and *XPA* mRNA expressions also support this. A decrease of DNA repair is associated with aging. Since CR can stimulate NER, it also stimulates anti-aging properties within the cells.

mRNA expression of all aging-associated genes was subject to donor variability in the CR samples. However, individuals displaying an increased *SOD2* or *SIRT3* mRNA expression may possess a stronger defense against oxidative stress. *FOXO3* is strongly associated with longevity in humans. Whereas grouped analyses of *FOXO3* and *LC3B* mRNA expression did

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not reveal significant influences of CR, some individuals displayed increased mRNA expressions nonetheless. In these individuals CR may have triggered autophagy. The hypothesis of an influence of CR on autophagy is supported by the significantly increased *AMPK $\alpha$*  expression in all individuals and in the “low” group. However, the influence of CR on autophagy must of course be examined separately for a straightforward statement. Taken together, these results suggest an improved anti-oxidant defense and an improved autophagy due to CR, although further experiments have to verify this assumption.

The CRMs resveratrol and spermidine influence mRNA expression of aging-associated genes similarly to CR. Spermidine triggers *SIRT1* and *SOD2* expression just like CR. In contrast to CR, spermidine also induced mRNA expression of *SIRT3* and *FOXO3* significantly. Contrary to the expectations and contrary to CR, resveratrol did not increase *SIRT1* mRNA expression, but *SIRT3*, *FOXO3* and *SOD2* expression. In conclusion, this firstly points to different mechanisms of action of the two CRMs with regard to their CR mimicking effects. Second, this leads to the hypothesis that CRMs may support CR, but cannot replace the holistic approach of CR. In future CRMs may thus be used additionally to CR. Further studies on the influence of CRMs on DNA repair capacity of human PBMCs have to be conducted in order to support the assumption of a similar influence of CR and CRMs on NER.

Not all individuals react equally to CR in the form of F.X. Mayr therapy. These inter-individual differences illustrate the necessity of *ex vivo* studies with human samples, since a reliable prediction for test persons with regard to an effect is only possible with a sufficient number of samples. By using CRMs, health-promoting interventions could in future be tailored more precisely to individuals or specific groups with the aim of achieving the best possible benefit. CRMs may be used additionally to a CR intervention in order to support individuals who otherwise would not benefit from CR or in order to boost CR effects. Here CR is suggested as a way to mitigate the aging-associated decline in DNA repair by increasing NER and thus counteracting aging.

An *ex vivo* spermidine treatment increases DNA repair capacity in human fibroblasts of an older donor. In a younger donor, spermidine even leads to a significant increase in NER capacity. Since only two donors were studied, a clear conclusion is difficult to be drawn.

However, in PBMCs the CRM spermidine also led to increases in the expression of aging-associated genes similar to CR. Keeping this in mind, spermidine could not only lead to increases in mRNA expression of aging-associated genes and NER capacity, but also act supportive during a CR, especially in individuals who do not experience an increase in DNA repair or mRNA expressions of aging-associated genes.

