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Age-related differences in stress response: Increase markers for oxidative stress and liver damage after heat stress in aged rats?

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Nomenclature

4-HNE	*4-hydroxynonenal*
8-OHdG	*8-hydroxydesoxyguanosin*
AIF	*apoptosis inducing factor*
ALD	*alcohol-induced liver disease*
ALT	*alanine-aminotransferase enzyme*
BMBF	*Federal Ministry of Education and Research*
BSA	*bovine serum albumin*
CAT	*catalase*
CCl_4	*carbon tetrachloride*
CR	*caloric restriction*
Cu^{2+}	*copper ion*
CYP2E1	*cytochrome P450 2E1*
ELISA	*enzyme-linked immunosorbent assay*
GC	*gas chromatography*
GPx	*glutathione peroxidase*
GR	*glutathione reductase*
GSH	*glutathione*
GSSG	*glutathione disulfide*
H&E-staining	*Hematoxylin & Eosin-staining*
H_2O_2	*hydrogen peroxide*
HCC	*hepatocellular carcinoma*
HPC	*heat stress preconditioning*
HPLC	*high performance liquid chromatography*
HRP	*horseradish peroxidase*
HSF	*heat stress transcription factor*
HSP	*heat shock proteins*
IPC	*ischemic preconditioning*
LDH	*lactate dehydrogenase enzyme*
LPO	*lipid peroxidation*
MDA	*malondialdehyde*
MLS	*maximum life span*

MS	*mass spectrometry*
mtDNA	*mitochondrial DNA*
O_2	*singlet oxygen*
O_2^{-}•	*superoxide anion*
OH•	*hydroxyl radical*
PUFA	*polyunsaturated fatty acid*
PTP	*permeability transition pore*
ROS	*reactive oxygen species*
SD	*standard deviation*
SOD	*superoxide dismutase*
TBA	*thiobarbituric acid*
TBARS	*thiobarbituric acid reactive substances*
TEP	*tetraehtoxypropane*

1. Introduction

1.1. Aging and age-related diseases

The process of aging affects every living creature on the planet and the death stands inevitably at the end of life. Since generations scientists try to find a way to encode and prevent the deleterious process. The aging of an organism is defined as a progressive decline of physiological functions with increased probability for disease and death within life span (Harman, 2003, Droge, 2002). The life expectancy of human beings has been constantly changing over the centuries. The number of people aged over 45 years is increasing, especially in the developed countries. In Germany as a developed country the shift towards an overaged population is clearly observed. By comparing the population pyramids of 1950 and 2015, the progressive change from 1950's clock-model with a large base of people under 30 years to an onion-shaped model in 2015 displaying a major group of people over 45 years is visible (Figure 1). This trend appears in both sexes as well as in other developed countries and even in underdeveloped countries (Nations, 2015).

Figure 1: Population pyramid of Germany from 1950 (left) and 2015 (right). Age distribution of males and females in 1950 is compared to 2015 visualizing the overaging of the population. [adopted from (Nations, 2015)]

During the last centuries the life expectancy of people has increased due to less infant mortality and improved health care conditions including treatment for infections and better management of chronic diseases and cancer (Mathers et al., 2015). Life expectancy is defined as the average life time that an individual in a certain environment will survive (Tosato et al., 2007). Aims concerning extended life expectancy are described as healthy longevity including maintenance of functional capacity up to an old age (Jin et al., 2015). Another view on the process of aging is the evaluation of the maximum life span defined as the maximum number of years that a human being can reach. The maximum life span of humans with a total of 125 years has not changed for 100.000 years and different approaches are made trying to explain how we age and why (Hayflick, 2000). It is hypothesized that different interacting factors exist that lead to aging of an organism (Cui et al., 2012). By observing epidemiological factors of a population such as mortality and morbidity, scientists were early trying to identify the “perfect survival conditions” for human beings but an explanation for the cause of aging is still missing (Ferrucci et al., 2008). With increased knowledge about molecular pathways, the theory of the accumulation of cellular damage as major cause for aging over time was developed (Gems and Partridge, 2013, Vijg and Campisi, 2008, Kirkwood, 2008). An increasing risk for protein, lipid and DNA damage as well as altered intracellular signal pathways and ineffective removal of damaged cells was identified in elderly individuals (López-Otín et al., 2013).

This thesis focuses on the understanding of accumulated oxidative cell damage in aging and age-related molecular changes in an organism. The degenerative aging process is characterized by a variety of universal traits such as impaired wound healing, poor vision and reduced hearing but it is also closely linked to certain diseases (Figure 2). It alters the function and structural integrity of the cell and weakens their regeneration abilities with increasing the risk for diseases mainly affecting the immune system, the central nervous system, the cardiovascular system and the liver (Poulose and Raju, 2014). The risk to suffer from Type 2 diabetes known as non-insulin-dependent diabetes increases

dramatically in age and up to 70% of patients with Type 2 diabetes are older than 55 years (Nathan et al., 1986).

In aged individuals different cells of the immune system are influenced in their function leading to a dysfunctional immune response. This increases the probability of infectious diseases like pneumonia and influenza, autoimmune disorders and cancer (Hawkley and Cacioppo, 2004, Castle, 2000). The acquired cell-mediated immunity is mainly affected by aging as the T-cell maturation and selection in the thymus is decreased. It is linked to the age-dependent involution of the thymus that is finally completed around the age of 60 (Gui et al., 2012, Grubeck-Loebenstein, 1997). It has been demonstrated that the amount of naïve T-cell production declines with an increase of memory T-cells. T-cell mediated signal cascades are altered weakening the specific immune protection against pathogens (Miller, 1996). The altered immune response is closely linked to malignant degeneration of cells. Certain types of neoplasm show a higher incidence in older people mainly affecting prostate gland, colon and breast tissue (Suen et al., 1974). Cancerous cells share similarities including cellular transformation, dysregulated apoptosis, uncontrolled cell proliferation and metastasis (Vasto et al., 2009). One explanation of the age-dependency of neoplasm is the increase of DNA mutations due to the cumulative exposure towards oxidative stress (DePinho, 2000). But also inefficient apoptosis in elderly people, normally useful to regulate the number of cells in tissues, enables the uncontrolled proliferation of cancerous cells increasing the resistance against their removal (Lowe and Lin, 2000).

Other age-dependent diseases are neurodegenerative diseases including Alzheimer's disease and Parkinson's disease that show a significantly increased incidence in older individuals (Schon and Przedborski, 2011). It is difficult to identify proper causes for brain aging in healthy elderly as diseases mainly contribute to the decline in cognitive function in aged individuals (Glorioso and Sibille, 2011). Properties of neurodegenerative diseases were also found in 'healthy' aged people and the theory of oxidative damage during aging develops with an accumulation of dysfunctional cells. The tissue of central nervous system can be characterized by a high metabolic rate, oxygen consumption and lipid

content making it more susceptible towards oxidative damage compared to other tissues (Kumar et al., 2012). Additionally, nervous cells contain mainly post-mitotic cells without further ability to divide and regenerate causing more severe damage in tissues compared to cells with a high turnover rate (Di Domenico et al., 2016). Patients suffering from Alzheimer's disease have shown increased rates of oxidized proteins underlining this theory (Smith et al., 1991).

Over many years diseases of the cardiovascular system remain the main cause of death in industrial countries with an increased risk in elderly for myocardial ischemia following arteriosclerosis and congestive heart failure (Sistino, 2003). Constitutional differences of the heart with advancing age could be identified even in apparently healthy elderly including increase in heart weight as a sign for left ventricular hypertrophy as well as altered diastolic filling periods (Fleg and Strait, 2012). Also the peripheral vascular system is affected as arteries elongate and the vascular wall gets more rigid due to structural changes and less elastic components (Lee and Oh, 2010). These are signs of early arteriosclerosis that in the final state includes focal lesions, stenosis of vessels and plaque formation (Ferrari et al., 2003). Both the brain and the heart are organs with a high metabolic rate that processes large amounts of oxygen (Dai et al., 2012). Therefore, the heart is prone to severe accumulation of oxidative damage and of special interest in determining an increase oxidative status in older individuals. Increased oxidative stress status was evaluated in the pathogenesis of arteriosclerosis (Mashima et al., 2001) as well as in patients suffering from congestive heart failure (Belch et al., 1991) and myocardial ischemia (Rodrigo et al., 2013).

Finally, the liver as a model organ in this study is known to be less affected by the aging process compared to other high-blood-flown organs like heart and brain. It has a high regenerative capacity and therefore is widely studied in the field of oxidative damage (Schmucker, 1998). The liver fulfills numerous functions that can be basically summed up as the ability to excrete harmful substances such as toxins and drugs, containing parts of carbohydrate, protein and lipid metabolism and the secretion of bile produced in the hepatocytes into the bile duct (Mangoni and Jackson, 2004, Mitra and Metcalf, 2009). With advancing age, the liver undergoes various changes with a loss of hepatic volume up to 20-40%

and reduced blood flow through the liver of up to 35-40% (Zeeh and Platt, 2002, Grizzi et al., 2013). This could be responsible for the reduced drug elimination rate in elderly leading to an altered metabolism for drugs and toxins. Further bile synthesis and secretion is halved indicating reduced transport mechanisms in aging (Zeeh and Platt, 2002). Finally, the regeneration capacity defined as loss of hepatic function declines and old individuals need more time to restore liver function after damage (Sanz et al., 1999).

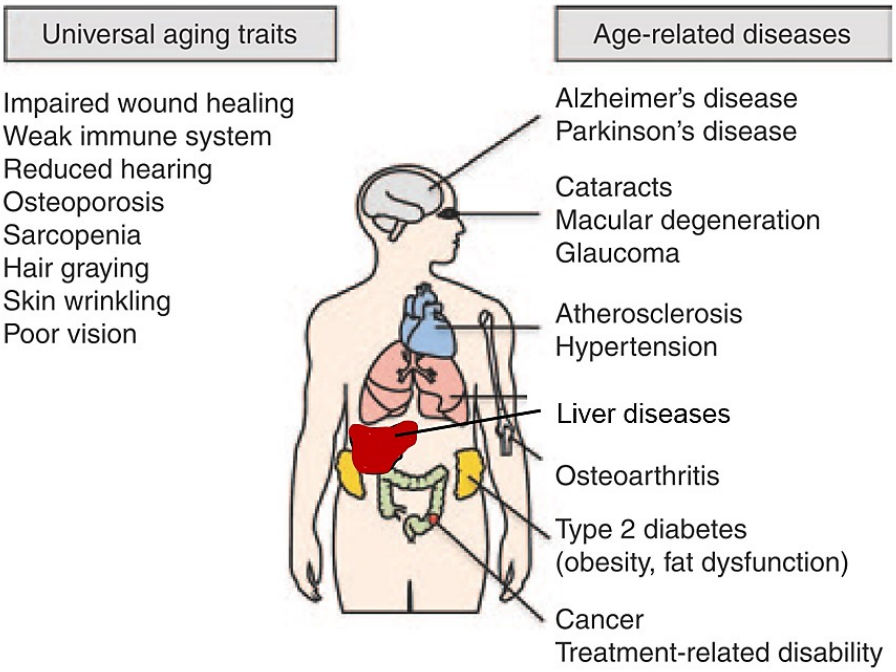


Figure 2: Universal traits of aging (left) and age-related diseases (right). Various general aging traits are identified monitoring the aging process including hearing loss, wrinkled skin, reduced vision and gray hair. Immune system response is weakened as well as wound healing. Age-related diseases interact with aging and display possible cellular alterations [modified from (Naylor et al., 2013)].

1.2. Oxidative stress, reactive oxygen species and mitochondria

One of the first biochemical models that links the process of aging and the accumulation of oxidative damage in an organism is the 'free radical theory of aging' of Denham Harman 50 years ago. According to Harman the impact of negative effects on the cell defined as free radicals could weaken their ability to react to certain levels of 'stress' (Harman, 1956). The accumulation of radicals inside the cell leads to further damage and finally cell death (Tosato et al., 2007).

The oxygen metabolism is identified as a major cause for the generation of radicals inside cells (Halliwell and Gutteridge, 2007). In an aerobic environment the oxygen is mainly processed into water in the respiratory chain of mitochondria producing ATP but small amounts of oxygen (up to 4%) are converted into oxygen radicals (Farber, 1994). These so-called reactive oxygen species (ROS) can accumulate during aging inside cells and alter the cells metabolism (Masoro, 2005). The imbalance that occurs between ROS formation and protection mechanisms of the cell with favor to the radical site is defined as oxidative stress (Sies, 1993). This could be caused either by an increased production of radicals or a decreased protection against radicals (Davies, 2000). Protection mechanisms were identified to extend the life span as the overexpression of genes encoding for elimination of radicals in the fruit fly *Drosophila melanogaster* was found to significantly increase the maximum life span, whereas the inactivation of protection mechanisms in the mouse is neonatal lethal (Melov, 2000). Controversial effects concerning radical formation and possible oxidative tissue injury have been described and physiological ROS levels are found to trigger signal transduction pathways (Halliwell and Gutteridge, 2007). The oxidative modification of kinases, phosphatases and transcription factors resulting in their activation regulates physiological functions inside cells as well as the ability to react towards stress (Hekimi et al., 2011) (Figure 3). ROS enhance signal cascades but it is also necessary to finally end the triggered signal. Therefore, it is essential for proper physiological responses to keep the sensitive balance between positive oxidative enforcement of signal cascades and reducing conditions for their termination (Droge, 2002). This so-called positive oxidative stress enables the adaptation of an organism to stressful conditions such as hypo-and hyperthermia, hypoxia and ischemia (Yan, 2014, Lu et al., 2014, Salido et al., 2013). Further studies have shown that ischemic tolerance after preconditioning was found to increase the protection of tissue against ischemic-induced injury (Eipel et al., 2005, Glanemann et al., 2004, Mori et al., 2000). However, by passing a sensitive threshold at higher levels ROS target different macromolecules of the cell mainly including proteins, nucleic acids and lipids. During aging this oxidative damage of macromolecules accumulates as

repair processes slow down and detection mechanisms decline leading to dysfunctional cells (Kumar et al., 2012). There are different techniques to determine the grade of oxidative stress in body fluids and tissues. The oxidation of proteins is measured as cross-linked protein aggregates (Stadtman, 2001). Alteration in nucleic acid include DNA strand breaks and base modifications which affect the genome stability and result in an increased rate of mutations (Mecocci et al., 1993) and the oxidation of lipids which initiates the chain reaction of lipid peroxidation with increased end-products indicate higher levels of oxidative stress (Niki, 2009).