## 6 References

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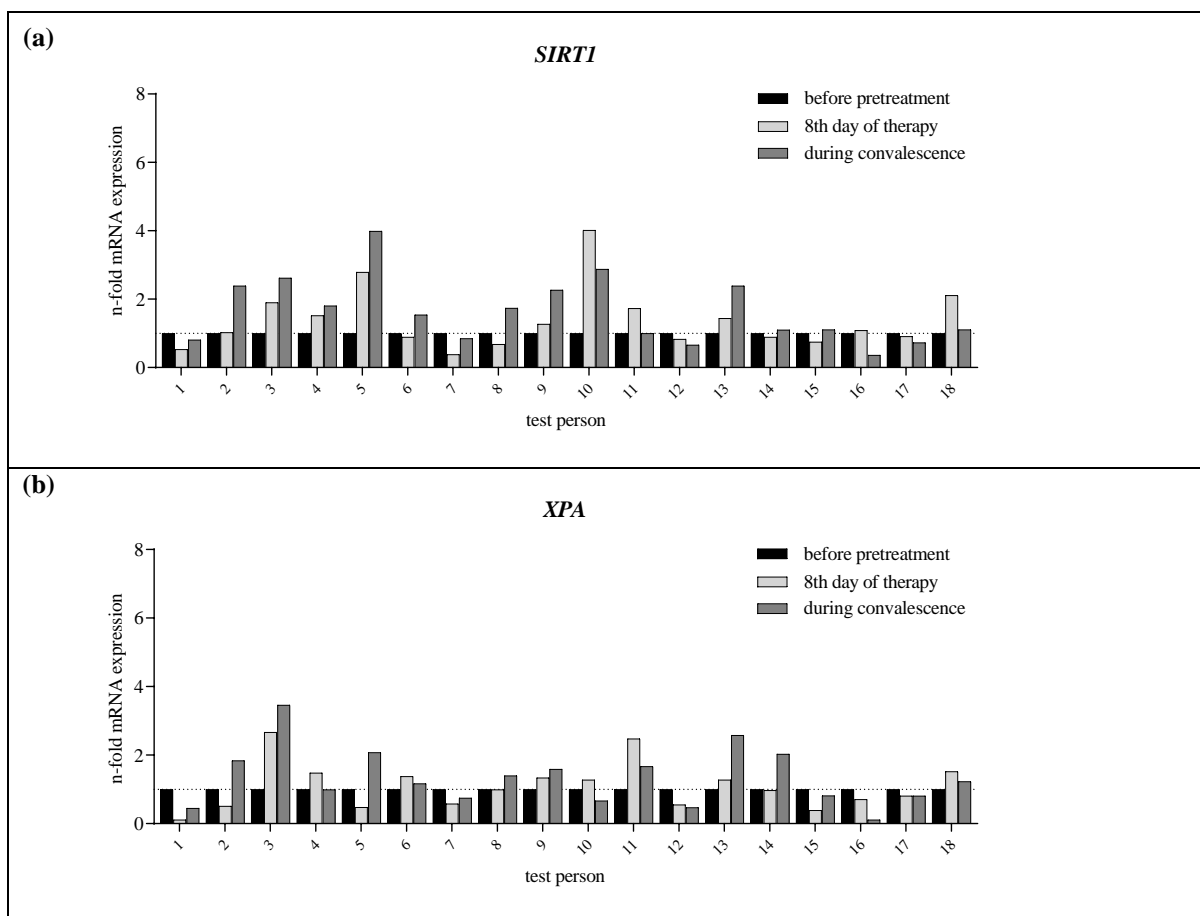
## 7 Appendix