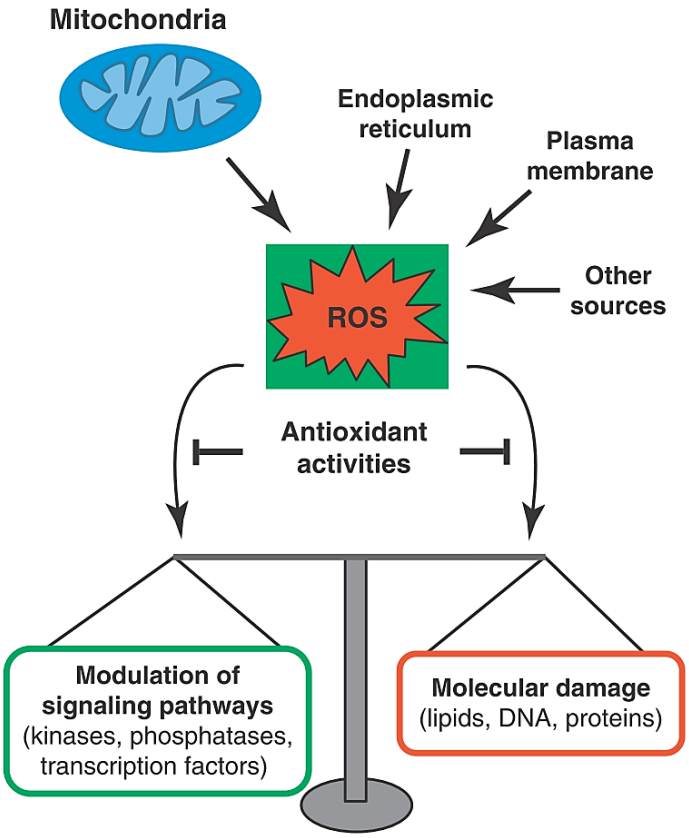


Figure 3: Sources and functions of ROS. Reactive oxygen species (ROS) generated in the mitochondria or at other sites fulfill two main functions in cell metabolism. At lower levels ROS serve as second messenger molecules and modulate important pathways but at higher levels they damage macromolecules including lipids, DNA and proteins. Antioxidants control the ROS generation, stop signal cascades and keep the redox balance [modified from (Hekimi et al., 2011)].

Reactive oxygen species (ROS) and mitochondria

ROS are generated in an organism from endogenous and exogenous sources. The main endogenous source is the respiratory chain of mitochondria but also enzymes like NADPH oxidases, lipoxygenases and cytochrome P450 can generate ROS (Finkel and Holbrook, 2000). Aging was found to increase the release of ROS from the respiratory chain and less energy in form of ATP is produced emphasizing the major impact of mitochondrial oxidative damage during aging (Moghaddas et al., 2003, Sastre et al., 2000). Exogenous sources are ionizing radiation and ultraviolet light as well as toxins and chemotherapeutics (Kudryavtseva et al., 2016). ROS include free radicals like superoxide anion (O_2^-•) and hydroxyl radical (OH•) as well as non-radicals like hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). They contain one or more unpaired electrons that make them highly reactive to other molecules (Shah et al., 2014, Ozcan and Ogun, 2015). ROS convert into stabilized molecules by stealing electrons from other nearby structures initiating the creation of more radicals or reacting with each other forming a stable end-product (Lee et al., 2004). O_2^-• is mainly generated in the respiratory chain of mitochondria that is located at foldings of the inner membrane. It consists of complex I-IV that build up an ionic gradient to enable energy production via ATP synthase (Figure 4A). During reactions, constantly O_2^-• leaks into the mitochondrial matrix as well as into the intermembrane space potentially damaging nearby macromolecules. Especially mitochondrial DNA (mtDNA), membrane lipids and proteins are vulnerable to ROS-mediated lesions leading to mitochondrial dysfunction with higher production of ROS and increased apoptosis.

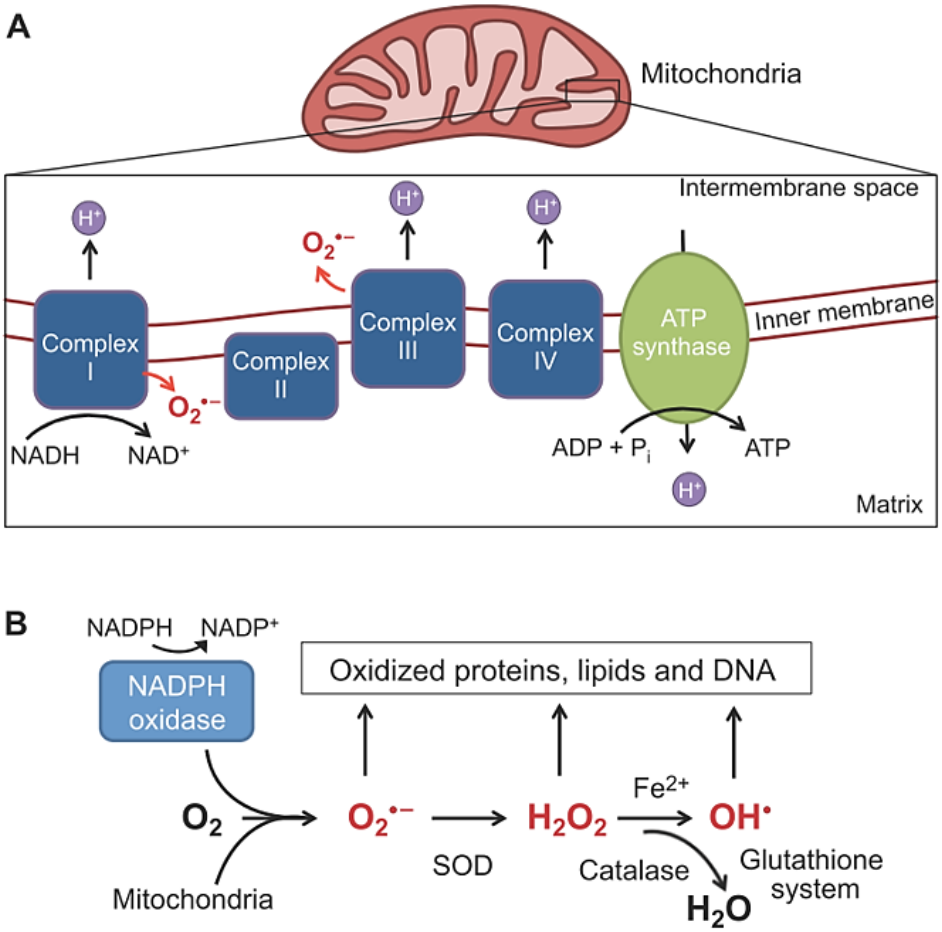


Figure 4: ROS generation in mitochondria and their metabolism inside cells. A Mitochondrial respiratory chain at the inner membrane consists of complex I-IV that build up a proton gradient (H^+) to generate energy via the ATP synthase. Superoxide anions (O_2^{-}•) leak in the matrix and in the intermembrane space. **B** ROS (O_2^{-}•, H_2O_2, OH•) metabolism inside cells. O_2^{-}• is processed via superoxide dismutase (SOD) into H_2O_2 and catalase as well as glutathione system buffers H_2O_2 into water. H_2O_2 can react with Fe^{2+} via Fenton reaction forming OH• [adopted from (Bigarella et al., 2014)].

O_2^{-}• is removed by the enzyme superoxide dismutase (SOD) creating oxygen and H_2O_2 (Figure 4B). H_2O_2 is a by-product of many reactions in the organism and can be decomposed in the presence of reduced iron ions either into highly dangerous OH• via Fenton reaction ($Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + OH + OH•$) (Cheng et al., 2002) or it is buffered by catalase (CAT) into water and oxygen (Slimen et al., 2014). H_2O_2 also targets distant cells as it can diffuse through membranes and has a relatively long half-life (Cadenas and Davies, 2000). OH• generated from H_2O_2 is known as the most damaging radical due to its extreme reactivity and

short half-life making it mainly responsible for nearby oxidative damage to DNA molecules such as base modifications and strand breaks (Zorov et al., 2014). OH• reacts with the guanosine base of DNA and forms 8-hydroxydesoxyguanosin (8-OHdG) that is found to increase in relation to oxidative stress (Haghdoost et al., 2005). It has been shown that mutations in mtDNA are 10 times higher compared to chromosomal DNA (Mecocci et al., 1993). In comparison to the nuclear DNA, mtDNA is not covered by histone proteins, has a high transcription rate and is located near the site of ROS production that makes them more susceptible for oxidative injury (Cai et al., 1998). With increasing age and accumulation of mtDNA damage the respiratory chain produces more ROS finally increasing the risk for diseases and enforcing the aging process (Van Remmen and Richardson, 2001).

Mitochondrial oxidative damage also increases the release of intermembrane space proteins into cytoplasm such as cytochrome c by making the outer membrane more permeable for these substances (Figure 5) (Murphy, 2009, Gellerich et al., 2000). Cytochrome c is part of the respiratory chain transferring electrons and when released out of the intermembrane space into cytoplasm it serves as a signaling molecule that activates a chain of caspases that finally induce apoptosis (Cai et al., 1998). Additionally, the formation of a permeability transition pore (PTP) in the inner membrane causes rapid movement of ions that lead to mitochondrial swelling and loss of mitochondrial membrane potential. This allows small molecules like the apoptosis-inducing factor (AIF) to be released more easily into cytoplasm inducing apoptosis (Murphy, 2009, Cai et al., 1998). Furthermore lipid peroxidation of the mitochondrial membrane as well as oxidization of mtDNA is induced making mitochondria more vulnerable and further affect the functionality of the respiratory chain (Sastre et al., 2003).

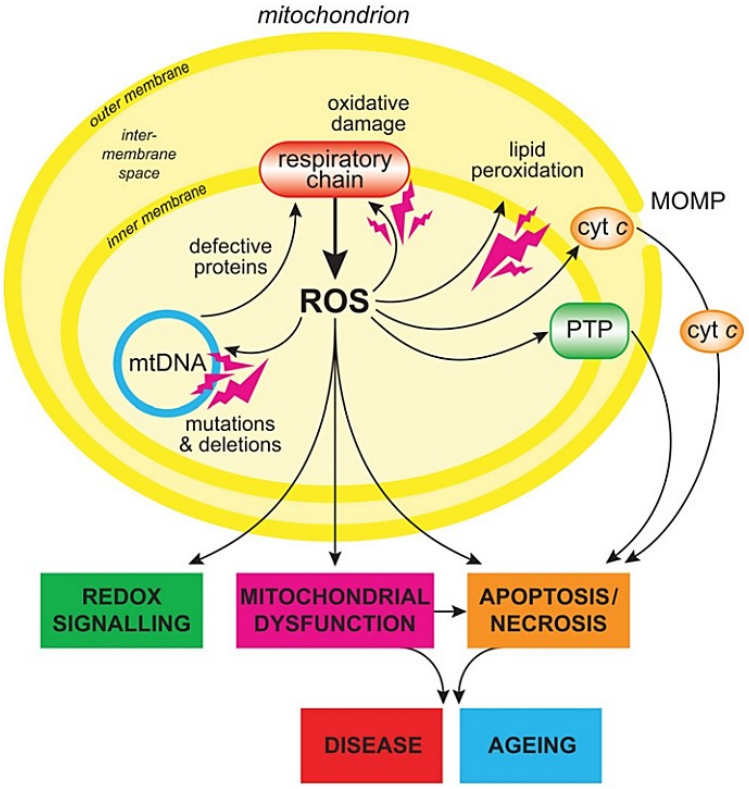


Figure 5: Mitochondrial ROS production and harmful effects of ROS. Mitochondrial ROS are produced in the respiratory chain at the inner membrane. ROS damage directly membranes via lipid peroxidation, attack mtDNA and alter the functionality of mitochondrial membranes. Outer membrane permeability (MOMP) is increased as well as opening permeability transition pore (PTP) leading to increased apoptosis and necrosis [adopted from (Murphy, 2009)].

Protection against oxidative stress

Organisms can react to increasing oxidative stress by upregulating certain protecting molecules such as antioxidative enzymes and non-enzymatic antioxidants that can buffer the ROS (Davies, 2000, Mezzetti et al., 1996). The antioxidant system consists of enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and non-enzymatic molecules such as vitamin E and glutathione (GSH) (Finkel and Holbrook, 2000, Yu, 1994, Lee et al., 2004). McCord and Fridovich discovered in 1969 the role of SOD as an enzyme scavenger protecting tissue against oxidative damage (McCord and Edeas, 2005, McCord, 1974). In humans exist three forms of SOD: cytoplasmic Cu/ZnSOD, mitochondrial MnSOD and extracellular SOD (Matés et al., 1999). All SOD forms have transition metals at their active side including copper, magnesium and zinc that are oxidized or reduced while SOD is

detoxifying O_2^{-}• (Davies, 2000). SOD activity in erythrocytes of healthy humans was found to decrease during aging drawing a link to oxidative injury as radicals could not be buffered effectively (İnal et al., 2001). SOD converts O_2^{-}• into oxygen and H_2O_2 which is further processed by CAT into water and oxygen (Figure 6). The glutathione redox system consists of GPx that also detoxifies H_2O_2 by oxidizing GSH to glutathione disulfide (GSSG) into water (Tian et al., 1998). GR is necessary to re-reduce GSSG into 2 molecules of GSH by oxidizing NADH to NAD^+ (Circu and Aw, 2010). GSH mainly synthesized in the liver is not only substrate for GPx but also antioxidative molecule itself as it buffers OH• and singlet oxygen (Sies, 1999). Another defense mechanism of the cell is the ability to undergo apoptosis instead of ending up with uncontrolled necrosis harming surrounding cells with a secondary stress. During apoptosis the cell is engulfed by phagocytes to prevent an immune reaction and re-use valuable nutrients (Davies, 2000). It is controversial discussed whether increased antioxidative capacity would increase life span as low levels of ROS serve as signaling molecules and former studies have shown that the excessive intake of antioxidants has severe side effects with increased mortality and even induction of cancer (Bjelakovic et al., 2004, Miller et al., 2005, Vivekananthan et al., 2003). Higher antioxidant levels can protect from oxidative damage but they also reduce the physiological adaptation of the cell towards stress that is necessary for metabolic pathways (Sena and Chandel, 2012).

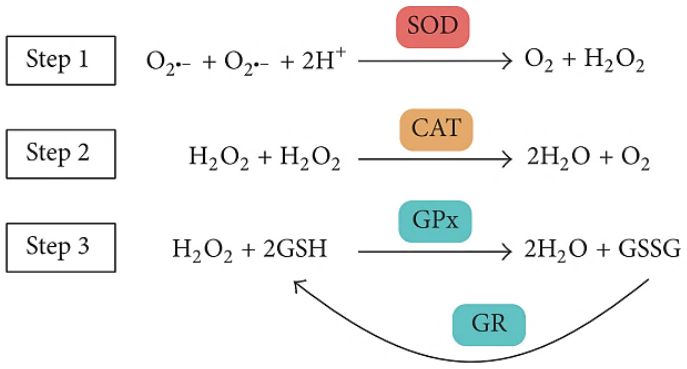


Figure 6: Scavenging reactions of antioxidative enzymes. Removal mechanisms for O_2^{-}• and H_2O_2 are schematically shown. SOD converts O_2^{-}• into O_2 and H_2O_2 and CAT further detoxifies H_2O_2 into H_2O and O_2. The glutathione redox system consists of GPx that transforms H_2O_2 into water by oxidizing 2 GSH into GSSG. GSSG is reduced into GSH by GR to form new substrate [adopted from (Peng et al., 2014)].