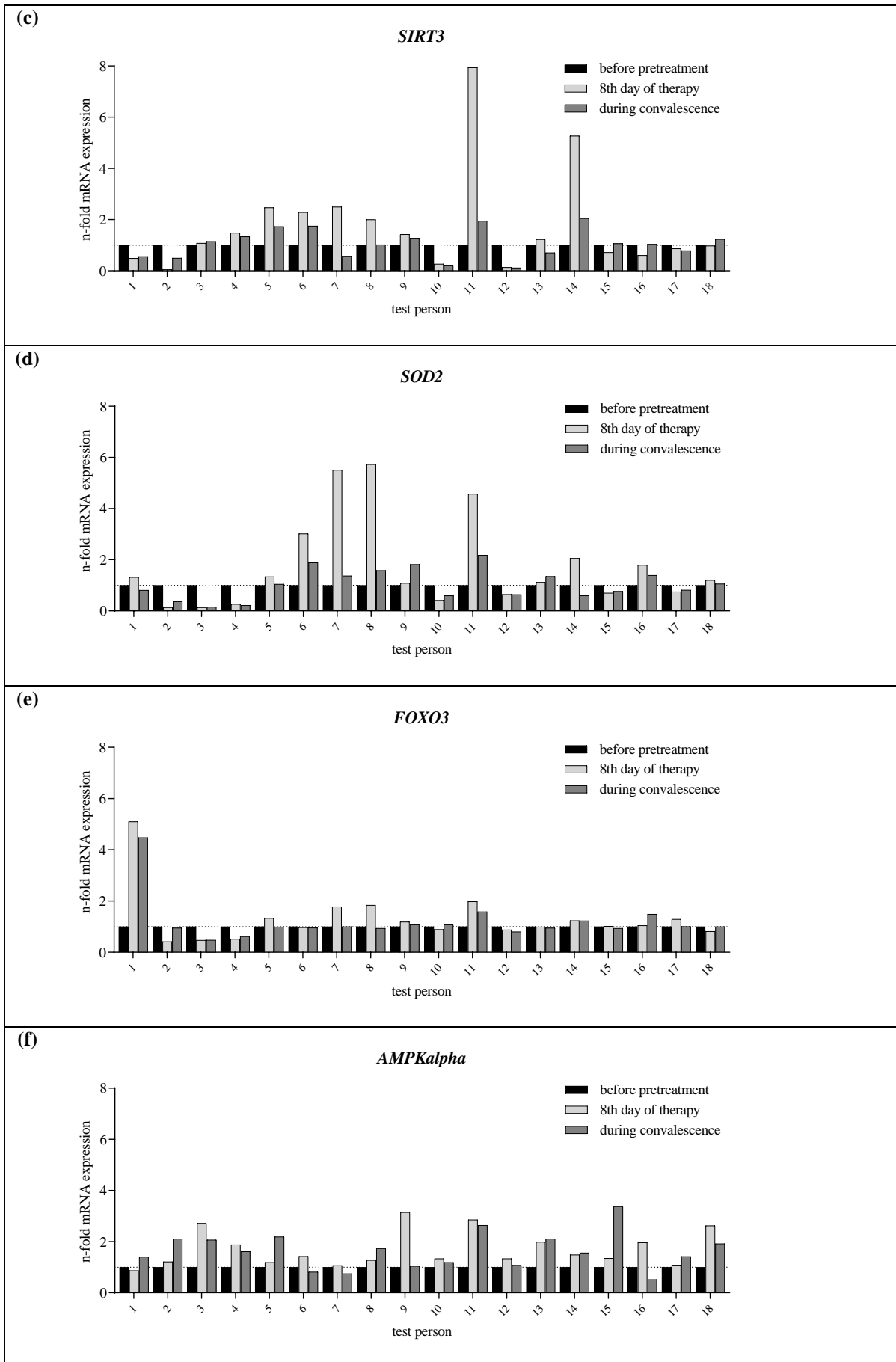
### 7.1 Attachment to results

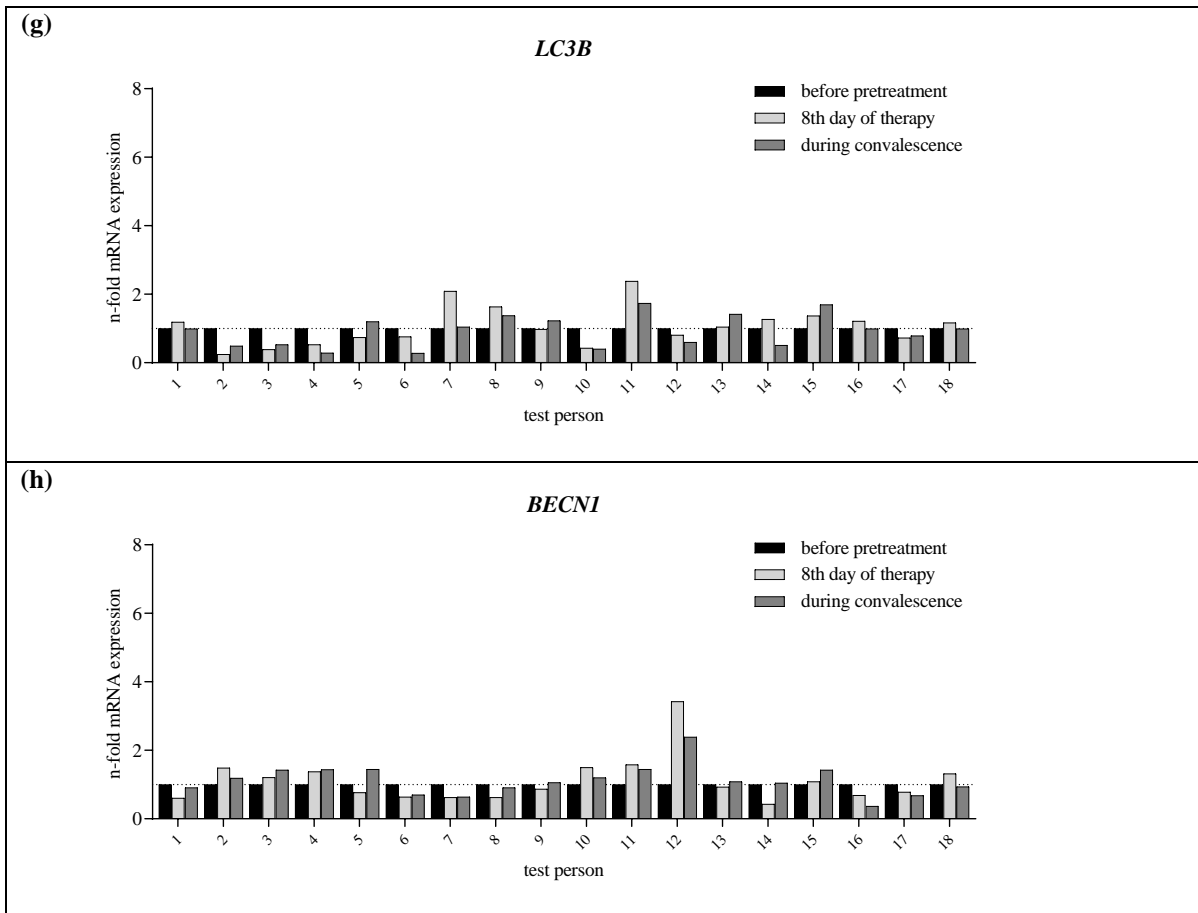
Additional illustrations of the results of this work are shown in the following two chapters of this appendix. mRNA expression patterns are displayed together in order to give an additional view on the individual mRNA expression patterns of the 18 test persons. For the correlations of two aging-associated genes each, the correlation analyses presented in this appendix resulted in low or negligible correlations and are therefore assigned to the appendix.

#### 7.1.1 Overview of mRNA expression patterns of all aging-associated genes

In order to give an additional summary of the mRNA expression patterns of the eight aging-associated genes, the bar graphs depicting the individual mRNA expression patterns of the 18 test persons are presented in the following **Figure 43 a-h**.







**Figure 43: mRNA expression patterns of eight aging-associated genes during F.X. Mayr therapy.**

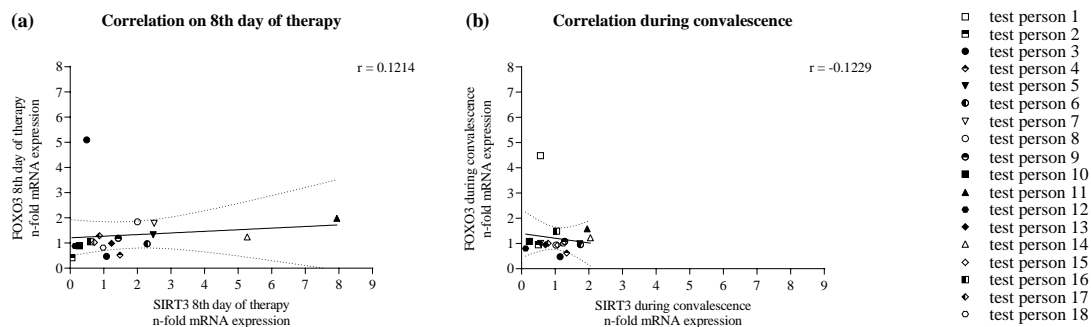
Special focus is on the individual mRNA expression of the test persons. Altogether, these results stress the inter-individual differences regarding the influence of CR on mRNA expression in human test persons (a) SIRT1 mRNA expression is particularly increased in test persons 5 and 10. (b) XPA mRNA expression is particularly increased in test person 5. (c) SIRT3 mRNA expression is particularly increased in test persons 11 and 14. (d) SOD2 mRNA expression is particularly increased in test persons 6, 7, 8 and 11. (e) FOXO3 mRNA expression is particularly increased in test person 1. (f) AMPK $\alpha$  mRNA expression is particularly increased in test persons 9, 11, 15 and 18. (g) LC3B mRNA expression is particularly increased in test persons 7 and 11. (h) BECN1 mRNA expression is particularly increased in test person 12.