1.3. Lipid peroxidation

The oxidative stress in form of ROS leads via a chain reaction that is called lipid peroxidation (LPO) to altered structure and function of lipids mainly in cell membranes (Kudryavtseva et al., 2016, Yu, 1994). To evaluate a possible oxidative damage, the amount of LPO is found to be significantly increased in stressful conditions. Lipids are made up of fatty acid chains with a carbon backbone mainly consisting of 14 to 24 carbon atoms that could be categorized into saturated or mono- and polyunsaturated fatty acids. Polyunsaturated fatty acids (PUFA) contain two or more carbon-carbon double bonds that weaken nearby carbon-hydrogen bonds and make them more susceptible for radical attacks because they more easily split off hydrogen atoms (Farber, 1994).

Membranes of aged individuals show an increased amount of long-chain PUFA that are more prone to peroxidation creating protein cross-links that increase the rigidity of membranes and make the membrane more vulnerable to oxidative stress (Laganiere and Yu, 1993). Previous studies have shown that short-living species like rats with maximum life span (MLS) of 4 years have more PUFA with higher numbers of double bonds that are more easily attacked by ROS in comparison to long-living species such as horses (MLS = 46 years) (Pamplona et al., 2000). Also naked mole-rats (MLS = 28 years) in comparison to mice (MLS = 3-4 years) have less PUFA that are highly affected by LPO underlining the theory that membrane composition has influence on the longevity of species (Hulbert et al., 2006). So long-living species show a decreased rate of unsaturation due to lower levels of highly reactive PUFA in membranes that decreases their sensitivity towards LPO and therefore fewer harmful products that damage cells are produced (Naudí et al., 2013).

Cell membranes work as selective barriers protecting the cell, support vital functions in signaling pathways and are composed of a phospholipid bilayer (30-80%) including proteins (20-60%) and some carbohydrates (0-10%) (Catalá, 2009). The high amount of PUFA in phospholipids of cell membranes are priority targets for LPO (Farber, 1994). Especially phospholipids located in the mitochondrial membrane are affected due to their near-by location to the respiratory chain (Andziak and Buffenstein, 2006). The membrane proteins can

be either located at the inner or outer part of the membrane as well as traversing the membrane. Together they create a so-called Fluid Mosaic Model, a two-dimensional fluid model of orientated proteins and lipids that allows molecules to move in the plane of the membrane (Nicolson, 2014). This is essential for the cellular function. Oxidative damage via LPO was found to affect the membrane fluidity and permeability for certain molecules leading to disturbed metabolic processes (Andziak and Buffenstein, 2006). The oxidized membrane lipids become rigid, lose selective permeability and in worst case lose their integrity (Davies, 2000). The membrane potential declines due to increased permeability for H^+ and other ions affecting ATP-generated and other ion transport mechanisms through membranes (Gutteridge, 1995). By altering the membrane structure the resistance to thermo-denaturation declines and end-products of LPO such as aldehydes are found to cause severe damage to various molecules by forming protein adducts with membrane proteins additionally altering their structure (Anzai et al., 1999, Lee et al., 2004). The process of lipid peroxidation includes three major steps and is outlined in Figure 7.

LPO is induced by a free radical (R•) and starts with the initiation step (①) by abstracting a hydrogen atom (H^+) from the fatty acid chain near a double bond leaving a lipid radical (LR•) behind. Under aerobic conditions the propagation (②) continues with the lipid radical (LR•) that combines with oxygen (O_2) forming a lipid peroxyl radical (LOO•). LOO• reacts with the next unsaturated fatty acid chain forming another LR• by abstracting H^+ and lipid hydroperoxide (LOOH). The LR• serves as a new substrate for the propagation phase and triggers a chain reaction creating more LOO• (③). The termination step (④) ends the chain reaction either by donation of H^+ from antioxidants to LOO• forming a non-radical and more stable product or with final decomposition of LOOH into end-products such as aldehydes (Dobrian et al., 2000). The highly reactive OH• can generate LR• and H_2O_2 out of LOOH. LOO• and LR• can restart the chain reaction of lipid peroxidation leading to the accumulation of radicals (Catalá, 2009).

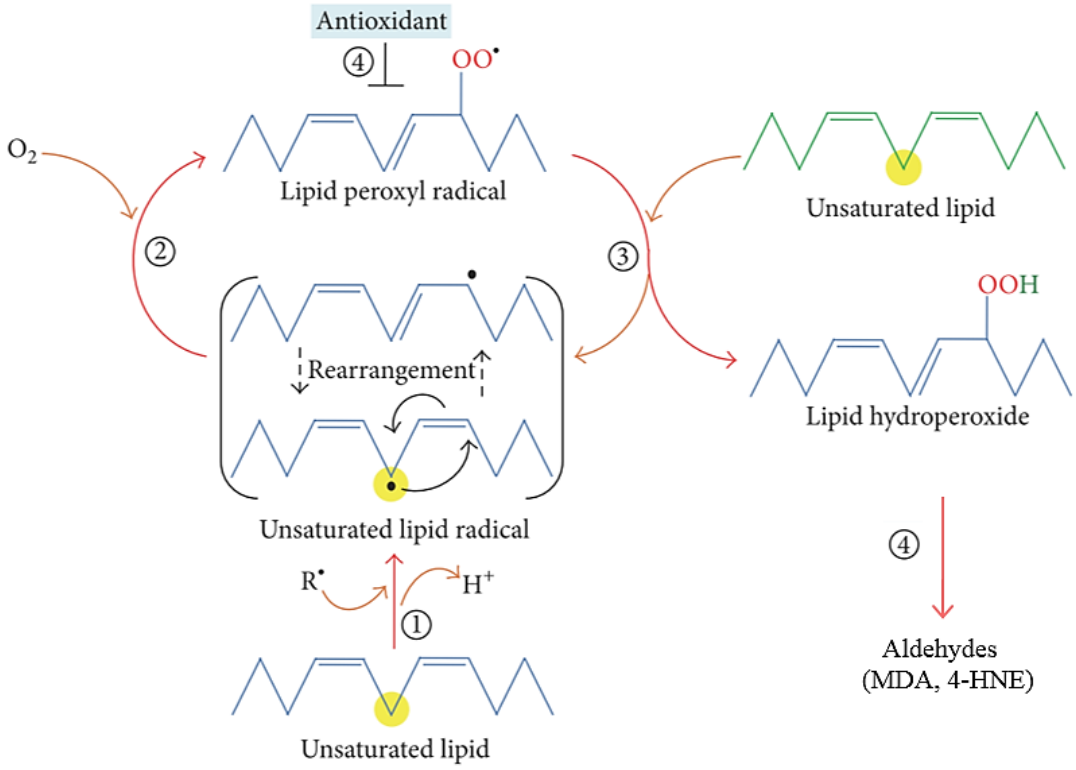


Figure 7: Process of lipid peroxidation. In initiation (1) radicals (R•) attack unsaturated lipids (LH) and form a lipid radical (LR•) by abstracting a hydrogen atom (H^+). LR• reacts with O_2 in propagation phase (2) forming lipid peroxyl radical (LOO•) which starts the chain reaction (3) by reacting with another LH and forms lipid hydroperoxide (LOOH). Termination (4) includes the decomposition of LOOH into aldehydes and donation of H^+ to LOO• from antioxidants [adopted from (Ayala et al., 2014)].

Marker of lipid peroxidation

Aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) mainly derive *in vivo* from PUFA with three or more double bonds. They are reactive end-products of lipid peroxidation that serve as toxic messengers by initiating the formation of free radicals leading to oxidative tissue damage (Catalá, 2009). The generation of MDA and 4-HNE as oxidative breakdown products of lipids in membranes via LOO• and LO• is shown in Figure 8. Both oxidative stress markers are widely studied for their role in tissue damage as well as signaling molecules in metabolism. In numerous age-dependent diseases increased amounts of MDA and 4-HNE were found including Alzheimer's disease (Markesbery and Lovell, 1998, Butterfield et al., 2006) and Parkinson's disease

(Navarro et al., 2009, Castellani et al., 2002), cancer (Gonenc et al., 2001, Cai et al., 2012), cardiovascular diseases (Walter et al., 2004), and liver diseases (Dou et al., 2012, Sampey et al., 2003). Further investigations are needed concerning cellular injury as it is not yet identified whether LPO is the cause for the damage or the outcome of earlier cell injury (Grotto et al., 2009). As aldehydes are relatively stable and water-soluble they diffuse through the altered oxidized membrane reaching targets even outside the membrane distant from their site of origin (Davies, 2000). To evaluate the amount of oxidative damage in cells, MDA and 4-HNE are determined as stable end-products of the chain reaction of LPO initiated by ROS attacks. MDA shows a great reactivity to molecules with free amino acid groups and forms partly stable products via cross-links (Janero, 1990). Further MDA is known to be mutagenic by forming different adducts with DNA bases (Esterbauer et al., 1991). These MDA-DNA adducts mainly induce base pair substitutions with increased mutation frequencies as seen in normal breast tissue of breast cancer patients with higher amounts of MDA-adducts compared to breast tissue of women without breast cancer (Wang et al., 1996).
4-HNE mainly derived from arachidonic and linoleic acid was identified to be even in small amounts highly toxic to cells with inhibition of protein and DNA synthesis as well as lowering GSH contents in membranes (Benedetti et al., 1980, White and Rees, 1984). The reactivity of 4-HNE is based on its reactivity to the amino acids cysteine, lysine and histidine forming 4-HNE-protein adducts (Poli et al., 2008). 4-HNE also affects the DNA as mutagenic agent by binding to guanosine bases of DNA forming 4-HNE-DNA adducts (Csala et al., 2015). Additionally, binding of 4-HNE to key signal proteins via sulfhydryl groups modulates physiological pathways also inducing pathological responses (van der Vliet and Bast, 1992). Increased levels of 4-HNE were found in oxidative stress-related pathologies such as during reperfusion of rat myocardium (Blasig et al., 1995), in plasma of premature infants with chronic lung disease (Ogihara et al., 1999) and in patients with alcohol-induced liver injury (Aleynik et al., 1998, Meagher et al., 1999).

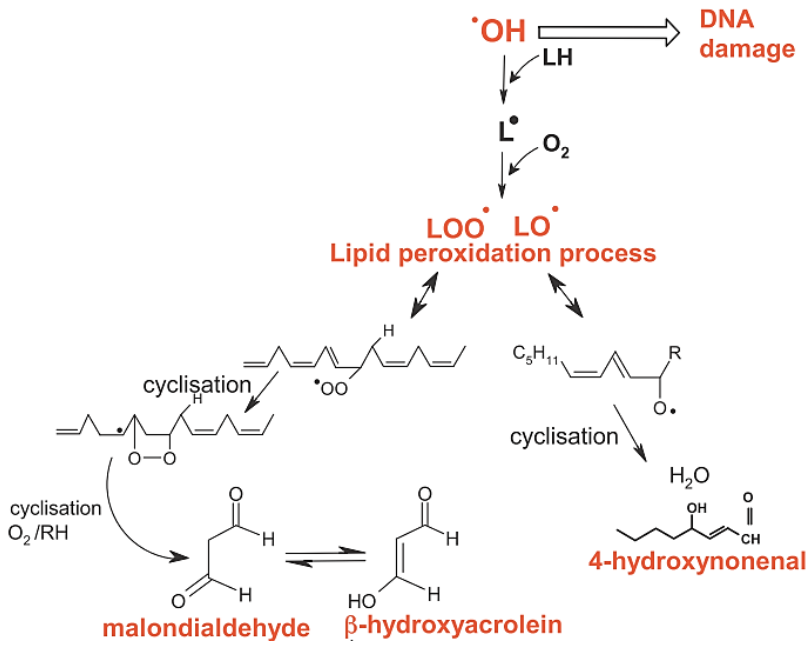


Figure 8: Formation of MDA and 4-HNE as end-products of lipid peroxidation. OH• attack unsaturated lipid (LH) and initiate the process of lipid peroxidation finally leading to formation of LOO• and LO• that further decompose into MDA and 4-HNE. These aldehydes indicate the amount of oxidative stress in an organism in form of lipid peroxidation [adopted from (Jomova and Valko, 2011)].

1.4. Oxidative stress-induced liver damage

The liver used as a model organ in this study is found to be highly affected by increased oxidative stress in form of ROS (Muriel, 2009). Liver tissue contains high amounts of PUFA that are priority targets of ROS as well as iron that in its free form can catalyze ROS-releasing reactions (Meng and Zhang, 2003). Increased age-dependent oxidative stress is linked to hepatic injury as old individuals are more likely to suffer from liver diseases with poorer clinical outcome due to reduced regeneration capacity (Tajiri and Shimizu, 2013). Figure 9 outlines the effects of oxidative stress on the liver that finally cause hepatic pathologies including fatty degeneration (steatosis), chronic inflammation (hepatitis), scar tissue formation (fibrosis) and further destruction of liver structure (cirrhosis) as well as hepatocellular carcinoma (HCC) (Li et al., 2015). ROS-induced pathways harm liver cells via oxidation of DNA that increases the risk for HCC and initiation of LPO with accumulation of fatty acids leading to steatosis. The ROS-induced formation of protein adducts and mitochondrial dysfunction

affect signaling molecules and increase inflammation reactions that lead to chronic hepatitis (Cichoz-Lach and Michalak, 2014). Hepatic stellate cells are particular sensitive towards ROS attacks and once activated secret collagen to form scar tissue that finally remodels liver structure into fibrotic and further cirrhotic tissue (Dunning et al., 2013). Oxidative stress-induced liver injury is well studied in animal models after the application of carbon tetrachloride (CCl_4) or ethanol as pro-oxidant agents (Kadiiska et al., 2005, Weber et al., 2003, Natarajan et al., 2006). CCl_4 has been identified as effective *in vivo* inducer of LPO in membranes causing steatosis in liver tissue (Recknagel et al., 1989). Various studies observed that the prior application of antioxidative substances including herbal extracts and dietary supplements prevents hepatotoxic effects of CCl_4 (Lin et al., 2008, Feng et al., 2011, Reyes-Gordillo et al., 2007).