### 7.1.2 Correlation of mRNA expression patterns

Linear regression analyses of *SIRT3* and *FOXO3*, *SIRT1* and *AMPK $\alpha$* , *SIRT1* and *LC3B*, *SIRT1* and *BECN1* as well as *AMPK $\alpha$*  and *BECN1* mRNA expressions did not reveal significant correlations. In order to condense the results-section of this work, the results of these correlation analyzes are presented in the appendix.

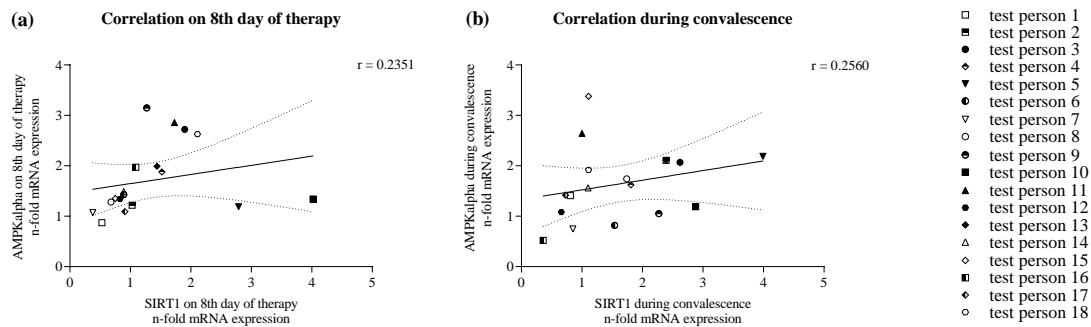
Linear regression analyses as well as resulting correlations of *SIRT3* and *FOXO3* mRNA expression are displayed in **Figure 44**. On the eighth day of therapy, a negligible correlation ( $r = 0.1214$ ) of *SIRT3* and *FOXO3* mRNA expression is evident, as displayed in **Figure 44 a**. During convalescence, this negligible correlation ( $r = -0.1229$ ) persists (see **Figure 44 b**).



**Figure 44: Correlation of *SIRT3* and *FOXO3* mRNA expression.**

(a) *SIRT3* and *FOXO3* mRNA expressions correlate negligibly ( $r = 0.1214$ ) on the eighth day of therapy.  
 (b) This negligible correlation ( $r = -0.1229$ ) of both mRNA expressions is still recognizable during convalescence.

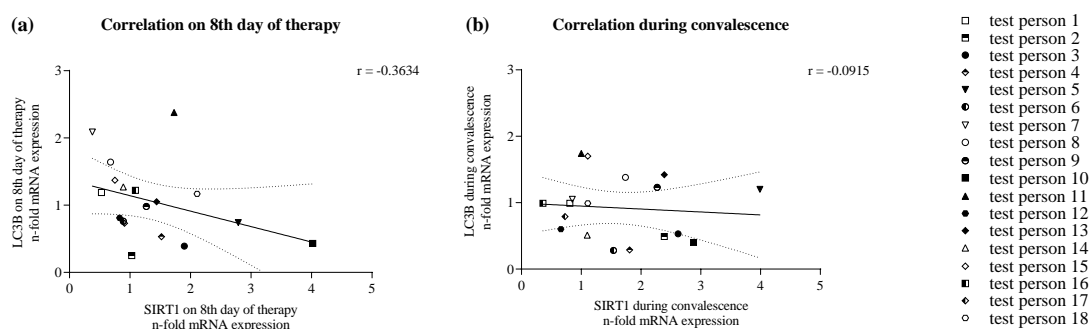
**Figure 45** displays linear regression analyses and resulting correlations of *SIRT1* and *AMPK $\alpha$*  mRNA expression. A negligible correlation is obvious on the eighth day of therapy ( $r = 0.2351$ ) and during convalescence ( $r = 0.2560$ ), as displayed in **Figure 45 a** and **Figure 45 b**.