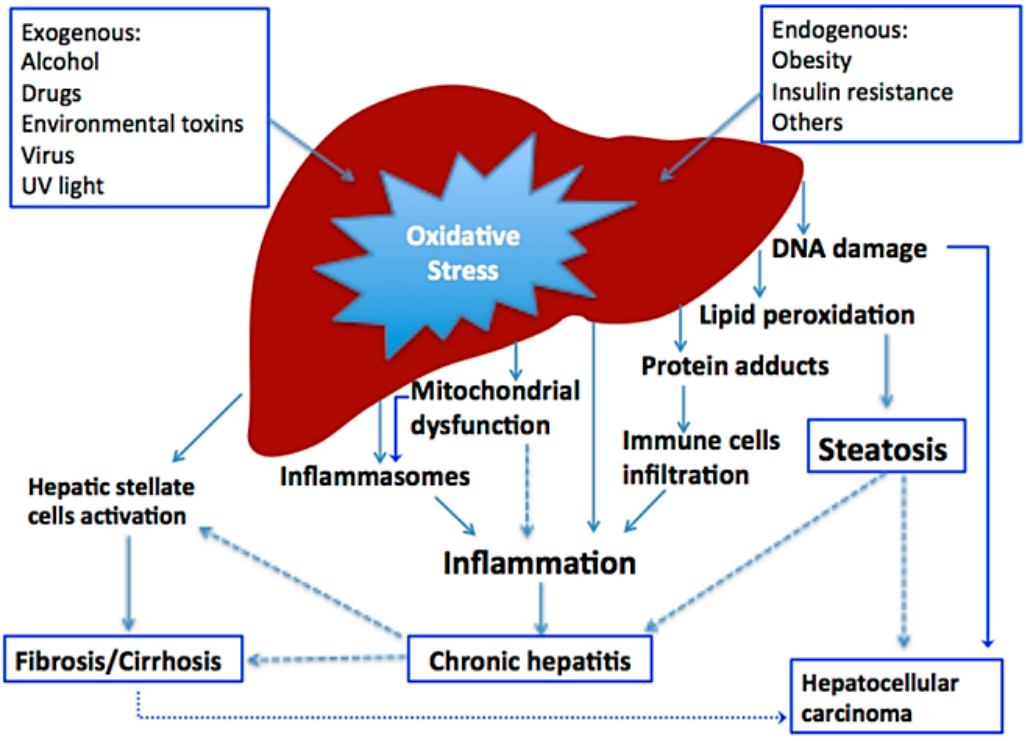


Figure 9: Oxidative stress in the liver causing liver diseases. Exogenous and endogenous sources lead to increased oxidative stress in the liver. ROS-induced pathways include oxidation of DNA resulting in hepatocellular carcinoma (HCC) and lipid peroxidation with accumulation of fatty acids that forms steatosis. Protein adduct formation and mitochondrial dysfunction affect signaling molecules and increase inflammation leading to chronic hepatitis. Hepatic stellate cells activated by ROS secret scar tissue and remodel liver structure into fibrotic/cirrhotic tissue [adopted from (Li et al., 2015)].

The alcohol-induced liver disease (ALD) is one of the leading causes of death and disability worldwide and oxidative stress is intensively studied in liver pathologies linked to excessive alcohol consumption (Louvet and Mathurin, 2015). Ethanol is mainly metabolized in the liver by alcohol dehydrogenases but also cytochrome P450 oxidases further metabolize toxins and drugs (Wu and Cederbaum, 2009). During these reactions in the liver increased ROS are generated that could be measured as increased LPO products and oxidative DNA damage markers displaying oxidative stress caused by ethanol (Seki et al., 2003, Albano et al., 1996). Especially the isoform cytochrome P450 2E1 (CYP2E1) is identified to be induced after alcohol ingestion and intragastrical feeding of ethanol to rats has been reported to increase CYP2E1 activity and LPO products underlining the theory of ROS-induced injury in ALD (French et al., 1993, Rouach et al., 1997, Takahashi et al., 1992). Further studies observed that chronic alcohol intake weakens the protection against oxidative stress in form of hepatic antioxidant defense system mainly measured as reduced antioxidative enzymes activities including SOD, CAT and GPx as well as reduced GSH levels (Husain et al., 2001, Molina et al., 2003). The prophylactic application of antioxidative substances including *Gingko bilboa* extract (Yao et al., 2006), quercetin (Nussler et al., 2010, Liu et al., 2010) and curcumin (Rong et al., 2012) prior to alcohol intake is observed to partially reduce ethanol-induced oxidative injury and displays a possible approach towards pharmaceutically preventing oxidative stress.

1.5. Heat stress in aging

During prolonged periods of environmental heat in form of heat waves especially people older than 65 years have a higher risk of mortality (Semenza et al., 1996, Vandentorren et al., 2006, Vanhems et al., 2003, Conti et al., 2005). Old individuals are less tolerant to hyperthermia and more affected by repeated heat stress (Semenza et al., 1996). To keep the physiological homeostasis in balance, the body has various adaptation mechanisms towards elevated temperatures. The production and evaporation of sweat is important to keep the core temperature within its range as well as the increase of cutaneous blood flow by

distributing the blood from central to peripheral vessels via vasodilatation (Kenney et al., 2014). Aging was found to decrease sweat production, prolong sweating during long heating times with loss of plasma volume and decrease cutaneous blood flow (Dufour and Candas, 2007, Armstrong and Kenney, 1993, Kenney et al., 2014). The plasma viscosity increases caused by the loss of fluids leading to a higher possibility of coronary blockage with increased risk of cardiovascular death (Kenney et al., 2014). To further investigate the relationship between the aging process and heat stress, changes that take place in an aged organism when confronted to increased temperatures need to be studied. As shown by Kenney and Munce in Figure 10, older individuals have a decreased sweat gland function with less sweat secretion and a lower cutaneous blood flow probably due to altered structure in aged skin compared to their younger counterparts in heat stress conditions (Kenney and Munce, 2003). Other studies comparing the cardiovascular system under passive heating investigated a reduced increase in cardiac output and less redistribution from visceral circulation (Minson et al., 1998). Former studies in gold miners suffering from severe heat stroke have also shown that the liver is highly affected from heat stress in form of liver injury (Kew et al., 1970, Kew et al., 1971).

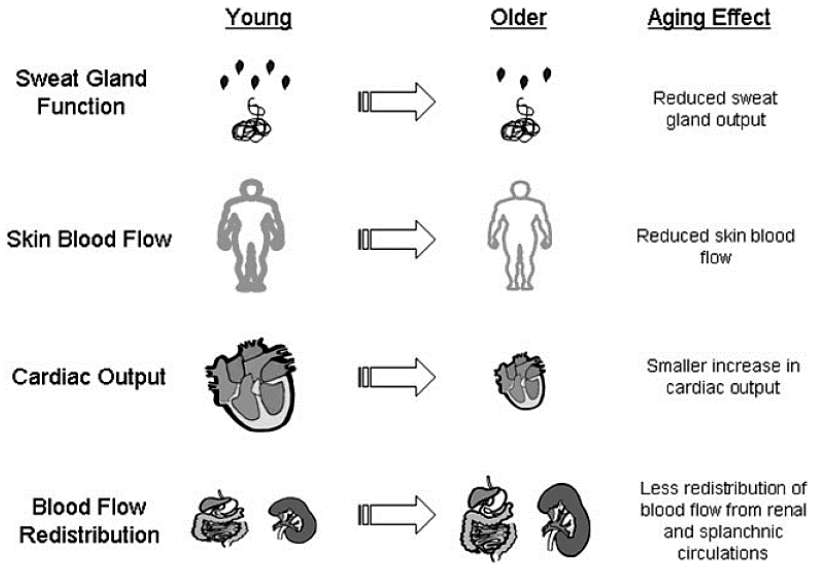


Figure 10: Age-related changes in heat stress conditions. Older people sweat less, have a reduced cutaneous blood flow, a smaller increase in cardiac output and the blood redistribution from renal and splanchnic circulations is reduced [adopted from (Kenney and Munce, 2003)].

By focusing on the molecular level, elevated temperatures as a form of external stress stimulate the ROS production and further increase the oxidative stress in an organism (Flanagan et al., 1998). Studies have shown that the respiratory chain of mitochondria gets uncoupled (i.e. loss of repspiratory control) when the temperature rises above a certain level and increased amounts of O_2^-• and H_2O_2 accumulate (Salo et al., 1991). Near-by structures inside the mitochondria are attacked and H_2O_2 additionally affects distant structures as it diffuses easily through cell membranes (Slimen et al., 2014). Different mechanisms have been identified as protection of the cell in heat stress. In hyperthermia the organsim produces so-called heat shock proteins (HSP) to repair denaturated proteins (Ristow and Schmeisser, 2014). The HSP synthesis and their function as chaperones for proteins is shown in Figure 11 (Slimen et al., 2014). Under the influence of physiological stressors like hyperthermia and oxidative stress protein degeneration occurs inside cells. Heat stress transcription factors (HSF) are activated and induce the production of mRNA encoding for HSP and finally lead to HSP synthesis. HSP serve as so-called molecular chaperones and facilitates the correct folding of degraded proteins. Different HSP have been identified and they are divided into groups according to their molecular masses. Especially the 70 kDa HSP family (HSP70 and HSP72) was found to be strongly induced in heat shock conditions (Kiang and Tsokos, 1998, Li and Srivastava, 2001). HSP70 supervises during heat stress the correct folding of intracellular proteins and detects misfolded or denatured proteins (Yamamoto et al., 2000). They form a complex with these proteins and either support the transformation into the correct form or assist with their removal (Hightower, 1991). The cell is protected from stress injury and the ability to recover from higher levels of stress is enhanced by HSP (Blake et al., 1991). Various studies on heat shock protein formation after heat stress in aging organism showed reduced HSP formation in old individuals that might be caused by a weakened response to stress signals (Kregel and Moseley, 1996, López-Otín et al., 2013).

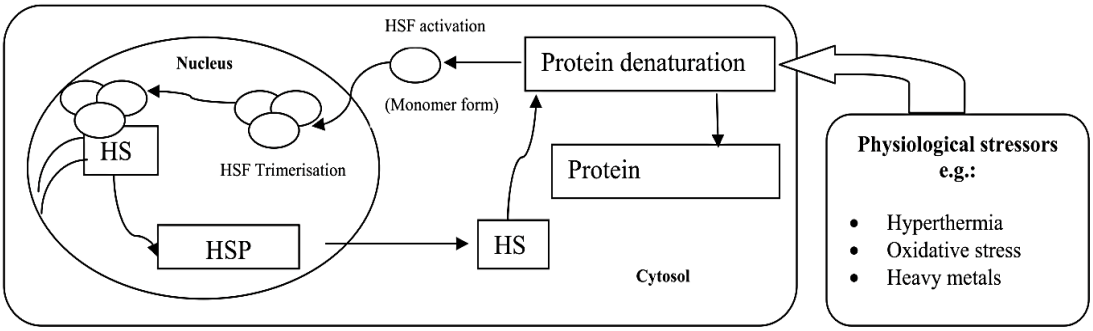


Figure 11: Schematic diagram of HSP synthesis. Physiological stressors (e.g. heat stress) induce the accumulation of denatured proteins and activate heat stress transcription factors (HSF). They are further processed in the nucleus and lead to transcription of HSP mRNA. HSP are synthesized and convert the denatured protein into is original formation [adopted from (Slimen et al., 2014)].

In heat stress conditions upregulated levels of protection mechanisms including GSH redox metabolism with increasing GSH production to buffer ROS are measured (Flanagan et al., 1998). Low intracellular GSH levels were identified to weaken the resistance of cells against hyperthermia (Flanagan et al., 1998) and increased levels of oxidized GSH as GSSG in the liver were found after ischemia indicating increased scavenging reactions of GSH (Abdalla et al., 1990).

1.6. BMBF joint research project OXISYS

This thesis is part of the subproject OXISYS that is integrated in the joint research project GerontoSYS II of the Federal Ministry of Education and Research (BMBF) under the title “Role of oxidative injury in aging and therapeutic implications” consisting of 6 partners. OXISYS research groups analyze aging processes through oxidative stress in the liver as model organ whereas other projects of GerontoSYS II examine various cells including brain tissue, fibroblasts of skin tissue and stem cells (BMBF, 2016). The aim of OXISYS subproject is to identify and further analyze potential biomarkers for clinical diagnosis involved in ROS-induced hepatic aging and to discover and evaluate their possible target structures in cell metabolism. The importance of these targets might further be proved by application of certain target-directed substances that interact and protect highly affected structures in the human liver during cellular aging. Therefore, the project is not only important for basic research of cell aging and

organ regeneration but also relevant for clinical usage. The understanding of key factors and metabolic pathways is necessary to improve diagnostic and therapeutic interventions resulting in higher quality of life allowing better prediction and prevention of aging processes. The experimental study of ROS-induced hepatic aging processes in rats and humans including *in vivo* and *in vitro* experiments in line with OXISYS subproject are performed by Charité Berlin, Pharmacelsus GmbH Saarbrücken, Eberhard Karls University Tübingen/Siegfried Weller Institut and University of Saarland. To mathematically analyze the generated data in the experiments and to establish *in silico* models that further allow simulation of aging processes and potential influencing factors two industrial partners, Hans-Knöll-Institut e.V. Jena and Insilico Biotechnology AG Stuttgart, are included as well.

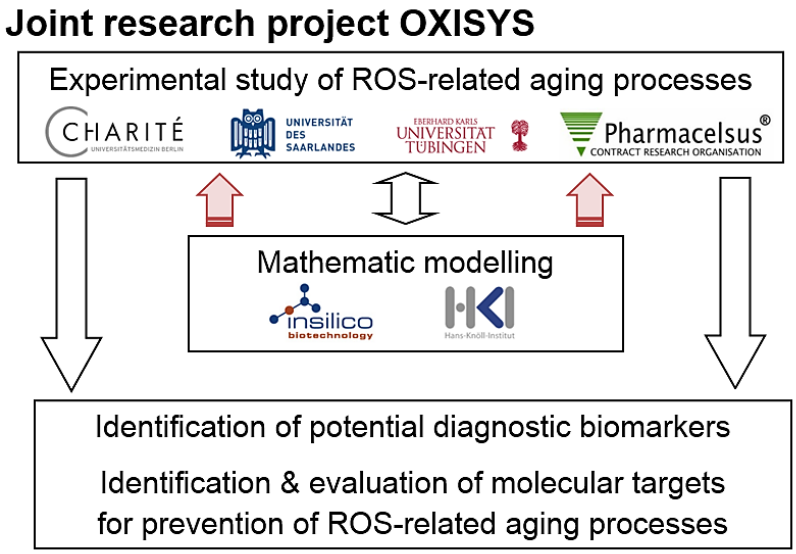


Figure 12: Structure of joint research project OXISYS [modified from (Kraft, 2012)].

1.7. Aim of the project

As part of the *in vivo* experimental setup of the OXISYS subproject, the aim of this thesis is to analyze the influence of oxidative stress in form of hyperthermia (up to 41°C) on differently aged male Wistar rats by measuring markers for lipid peroxidation (MDA, 4-HNE) and cell damage (LDH, ALT) in serum and liver tissue before and after two heat stress episodes. The hypothesis is an age-related increase of liver damage in old rats caused by oxidative stress after heat stress.

Untreated control groups divided into young, middle-aged and old rats were included to display the basal level of markers. The heat-stressed group consists of middle-aged and old rats that were liquidated at four points in time after the heating (0 h, 2 h, 6 h, 24 h). The liver was chosen as model organ as it has various diverse functions in the body and was identified to be affected by ROS-induced tissue damage from environmental stressors such as heat stress. Further H&E-stained liver sections before and after heat stress were histologically analyzed to visualize age-dependent hepatocellular damage. Focus lays on adaptation as well as protection mechanisms against tissue injury from higher stress levels after hyperthermia. The animal model is an important step towards the identification of biomarkers *in vivo* as diagnostic tools for detecting liver aging processes that later might be therapeutically used in humans to display and prevent the aging of an organism.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Table 1: List of used solutions, solids and buffers.