**Figure 45: Correlation of *SIRT1* and *AMPK $\alpha$*  mRNA expression.**

(a) A negligible correlation of *SIRT1* and *AMPK $\alpha$*  mRNA expression is visible on the eighth day of therapy ( $r = 0.2351$ ). (b) During convalescence, a negligible correlation is observable for both mRNA expressions ( $r = 0.2560$ ).

Linear regression analyses and correlations of *SIRT1* and *LC3B* mRNA expression are shown in **Figure 46**. Both time points show a negative correlation of both mRNA expressions. **Figure 46 a** displays a low negative correlation ( $r = -0.3634$ ) on the eighth day, whereas **Figure 46 b** shows a negligible negative correlation ( $r = -0.0915$ ) during convalescence.

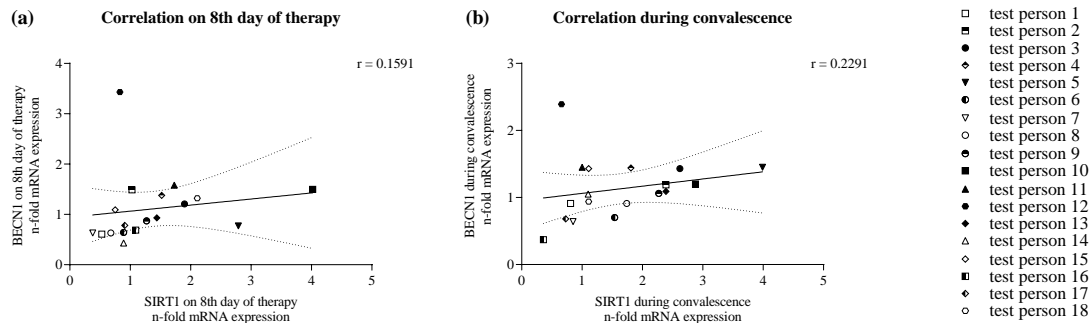


**Figure 46: Correlation of *SIRT1* and *LC3B* mRNA expression.**

(a) *SIRT1* and *LC3B* mRNA expressions correlate lowly in a negative way ( $r = -0.3634$ ) on the eighth day of therapy. (b) During convalescence, a negligible negative correlation of *SIRT1* and *LC3B* expression persists ( $r = -0.0915$ ).

Linear regression analyses and resulting correlations of *SIRT1* and *BECN1* mRNA expression are displayed in **Figure 47**. On the eighth day as well as during convalescence, *SIRT1* and

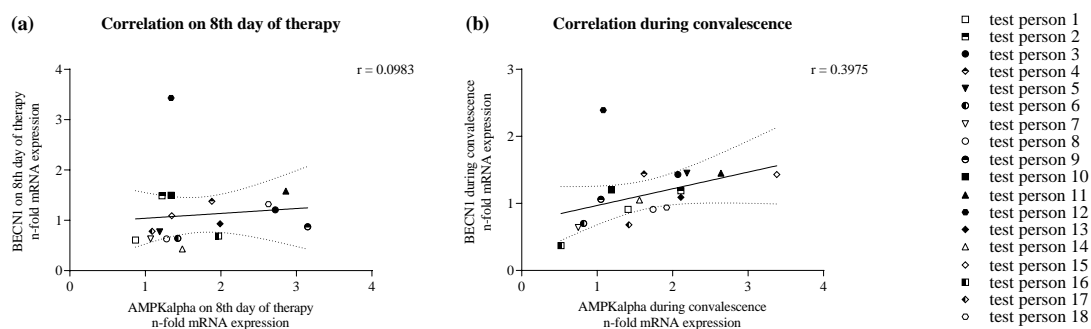
*BECN1* expression correlate negligibly ( $r = 0.1591$  and  $r = 0.2291$ , respectively), as displayed in **Figure 47 a** and **Figure 47 b**.