1,1,3,3-Tetraehtoxypropane (TEP)	Sigma-Aldrich Chemie GmbH
2-Thiobarbituric acid (TBA)	Sigma-Aldrich Chemie GmbH
4-HNE ELISA Kit	Cusabio Biotech Co. Ltd.
Acetic Acid	Carl Roth GmbH + Co. KG
Bovine Serum Albumin (BSA)	Carl Roth GmbH + Co. KG
$CuSO_4$ stock solution (C_{final} 1%)	1 g $CuSO_4$*5 H_2O 100 ml ddH_2O
Copper(II) sulfate pentahydrate ($CuSO_4$*5 H_2O)	Carl Roth GmbH + Co. KG
Deoxycholic acid sodium salt (DOC)	Carl Roth GmbH + Co. KG
Ethylenediamine tetraacetic acid disodium salt (EDTA)	Carl Roth GmbH + Co. KG
Fluitest GPT/ALT Kit Order No. 1186	Analyticon Biotechnologies AG
Fluitest LDH-L Order No. 2222	Analyticon Biotechnologies AG
Folin & Ciocalteu's phenol	Sigma-Aldrich Chemie GmbH
Na-K-Tartrate stock solution (C_{final} 2%)	2 g Na-K-Tartrate 100 ml ddH_2O
Na-K-Tartrate	Sigma-Aldrich Chemie GmbH
Na_2CO_3 stock solution (C_{final} 2%)	20 g Na_2CO_3 950 ml ddH_2O 50 ml 2 M NaOH
n-Butanol	Sigma-Aldrich Chemie GmbH
Pyridine	Carl Roth GmbH + Co. KG
RIPA stock solution	0.121 g TRIS Base 0.58 g NaCl 500 µl Tergitol solution 0.3 g DOC 0.372 g EDTA
Sodium carbonate (Na_2CO_3)	Carl Roth GmbH + Co. KG
Sodium chloride (NaCl)	VWR International GmbH
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG
Tergitol solution	Sigma-Aldrich Chemie GmbH
TRIS Base	Sigma-Aldrich Chemie GmbH

2.1.2. Equipment

Table 2: List of used equipment.

AF80 Ice flaker	Scotsman Ice Systems
Aqualine AL 25	Lauda Dr. R. Wobser GmbH & Co. KG
Cellstar 96 Well Microplate	Greiner Bio-One International GmbH
Cellstar Tube 15 ml	Greiner Bio-One International GmbH
Cellstar Tube 50 ml	Greiner Bio-One International GmbH
Costar Stripettes 5 ml	Corning Incorporated
Costar Stripettes 10 ml	Corning Incorporated
Costar Stripettes 25 ml	Corning Incorporated
Costar Stripettes 50 ml	Corning Incorporated
Digital Disruptor Genie	Scientific Industries, Inc.
Disposable Scalpel	Feather Safety Razor Co., Ltd.
FLUOstar Omega Microplate Reader	BMG Labtech GmbH
Fridge +4°C	Liebherr-International Deutschland GmbH
Fridge -20°C	Liebherr-International Deutschland GmbH
Fridge -80°C	Thermo Fisher Scientific Inc.
Heraeus Fresco 17 Centrifuge	Thermo Fisher Scientific Inc.
Heratherm Oven	Thermo Fisher Scientific Inc.
Incubator	Binder GmbH
Lambda Multichannel Pipettor 5 - 50 µl	Corning Incorporated
Lambda Multichannel Pipettor 20 - 200 µl	Corning Incorporated
Lambda Multichannel Pipettor 50 - 300 µl	Corning Incorporated
Lambda Single-channel Pipettor 2 - 20 µl	Corning Incorporated
Lambda Single-channel Pipettor 10 - 100 µl	Corning Incorporated
Lambda Single-channel Pipettor 20 - 200 µl	Corning Incorporated
Lambda Single-channel Pipettor 100 - 1000 µl	Corning Incorporated
LSE Vortex Mixer	Corning Incorporated
Megafuge 40 R	Thermo Fisher Scientific Inc.
Microscope Primo Vert	Carl Zeiss AG
Pipette Tips 0.1 - 10 µl Colorless	Sorenson BioScience, Inc.
Pipette Tips 2 - 200 µl Yellow	Sarstedt AG & Co.
Pipette Tips 100 - 1000 µl Blue	Ratiolab GmbH
Precellys Ceramic Kit 1.4/2.8 mm	Peqlab GmbH
Rotilabo-microcentrifuge tubes 1.5 ml	Carl Roth GmbH + Co. KG
Sky Line Digital Orbital Shaker	ELMI Ltd.
Spatula Set	Carl Roth GmbH + Co. KG
Sterile Bench Safe 2020	Thermo Fisher Scientific Inc.
Thermo-Shaker TS-100	Biosan

Weight ABJ 120-4M	KERN & SOHN GmbH
Weight PCB 250-3	KERN & SOHN GmbH

2.1.3. Animals

72 male Wistar rats (RjHan: WI) of three different age groups were obtained from Janvier Labs (Genest-St-Isle, France). They were housed in a temperature-controlled room (20-24°C) and adapted to a 12 h light/ 12 h dark cycle. The animals were given free access to food (ssniff ® R/M-H, 10 mm) and water before and during the study. Experiments were started after an acclimatization period of at least 1 week. All experimental procedures employed were approved by and conducted in accordance with the regulations of the local Animal Welfare authorities (Landesamt für Gesundheit und Verbraucherschutz, Abteilung Lebensmittel- und Veterinärwesen, Saarbrücken, file number C1 2.4.2.2. Nr. 12/2012). The heat stress experiments as well as the sampling of serum and tissue samples were performed by Pharmacelsus GmbH (Saarbrücken, Germany). The rats were divided into two groups: 28 young, middle-aged and old rats in the untreated control group as shown in Figure 13 and 44 middle-aged and old rats in the heat-stressed group that were liquidated at four points in time after heat stress as presented in Figure 14. Untreated young rats are 7 weeks old, middle-aged rats are 6 to 7 months old and old rats 23 months. The average body weight constantly increases from young to middle-aged rats with highest weight in old rats. Heat-stressed middle-aged rats are 7 months old and old rats aged 23 months. The average body weight in old heat-stressed rats is higher in every time group compared to middle-aged heat-stressed rats.

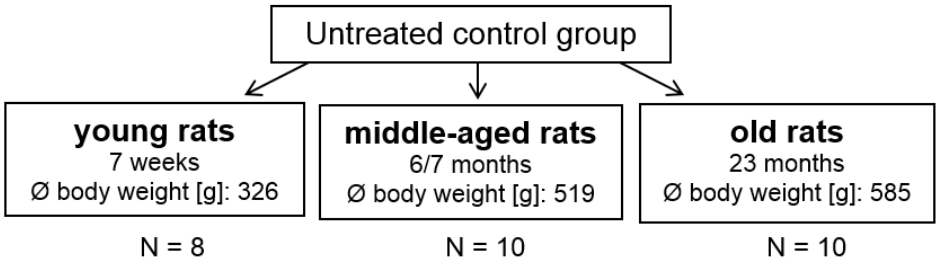


Figure 13: Overview of untreated control group. Young (7 weeks), middle-aged (6/7 months) and old (23 months) rats are included in the untreated control group. This figure shows the average body weight and number of individuals per age group.

2.1.4. Heat stress experiments

Middle-aged and old male Wistar rats were heat-stressed according to a modified protocol of the described heat stress experiment of Zhang et al. (Zhang et al., 2003). To record and supervise the exact body temperature during the heat stress, a temperature transponder (IPTT-300, Plexx B.V., The Netherlands) was implanted subcutaneously in the neck region under a short anaesthesia using isoflurane. To determine baseline data, a serum sample (300 µl) was simultaneously taken from the tail artery. After 3-5 days of implanting the transponder, the heat stress sessions were performed. Therefore, two rats with nearly the same weight at a time were put into a warming cabinet (small animal recovery chamber, Harvard Apparatus GmbH, Germany) provided with bedding, but no food or water. To minimize other stress factors, the animals were familiarised one day before the actual heat stress sessions to the warming cabinet without any heating by keeping them in the experimental environment for one hour. Each rat of the heat-stressed group was exposed to two periods of identical heat stress in the morning of two consecutive days. During the heat stress, their body temperature was registered every 5 minutes via the subcutaneous temperature transponder. The animals were heated in the warming cabinet for exactly 60 minutes to achieve a body temperature above 40°C (target heating rate: 0.06°C/min), followed by exactly 30 minutes keeping the body temperature between 40 and 42°C. After the second heat stress on day 2, each rat was eliminated using an overdose of isoflurane anaesthesia after a certain defined point in time (0 h (i.e. directly after heat stress), 2 h, 6 h, 24 h) and blood was drawn by cardiac puncture for preparation of serum. At the end, livers were dissected for preparation of tissue samples. All serum and liver tissue samples were stored in a freezer at -80°C and aliquoted to prevent repeated freeze-thaw cycles, which would affect measured enzyme activities due to altered protein conformation.

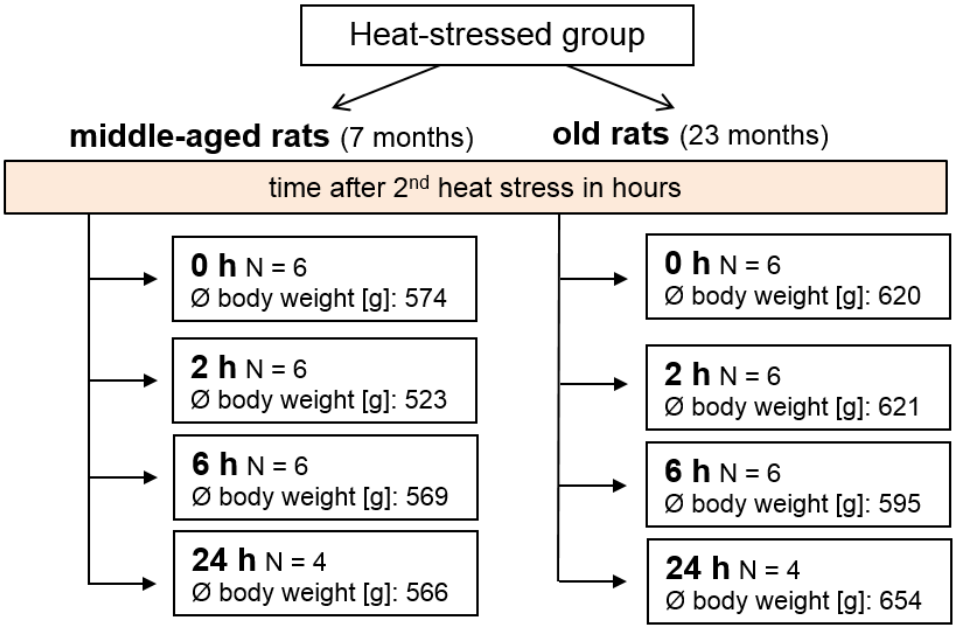


Figure 14: Overview of middle-aged and old rats after heat stress. 44 middle-aged (7 months) and old (23 months) male Wistar rats are included in the heat stress experiment, subdivided into four groups after heat stress according to time of liquidation (0 h, 2 h, 6 h, 24 h). This figure shows the average body weight and number of individuals per age group at four points in time.

2.2. Methods

2.2.1. Human serum for preliminary experiments

The serum of young people (Ø age: 27 years) and the serum of old patients (Ø age: 72 years) was used for preliminary experiments trying to proof the experimental setup. The young control group (N = 8, 4 men and 4 women) were healthy non-smokers whereas all in the group of older patients (N = 8, 4 men and 4 women) were suffering from liver diseases such as hepatocellular carcinoma, liver metastasis of colorectal carcinoma and cholangiocarcinoma.

2.2.2. Isolation of cytosol from liver tissue

The homogenization of liver tissue was performed using a disruptor, pre-cooled tubes with ceramic beads and always chilling the tubes on ice immediately to avoid loss of enzymatic activity. The liver tissue samples stored at -80°C were thawed on ice. After thawing a piece of liver tissue (about 50 mg) was chopped using a scalpel. The exact weight was noted and the tissue was further cut into little pieces with the scalpel and then transferred to pre-cooled tubes filled with

ceramic-beads (Precellys Ceramic Kit Ø 1.4 and 2.8 mm, Peqlab). 300 µl of RIPA stock solution was added per tube and spinned down shortly in the microcentrifuge at 4°C. Afterwards the tissue samples were homogenized in a disrupter at 2850 rpm for 1 minute and immediately chilled on ice for another 3 minutes. This disruption cycles with cooling time were repeated three times for each sample until the solution appeared homogeneous. The homogeneous lysates were transferred to new 1.5 mL Eppendorf tubes and then centrifuged for 30 minutes (800 g, 4°C) to separate cell nuclei and other cell particles. The supernatant containing cytosol was frozen immediately by using liquid nitrogen and stored in the freezer at -80°C.

2.2.3. *Lowry protein assay*

Every cell in an organism contains various proteins, including enzymes. The determination of total protein levels in a sample is necessary to compare measured protein amounts in liver tissue. Lowry protein assay was used in a modified method based on Lowry et al. 1951 and generally consists of two steps outlined in Figure 15. Step 1 is the Biuret reaction including the reaction of copper ions (Cu^{2+}) with peptide bonds of proteins in an alkaline solution by reducing Cu^{2+} into Cu^{+} forming a purple colored complex (Waterborg, 2009). The Folin & Ciocalteau's reaction in step 2 contains phosphomolybdate and phosphotungstate that reduce the copper-protein complex, enhance the color and turn it into blue (Lowry et al., 1951, Smith et al., 1985).

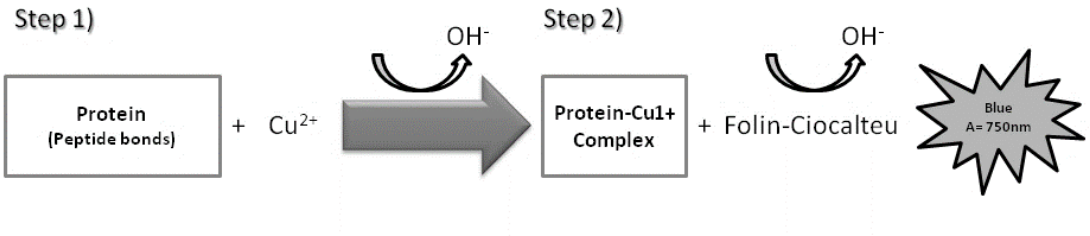


Figure 15: Lowry protein assay reactions step by step. Cu^{2+} ions react with peptide bonds of proteins forming a complex that is further processed with Folin & Ciocalteau's reagent creating a blue colored product [adopted from (Johnson, 2012)].

The Standard operating procedure of the lab served as guideline. Briefly, the protein content was measured photometrically as intensity of blue color.

Bovine Serum Albumin (BSA) was used in the following concentrations to generate the standard curve.

Table 3: BSA standard curve mass concentrations.