**Figure 47: Correlation of *SIRT1* and *BECN1* mRNA expression.**

(a) A negligible correlation of *SIRT1* and *BECN1* mRNA expression is visible on the eighth day of therapy ( $r = 0.1591$ ). (b) During convalescence, this negligible correlation persists ( $r = 0.2291$ ).

**Figure 48** shows linear regression analyses as well as correlations of *AMPK $\alpha$*  and *BECN1* mRNA expression. **Figure 48 a** displays a negligible correlation ( $r = 0.0983$ ) for the expression of *AMPK $\alpha$*  and *BECN1* on the eighth day, whereas **Figure 48 b** displays a low positive correlation ( $r = 0.3975$ ) during convalescence.



**Figure 48: Correlation of *AMPK $\alpha$*  and *BECN1* mRNA expression.**

(a) *AMPK $\alpha$*  and *BECN1* mRNA expressions correlate negligibly ( $r = -0.3634$ ) on the eighth day of therapy. (b) During convalescence, both mRNA expressions correlate lowly ( $r = 0.3975$ ).

## 7.2 Acknowledgements

At first, I would like to thank my supervisor at the Albstadt-Sigmaringen University, **Prof. Dr. Jörg Bergemann**, for his support, encouragement and the many helpful scientific discussions. I am grateful for his input and enthusiasm for the topic of calorie restriction and DNA repair and for giving me the opportunity to work on this exciting topic for my PhD thesis.

I would like to thank my supervisor at the University of Konstanz, **Prof. Dr. Alexander Bürkle**, for supervising this thesis. His scientific input, enthusiasm and advices during thesis committee meetings greatly helped in driving this thesis forward.

I would also like to thank **Prof. Dr. Christof Hauck** for agreeing to be the third member of my thesis committee.

For the provision of blood samples and information on F.X. Mayr therapy, I thank Dr. med. Karin Rupprecht, Dr. med. Michael Rupprecht and the entire team at the center for Traditional Chinese Medicine (TCM) in Sigmaringen.

I especially want to thank the members and former members of the Bergemann Lab in Sigmaringen. My fellow PhD students **Alica Schöller-Mann** and **Barbara Hochecker**, **Dr. Daniel Schniertshauer**, **Sonja Müller** and **Dr. Daniel Gebhard** for being excellent lab mates and creating a great working atmosphere. Thank you for many many interesting and helpful conversations and discussions - not only the scientific ones - and for the fun we had during work. You really cut down long lab days!

Finally, and most importantly, I would like thank my **family, especially my parents, my sisters and Denis**. Without the family background that I was lucky to have, I wouldn't be who I am today. You have taught me to work hard to achieve my goals, to persevere even when it gets difficult, not to give up, but most importantly to never lose the right perspective and fun in what I am doing. Thank you for your continuous love, support, encouragement and patience and for keeping me grounded.

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### 7.3 Publications

Schöller-Mann A, **Matt K**, Hochecker B, Bergemann J. Ex vivo assessment of mitochondrial function in human peripheral blood mononuclear cells using XF Analyzer. Accepted for publication in *Bio-protocol* in February 2021

**Matt K**, Hochecker B, Schöller-Mann A, Bergemann J. mRNA expression of ageing-associated genes in calorie reduction is subject to donor variability and can be induced by calorie restriction mimetics. *Nutr Health*. 2020;26(3):253-262. doi:10.1177/0260106020932732

Schöller-Mann A, **Matt K**, Schniertshauer D, Hochecker B, Bergemann J. 12 days of in vivo caloric reduction can improve important parameters of aging in humans. *Mech Ageing Dev*. 2020;188:111238. doi:10.1016/j.mad.2020.111238

**Matt K**, and Bergemann, J. (2019). *Ex vivo* Analysis of DNA Repair Capacity of Human Peripheral Blood Mononuclear Cells by a Modified Host Cell Reactivation Assay. *Bio-protocol* 9(15): e3325.

**Matt K**, Burger K, Rupprecht K, Gebhard D, Bergemann J. Stimulierung körpereigener Reparaturprozesse durch Kalorienrestriktion. *OM & Ernährung*. 2016; Nr. 157: F02-07

**Matt K**, Burger K, Gebhard D, Bergemann J. Influence of calorie reduction on DNA repair capacity of human peripheral blood mononuclear cells. *Mech Ageing Dev*. 2016 Mar; 154:24-9. doi: 10.1016/j.mad.2016.02.008. Epub 2016 Feb 13.

## 7.4 Poster and oral presentations

### Poster presentations

**Matt K**, Burger K, Rupprecht K, Gebhard D, Bergemann J. Ex vivo approach for analyzing DNA repair capacity. 30th Ernst Klenk Symposium in Molecular Medicine DNA Damage Response and Repair Mechanisms in Aging and Disease; September 21–23, 2014 in Cologne

**Matt K**, Gebhard D, Burger K, Schniertshauer D, Bergemann J. Mitochondrial biomarkers for UV exposure. 4. Dreiländer-Kongress Mitochondriale Medizin; July 09-10, 2015 in Salzburg, Austria

**Matt K**, Burger K, Bergemann J. Calorie reduction and DNA repair in human blood cells. Cell Symposia Aging and Metabolism; July 10–12, 2016 in Sitges, Spain

**Matt K**, Schöller-Mann A, Bergemann J. Kalorienreduktion steigert die körpereigene DNA-Reparaturkapazität. Healthy Ageing Conference 2017; March 16-17, 2017 Hochschule Fulda

**Matt K**, Bergemann J. Increase of DNA repair capacity by calorie reduction. 4<sup>th</sup> German-French DNA Repair Meeting; September 21-23, 2017 in Cologne

Schöller-Mann A, **Matt K**, Schniertshauer D, Bergemann J. Influence of Caloric Reduction on Mitochondrial Function. 8<sup>th</sup> World Congress on Targeting Mitochondria; October 23-24, 2017 in Berlin

**Matt K**, Schöller-Mann A, Bergemann J. Influencing human DNA Repair Capacity. BW-CAR Forschungstag; July 25th, 2018 in Stuttgart

Schöller-Mann A, **Matt K**, Schniertshauer D, Bergemann J. Caloric Reduction affects DNA Repair Capacity and Mitochondrial Function. Poster presentation, 9<sup>th</sup> World Congress on Targeting Mitochondria; October 23-25, 2018 in Berlin

**Matt K**, Schöller-Mann A, Bergemann J. Establishment of Laboratory Diagnostic Methods to investigate cellular Effects of Caloric Restriction. Diagnostics-4-Future Conference; November 28th, 2018 in Konstanz

### Oral presentation

**Matt K**. Mitochondrial respiration in human PBMCs. Seahorse Workshop - Measuring Metabolic Engines and Fuels with the Seahorse XF Analyzer, December 8-9, 2016

## 7.5 Awards

### **Poster Award**

Matt K, Schöller-Mann A, Bergemann J. Kalorienreduktion steigert die körpereigene DNA-Reparaturkapazität. Healthy Ageing Conference 2017; March 16-17 2017 Hochschule Fulda

### **Junior-IMMA Award - International Mitochondrial Association (IMMA) 2017**

“für besondere experimentelle und klinisch relevante wissenschaftliche Leistungen auf dem Gebiet der Mitochondrialen Medizin“ – Sie hat insbesondere einen Beitrag zu folgendem Thema geleistet: „Mechanismen des biologischen Alterns und deren Beeinflussung durch QuinoMit Q10 fluid“