BSA standard [µg/µl]						
10	**8**	**6**	**4**	**2**	**1**	**0**

Solution A (20 µl Na-K-Tartrate stock solution, 20 µl $CuSO_4$ stock Solution, 1960 µl Na_2CO_3 stock solution) and B (500 µl Folin & Ciocalteu's phenol, 1000 µl ddH_2O) were prepared freshly each time before measurement. 2 µl of each BSA standard concentration as well as 2 µl of each sample were pipetted into a 96-well-plate in triplicates. 150 µl of Solution A was added per well and incubated shaking for 10 minutes at room temperature. Then 30 µl of Solution B is added per well and again incubated shaking at room temperature for 2 h. The adsorption was measured at 750 nm using a microplate reader. The measured optical density of the samples was calculated from the generated standard curve. As samples were diluted (1:3) and cytosol of liver tissue (1:6) the results of the calculation were multiplied by the dilution factor.

2.2.4. Lactate dehydrogenase activity assay

The lactate dehydrogenase enzyme (LDH) is a cytosolic enzyme present in various body fluids and tissues, mainly in the heart, liver, muscles and kidneys (Kopperschlager and Kirchberger, 1996). LDH is a tetramer composed of two types of subunits (heart: H or muscle: M) that can be randomly matched forming five isoenzymes (Bais and Philcox, 1994b). LDH catalyzes in anaerobic conditions the reversible reaction of lactate to pyruvate by using the reduction of NAD^+ into NADH as electron transfer.

$$\text{L-lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

Figure 16: Principle of LDH activity assay. [adopted from (Bais and Philcox, 1994a)].

The formation of NADH is proportional to the LDH activity and can be photometrical measured. As LDH is located in the cytosol of cells, high LDH serum levels indicate leakage through the cell membrane after certain cell

damage. An increased LDH activity is not specific for liver injury and other markers for liver damage should be measured additionally to identify other sources for increased LDH in serum. High levels of increased serum LDH could be measured in megaloblastic anemia and disseminated carcinoma (Kopperschlager and Kirchberger, 1996). The increase of NADH is directly proportional to LDH activity and can be detected photometrically at 340 nm. To quantify LDH activity in serum and liver tissue a commercial test kit was used following manufacturers' instructions (Fluitest LDH-L Kit, Analyticon Biotechnologies AG). According to the user manual five parts of reagent 1 (2-Aminomethylpropanol pH 9.4 and Lithium lactate) and one part of reagent 2 (Imidazole and NAD^+) were mixed freshly each time before usage. First 5 µl of sample was pipetted onto a 96-well-plate in duplicates followed by 180 µl of working solution (5 R1 + 1 R2) per well. After an incubation time of 90 seconds the absorbance was measured at a wavelength of 340 nm once every minute over 10 minutes using a microplate reader. The liver tissue samples were diluted (1:100) preventing too high concentration that interferes with photometric measurements and the results were multiplied by the dilution factor. The LDH activity was calculated by using the following formula:

$$A = \frac{\frac{\Delta E}{min} \cdot dilution\ factor \cdot V_{total}}{\Delta\varepsilon \cdot d \cdot V_{sample}} \qquad A = \frac{\Delta E}{min} \cdot 0.006426 \frac{mol}{l}$$

$$A = \Delta E \cdot 6426 \cdot \frac{\mu mol}{min \cdot l}$$

$$A = \frac{\frac{\Delta E}{min} \cdot 185 \mu l}{6300 \frac{l}{mol \cdot cm} \cdot 0.914 cm \cdot 5 \mu l} \qquad A = \Delta E \cdot 6426 \cdot \frac{U}{l}$$

Figure 17: Formula for calculation of LDH activity. A is activity, Δε is molecular extinction coefficient, V_{total} is total volume per well, V_{sample} is sample per well and *d* is liquid layer thickness.

2.2.5. Alanine-aminotransferase activity assay

The alanine-aminotransferase enzyme (ALT) (formerly named glutamate-pyruvate-transaminase, GPT) is known as liver specific enzyme mainly located in hepatocytes but also minor amounts in kidneys and heart tissue (Dufour et al., 2000). As ALT activity is about 3000 times higher in cytoplasm of hepatocytes compared to serum, increased serum levels of ALT are diagnostically used to

detect liver parenchymal damage (Kim et al., 2008). ALT belongs to aminotransaminases and transfers the amino group of L-alanine to 2-oxoglutarate revealing L-glutamate and pyruvate as described in Figure 18 (Senior, 2012). Pyruvate formed in reaction (I) is immediately further processed in the indicator reaction (II) and ALT activity is measured via the rate of oxidation of NADH by LDH (Bergmeyer et al., 1986). The decrease of NADH is directly proportional to ALT activity and is measured photometrically at a wavelength of 340 nm.

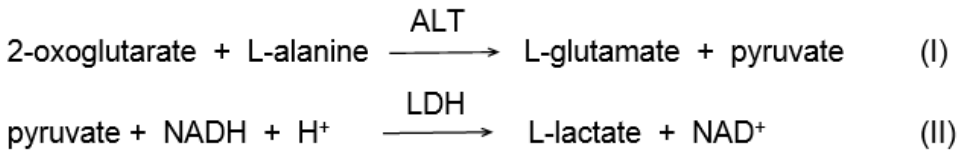


Figure 18: Principle of ALT activity assay. Two coupled reactions lead to decreased levels of NADH proportional to ALT activity in the sample that are measured photometrically at 340 nm.

For quantification of ALT activity in serum a commercial test kit was used following manufacturers' instructions (Fluitest GPT/ALT Kit, Analyticon Biotechnologies AG). According to the user manual five parts of reagent 1 (Tris buffer pH 7.8, L-alanine and LDH) and one part of reagent 2 ($NADH_2$ and 2-oxoglutarate) were mixed each time freshly before usage. First 20 µl of sample was added onto a 96-well-plate followed by 165 µl of working solution (5 R1 + 1 R2) per well. After an incubation time of 60 seconds the absorbance was measured at a wavelength of 340 nm once every minute over 10 minutes using a microplate reader. The ALT activity was calculated by using the following formula:

$$A = \frac{\frac{\Delta E}{min} \cdot dilution\ factor \cdot V_{total}}{\Delta\varepsilon \cdot d \cdot V_{sample}} \qquad A = \frac{\Delta E}{min} \cdot 0{,}001606\ \frac{mol}{l}$$

$$A = \Delta E \cdot 1606 \cdot \frac{\mu mol}{min \cdot l}$$

$$A = \frac{\frac{\Delta E}{min} \cdot 185\mu l}{6300\frac{l}{mol \cdot cm} \cdot 0.914cm \cdot 20\mu l} \qquad A = \Delta E \cdot 1606 \cdot \frac{U}{l}$$

Figure 19: Formula for calculation of ALT activity. A is activity, $\Delta\varepsilon$ is molecular extinction coefficient, V_{total} is total volume per well, V_{sample} is sample per well and *d* is liquid layer thickness.

2.2.6. Malondialdehyde assay

To determine the degree of oxidative stress, MDA is measured as marker for increased lipid peroxidation. MDA is a decomposition product mainly of arachidonic acid that is generated via lipid peroxidation processes and can be mutagenic by reacting with DNA forming MDA-DNA adducts (Lykkesfeldt, 2007, Marnett, 1999). The MDA Assay used in this setting is based on the thiobarbituric acid (TBA)-Method outlined in Figure 20 according to Ohkawa et al. with modifications (Ohkawa et al., 1979). Two molecules of TBA react under heating and acidic conditions with one molecule of MDA to molecule-bound MDA forming a fluorescent red product that could be photometrically measured (Spiteller, 2001).

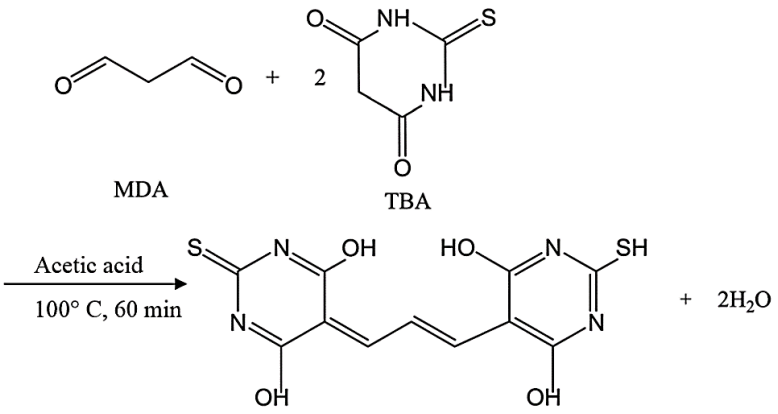


Figure 20: Principle of TBA-method for determination of MDA level in serum and liver tissue. One molecule of MDA reacts with two molecules of TBA forming a fluorescent red product [modified from (Yahyavi et al., 2016)].

The TBA-Method for determination of MDA level in serum was performed in triplicates and distilled water was used as blank value. The breakdown product of Tetraehtoxypropane (TEP) via acid hydrolysis as form of pure MDA was used to generate the standard curve (Seljeskog et al., 2006). First 45 µl of serum sample and 45 µl of TEP solution with highest concentration of 25 µM were transferred to separate Eppendorf tubes. Then 5 parts of 0.6% TBA (0.12 g TBA in 20 ml ddH_2O) and 4 parts of 25% acetic acid (10 ml acetic acid in 30 ml ddH_2O) were mixed. 135 µl of TBA-acetic acid mixture (5:4) was added to each sample and standard. The samples and standard were heated at 100°C shaking at 250 rpm for 1 h. After heating all tubes were immediately kept on ice to stop the

reaction. After that, 125 µl of n-Butanol mixed with pyridine in a 15:1 ratio was added per sample and 250 µl of this solution per standard. From now on the tubes needed protection from daylight and were covered with aluminum foil. After 10 minutes of incubation on ice the tubes were vortexed thoroughly for 15 seconds per tube and then centrifuged for 5 minutes (7000 g, 4°C). Now the upper phase which appears red and contains organic material is loaded with a quantity of 50 µl per sample and 100 µl per standard on the plate. The TEP standard (25 µM) is diluted with n-Butanol via a two-fold dilution series as described in Table 4 to generate the standard curve.

Table 4: Standard curve for TBA-Method generated as two-fold dilution series.

Standard TEP solution	Applied chemicals
25 µM	100 µl of standard
12.5 µM	50 µl n-Butanol + 50 µl of 25 µM standard
6.25 µM	50 µl n-Butanol + 50 µl of 12.5 µM standard
3.13 µM	Continue as above
1.56 µM	Continue as above
0.78 µM	Continue as above
0.39 µM	Continue as above
0 µM	50 µl n-Butanol

The fluorescence was measured at excitation wavelength of 544 nm and emission wavelength of 590 nm using a microplate reader. For the analysis of results the software GraphPad Prism 6.0 was used to plot the fluorescence intensities of each standard concentration against the corresponding concentrations and perform linear regression to determine the sample concentrations by using the resulting linear equation. For each MDA assay a standard curve was created to validate that sample concentrations of MDA are in the linear range of the standard curve (Zhang et al., 2003).

For determination of MDA in liver tissue samples the isolation of cytosol from liver tissue was necessary as described above. Instead of distilled water RIPA stock solution was used as blank value. First 30 µl of supernatant and 30 µl of TEP solution (25 µM) were transferred to separate Eppendorf tubes. Then 150 µl of TBA-acetic acid mixture (5:4) was added to each sample and standard. The following steps were identical to determination of MDA in serum.

2.2.7. 4-Hydroxynonenal ELISA

Derived as relatively stable end-product of lipid peroxidation from PUFA in membranes, 4-HNE is a well-studied marker for oxidative stress (Uchida et al., 1999). For quantification of 4-HNE concentrations in this setting, the 4-HNE Sandwich enzyme-linked immunosorbent assay (ELISA) was carried out using a commercial 4-HNE ELISA kit (Cusabio Biotech Co. Ltd, China). The assay was performed following the instructions in the manufacturers' manual. The principle of 4-HNE ELISA is outlined in Figure 21. An antibody specific for 4-HNE has been pre-coated onto a microplate. Any 4-HNE present in the sample or standard is bound to the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for detection of 4-HNE is added to the wells. After washing, avidin-conjugated Horseradish Peroxidase (HRP) is added to the wells. Following another wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of 4-HNE bound in the initial step. The color development is stopped using stop solution and the intensity of the color is measured.

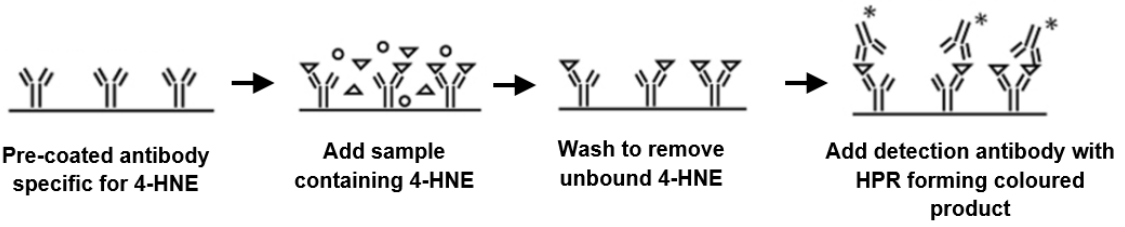


Figure 21: Principle of 4-HNE Sandwich ELISA method. Pre-coated antibodies specific for 4-HNE react with 4-HNE in sample and bound 4-HNE is detected via detection antibody conjugated with HPR that forms a coloured product with substrate solution [modified from (Technology, 2016)].

First Biotin-Antibody, HRP-avidin and wash buffer were prepared freshly before each usage. The standard curve was created with stock solution (40 ng/ml) and sample diluent provided in the kit. Therefore, 250 µl of sample diluent were pipetted into each tube S0-S6 and a two-fold dilution series was produced with stock solution as described in Table 5. The sample diluent serves as a blank value (S0).

Table 5: Standard curve for 4-HNE ELISA generated as two-fold dilution series.

Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	40	20	10	5	2.5	1.25	0.625	0

First 100 µl standard and cytosol of liver tissue were added to each well that is pre-coated with HNE specific antibody. After covering with an adhesive strip incubation for 2 h at 37°C followed. Afterwards the liquid of each well was removed without washing. 100 µl of the Biotin-antibody is added to each well, covered again with a new adhesive strip and incubated for 1 h at 37°C. Then each well was aspirated and washed three times in total applying 200 µl of the enclosed wash buffer per wash leaving buffer in wells for 2 minutes of soaking time. After the last wash, the liquid was removed completely by inverting the plate and blotting it against clean paper towel. For the next step 100 µl of HRP-avidin is added to each well, again covered with an adhesive strip and incubated for 1 h at 37°C. The aspiration-washing process was repeated for five times in total as described above. Then 90 µl of TMB substrate is added per well and from now on the plate was protected from light by a wrapping of aluminum foil followed by incubation for 25 minutes at 37°C. Afterwards the reaction is stopped by pipetting 50 µl of stop solution into each well and the color appeared blue. To determine the optical density, the absorbance is measured at a wavelength of 450 nm using a microplate reader. The readings at 540 nm were subtracted from the readings at 450 nm to correct optical imperfections in the plate. GraphPad Prism version 6.0 was used to create a dose-response curve and concentration of 4-HNE in samples was calculated from the generated standard curve. The tissue samples were diluted (1:2000) preventing too high concentration that interferes with photometric measurements and the results of the calculation were multiplied by the dilution factor.

2.2.8. H&E-staining of liver sections

The Hematoxylin & Eosin-staining (H&E-staining) of liver sections was performed to visualize possible damage in liver tissue of untreated and heat-stressed rats comparing different ages and points in time after heat stress (0 h, 2 h, 6 h, 24 h). Basic hematoxylin stains the basophile DNA blue/violet and the acidophil

cytoplasm is stained red by eosin (Fischer et al., 2008). After cutting paraffin slices from liver tissue, the slides were stained according to the protocol of the Department of Pathology from University of Tuebingen in an automatic staining machine (Feldman and Wolfe, 2014). Histological images of liver sections were made using Microscope Primo Vert (Carl Zeiss AG). Focus was on examination of hepatic lobules with central vein for various signs of hepatocellular damage such as fatty degeneration, hepatocellular vacuolization, ballooning degeneration and apoptosis (Grizzi et al., 2013). For quantitative analysis, three liver sections were used per age group and at each point in time after heat stress.

2.2.9. Statistical analysis

All Data were expressed as mean ± standard deviation (SD). All calculations and graphics were created using the statistic software GraphPad Prism 6.0 (San Diego, CA, USA). Means of age groups within heat-stressed and control animals were compared by one-way analysis of variance (ANOVA). Differences between young and old human serum were calculated by Mann-Whitney U t-test. Statistical outliers were determined with ROUT outlier test and excluded from calculations. Differences were considered statistically significant at values of $p<0.05$.

3. Results

By measuring markers for cell damage and oxidative stress, this thesis analyzes age-related differences in stress response after *in vivo* heat stress. Young (7 weeks), middle-aged (6/7 months) and old (23 months) rats are included as control group. Middle-aged (7 months) and old (23 months) rats were exposed to two-phase heat stress and liquidated at four points in time after the second heat stress (0 h, 2 h, 6 h, 24 h). For preliminary experiments and to proof measurements human serum of young (Ø 27 years) and old (Ø 72 years) individuals is included. General cell damage is measured using LDH activity and ALT activities display specifically possible hepatic injury. Further H&E-staining of liver sections are examined to visualize hepatocellular damage. Lipid peroxidation markers MDA and 4-HNE are determined as indicators for increased oxidative stress.

3.1. Human serum for preliminary experiments

The serum of young individuals (Ø 27 years) obtained from healthy non-smokers and the serum of old patients (Ø 72 years) with liver diseases were compared in their amount of LDH and ALT activity as well as MDA (Figure 22). LDH activity increases significantly in old patients indicating higher general cell damage. The LDH activity is nearly two times higher in older patients compared to the young group. ALT as an indicator of hepatic damage is measured in both groups. The mean value of old individuals is slightly higher compared to young but there is high variation in the old group indicating fluctuations that could be caused by different liver diseases. The serum of the young group is directly processed after blood withdrawal, whereas the serum of older patients is taken during liver surgery and processed after a transport period. Due to the transport period of the serum of old patients the enzyme activities could be affected and measured activities might not display the actual *in vivo* situation. Results of MDA assay indicates slightly higher MDA levels for old individuals compared to their younger counterparts. Prior to the experiments a significant increase of markers for cellular damage (LDH), especially liver damage (ALT) and increased levels of oxidative stress marker MDA in older patients were suggested.

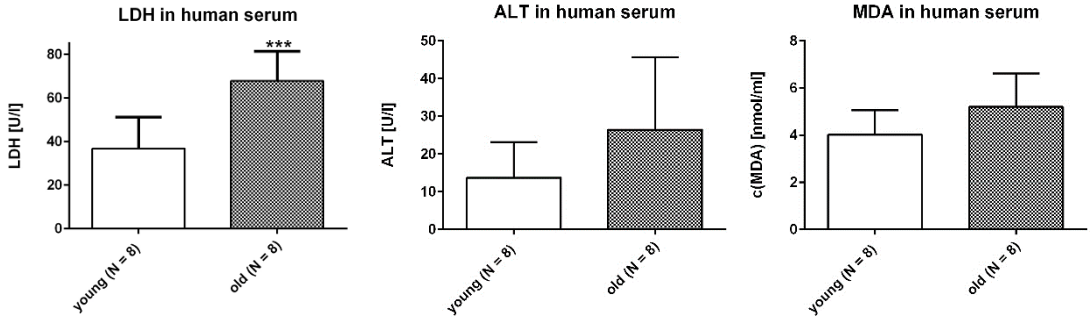


Figure 22: LDH, ALT and c(MDA) in human serum of young and old individuals. Serum levels of LDH significantly increase in old patients whereas ALT activity and MDA levels show mild elevated amounts in old. ***p<0.01 (young versus old group).

Table 6: Serum levels of LDH, ALT and MDA in serum of young and old patients. Values are presented as mean ± SD. ns (not significant).

	Young patients (N = 8)	**Old patients (N = 8)**
Protein content [mg/ml]	84.89 ± 13.05	71.11 ± 7.16 (ns)
LDH activity [U/L]	36.82 ± 14.38	67.74 ± 13.54 (***p<0.01)
ALT activity [U/L]	13.72 ± 9.50	26.47 ± 19.17 (ns)
MDA Assay [nmol/ml]	4.02 ± 1.04	5.20 ± 1.41 (ns)

3.2. H&E-staining of liver sections

Images for the histological evaluation of Hematoxylin & Eosin-stained liver sections of untreated rats are shown in Figure 23. The liver morphology appears homogenous in young rats (A+B) with normal configurated nuclei. In liver sections of old rats (E+F) forms of severe liver injury including hepatocellular vacuolization, lipid-filled hepatocytes and sinusoidal congestion are observed that indicate increased liver damage in aged individuals. In comparison to old rats show middle-aged rats minor liver injury with signs of apoptosis and few fatty infiltrates (C+D). The liver sections of different aged untreated rats show a slight increase of liver damage during aging.

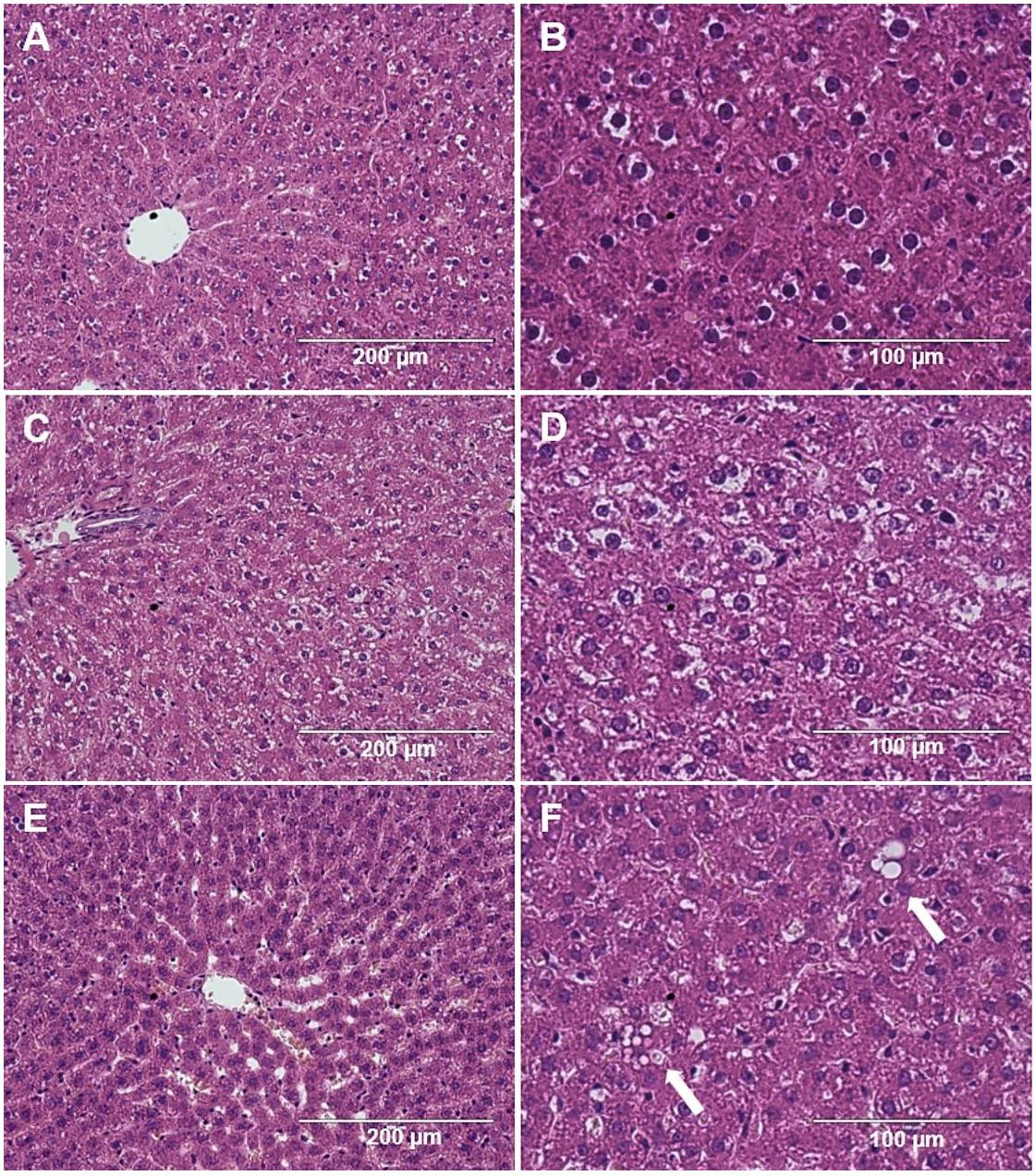
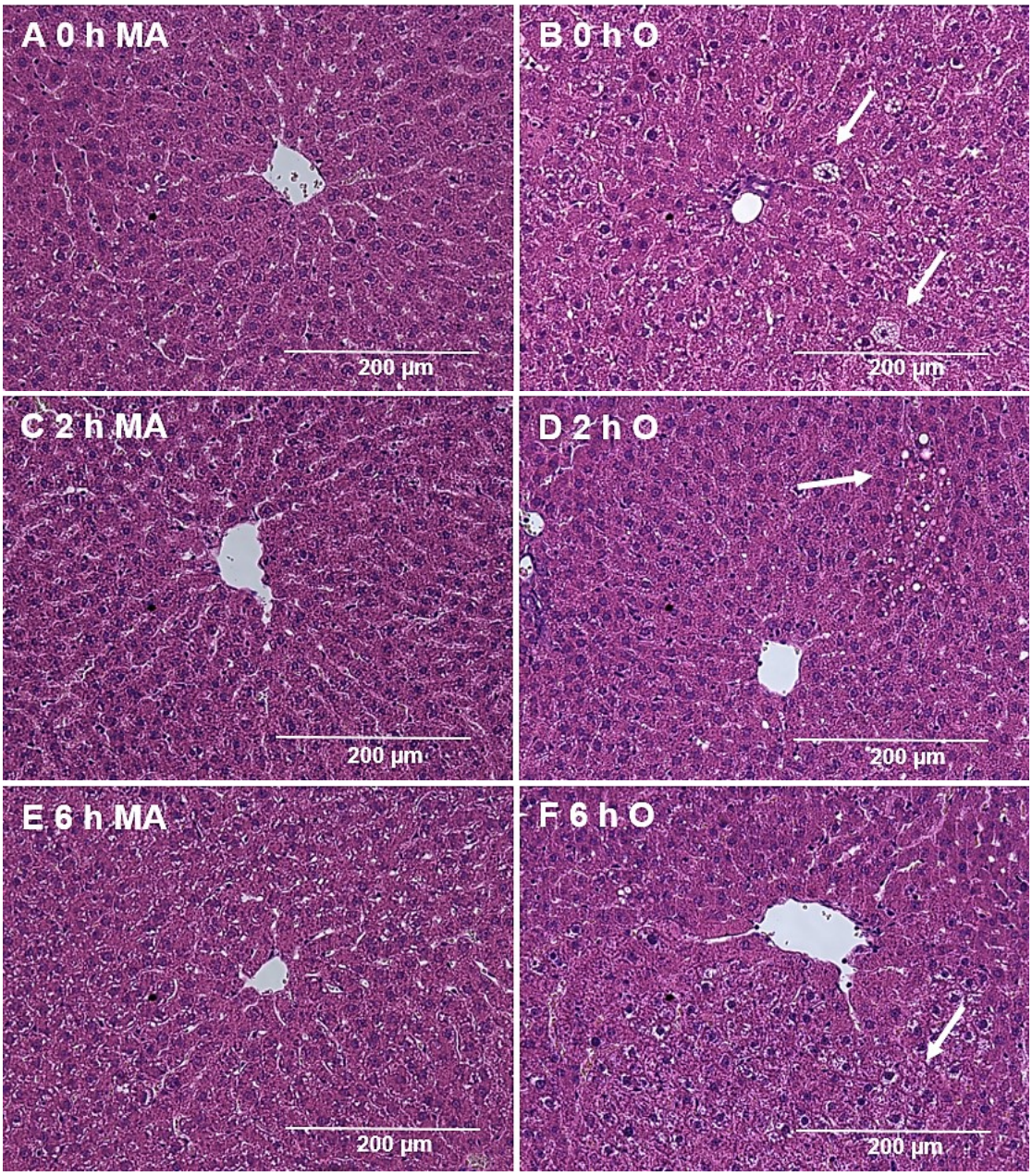


Figure 23: H&E-stained liver sections of young, middle-aged and old untreated rats. Liver morphology was normal in young rats (A+B) and more severe liver injury was found in old rats (E+F, arrows) including fatty degeneration and hepatocellular vacuolization. Middle-aged rats (C+D) show only slight liver damage. Scale bars A, C, E: 200 µm and B, D, F: 100 µm.

Images for the histological evaluation of H&E-stained liver sections of middle-aged and old rats after heat stress are shown in Figure 24.The middle-aged rats (MA) show few signs of liver damage at all points in time (A: 0 h, C: 2 h, E: 6 h, G: 24 h). More severe liver damage appears in old rats (O) compared to MA at all points in time including fatty infiltration, sinusoidal congestion, hepatocellular

vacuolization and ballooning degeneration (B: 0 h, D: 2 h, F: 6 h, H: 24 h, arrows). By comparing old rats at different points in time after heat stress no significant increased damage is found. There is a higher grade of liver damage with increased rate of necrosis, fatty infiltration and ballooning degeneration in old rats after heat stress compared to untreated control group.



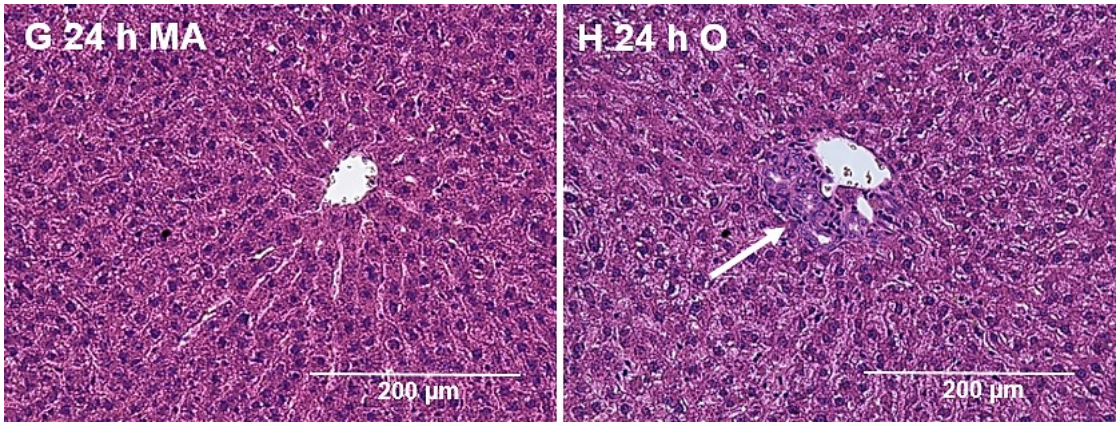


Figure 24: H&E-stained liver sections of middle-aged and old rats at different points in time (0 h, 2 h, 6 h, 24 h) after heat stress. Middle-aged rats (MA) show few signs of liver damage at all points in time (A: 0 h, C: 2 h, E: 6 h, G: 24 h). More severe liver damage appears in old rats (O) compared to MA at all points in time including fatty infiltration, sinusoidal congestion, hepatocellular vacuolization and ballooning degeneration (B: 0 h, D: 2 h, F: 6 h, H: 24 h, arrows). Scale bars: 200 µm.

3.3. Serum and liver tissue of untreated rats

3.3.1. Protein content

The results of the Lowry protein assay in serum and liver tissue of young, middle-aged and old untreated rats are shown in Figure 25. The protein content in serum and liver tissue comparing the different age groups is nearly equal with slightly increased mean liver tissue protein levels. The protein mass concentrations are used to normalize measured enzyme activities of LDH and ALT as well as levels of MDA and 4-HNE in liver tissue.

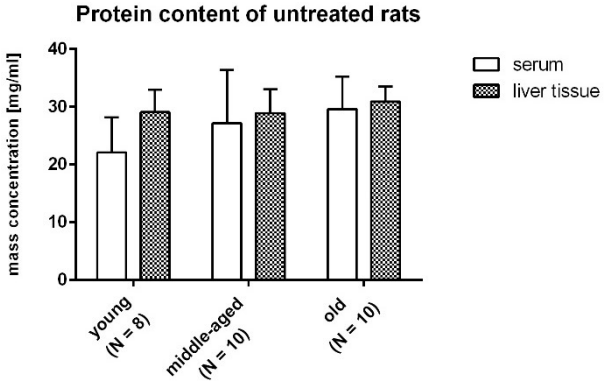


Figure 25: Protein mass concentration [mg/ml] in young, middle-aged and old untreated rats. The protein content of serum and liver tissue is nearly equal in the different age groups. There is no significant difference calculated between the age groups using statistical analysis. Values are represented as mean ± SD.

3.3.2. Markers for cell damage

LDH activity assay

The results of LDH activity assay in serum and liver tissue of young, middle-aged and old untreated rats are shown in Figure 26. The graph shows equal activities of LDH in serum by comparing different aged rats with higher error bars in the middle-aged group. Regarding LDH activity in liver tissue in contrast to serum, the present results show a decline from young to middle-aged and significantly lower levels in old rats. These differences in LDH activity may be explained by underlying influencing factors including grade of exercise and diseases that might affect the measured levels. High LDH activities have been documented in pathological conditions such as different types of cancer, hemolysis and infections (Erez et al., 2014). The results of the present study indicate that LDH activity as marker for general cellular damage is not specific for measuring the level of oxidative stress injury in the liver. In addition to reduced specificity of LDH, display the high error bars difficulties regarding preservation of enzyme activity over time and LDH might get inactivated during storage and not displaying the actual situation.

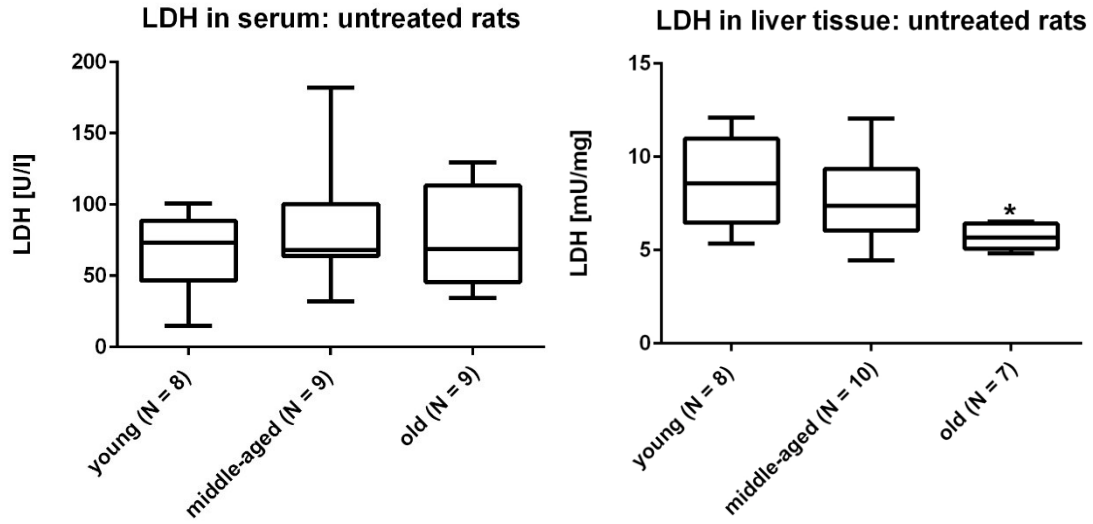


Figure 26: LDH activities in serum [U/l] and liver tissue [mU/mg] of young, middle-aged and old untreated rats. LDH activity is almost equal in serum of all age groups. The LDH in liver tissue shows a decline from young to middle-aged with lowest amount in old rats. *p<0,05 (young versus old rats per one-way ANOVA).

ALT activity assay

The results of ALT activity assay in serum of young, middle-aged and old untreated rats are shown in Figure 27. In comparison to serum LDH activities which stay almost equal in aging, the ALT activities in serum show a clear age-dependent increase. The old rats have the highest level of ALT activity followed by the middle-aged group. The lowest amount of ALT was detected in the serum of young rats. The measured ALT activities differ significantly between young, middle-aged and old rats that is consistent with the histological findings that visualize higher liver damage in old rats compared to young and middle-aged.

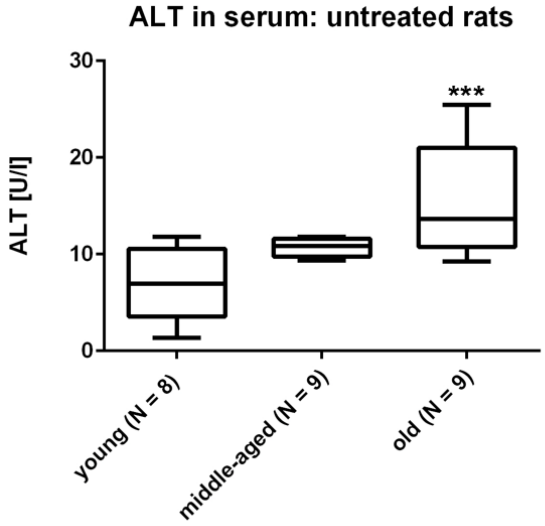


Figure 27: ALT activities [U/l] in serum of young, middle-aged and old untreated rats. ALT activities in old rats increase significantly compared to young and middle-aged rats. Lowest amount of ALT was detected in young rats. ***p<0.01 (young versus old rats per one-way ANOVA).

3.3.3. Lipid peroxidation marker

MDA assay

The results of MDA assay in serum and liver tissue of young, middle-aged and old untreated rats are shown in Figure 28. The serum concentration of MDA stays nearly equal by comparing the different age groups. In the serum of old rats slightly increased MDA concentrations are observed. The values in the middle-aged group show high fluctuations. In the liver tissue the MDA concentration is almost similar in every age group and high fluctuations appear in all ages.

No clear trend for increased MDA content could be evaluated in serum and liver tissue of different aged untreated rats.

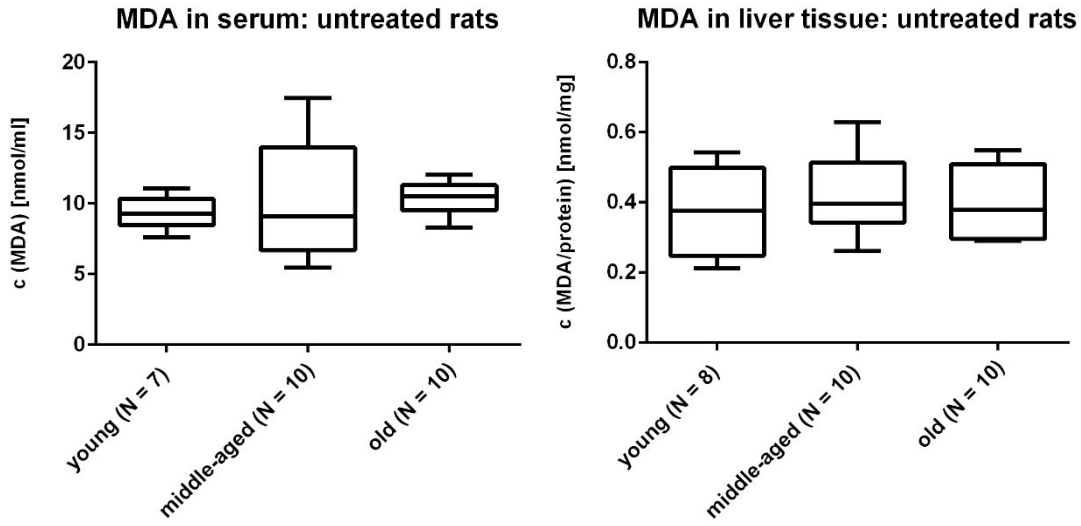


Figure 28: c(MDA) in serum [nmol/ml] and liver tissue [nmol/mg] of young, middle-aged and old untreated rats. There could be no significant difference calculated between the age groups using statistical analysis.

4-HNE ELISA

The results of 4-HNE ELISA measured in liver tissue of young, middle-aged and old untreated rats are shown in Figure 29. Before measurements were carried out different dilutions were tested. The values that fitted best in the standard curve were seen by diluting the sample 1:2000. The lowest level of 4-HNE is measured in old rats followed by young and with highest amount in the middle-aged group. In contrast to the MDA concentrations in liver tissue, the 4-HNE amounts rise from young to middle-aged and fall to the lowest amount in old rats. As 4-HNE increases in relation to oxidative damage the results do not verify earlier findings. The serum levels of 4-HNE could not be measured due to high interference of serum proteins that could not be removed using common methods.

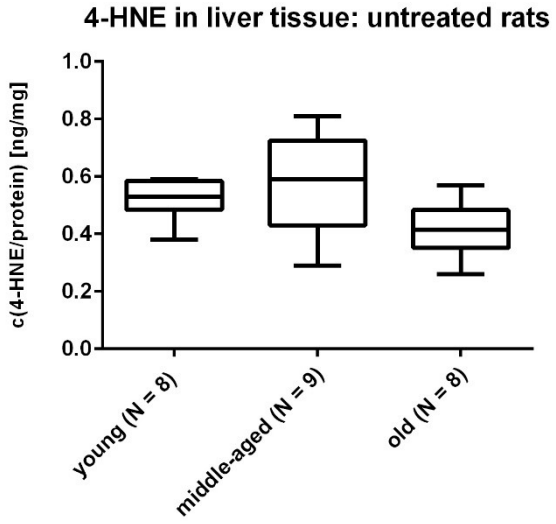


Figure 29: c(4-HNE) [ng/mg] in liver tissue of young, middle-aged and old untreated rats. Lowest level of 4-HNE appears in old rats and there is an increase from young to middle-aged rats. There could be no significant difference calculated between the age groups using statistical analysis.

3.4. Serum and liver tissue after the heat stress

3.4.1. Protein content

The results of Lowry protein assay of middle-aged and old rats after heat stress in serum and liver tissue are shown in Figure 30. The protein content of old rats before and after the heat stress is slightly higher compared to the middle-age group with higher error bars. By looking at the different points in time after heat stress (0 h, 2 h, 6 h, 24 h), the protein content in serum and liver tissue of old and middle-age rats is almost similar. The protein mass concentrations are used to normalize enzyme activities of LDH and ALT as well as levels of MDA and 4-HNE after heat stress at different points in time in liver tissue samples.

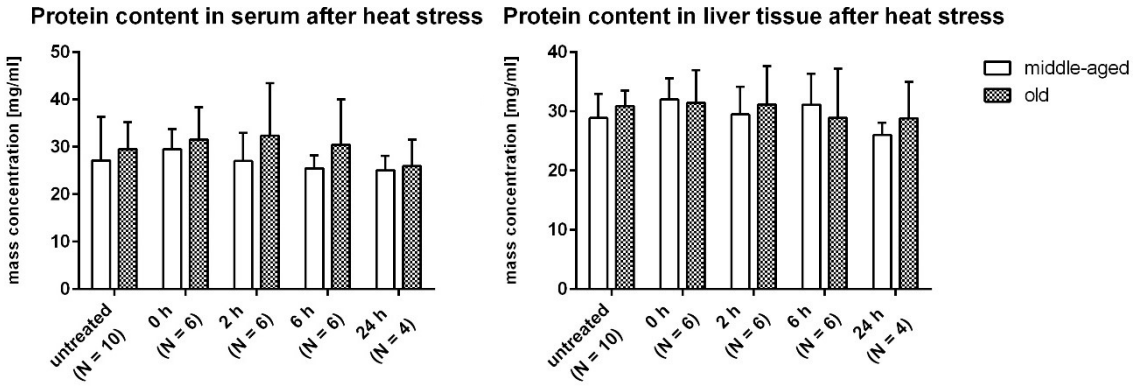


Figure 30: Protein mass concentration [mg/ml] in serum and liver tissue of untreated and heat-stressed middle-aged and old rats. Almost equal protein amounts in serum and liver tissue of old and middle-aged rats are shown at different points in time after heat stress (0 h, 2 h, 6 h, 24 h). There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

3.4.2. Markers for cell damage

LDH assay

The results of the LDH activity assay in serum of middle-aged and old rats before and at four points in time after heat stress are shown in Figure 31. The LDH activities in serum of old rats slightly increase directly after heat stress (0 h) and decline until the lowest level 6 h after heat stress. 24 h after heat stress is the level of LDH activities in old rats nearly the same as in untreated old rats. In the middle-aged group the LDH activities first drop directly after the heat stress (0 h) and then rise again in 2 h and 6 h until reaching the peak at 24 h after heat stress. The mean values for older rats are in all points in time except for 24 h-group higher compared to middle-aged rats. The high error bars indicate a great variance of values, whereas there is no significant difference between the age groups detected. In comparison to the untreated control group, the heat-stressed rats reveal no further detectable cell damage measured as higher LDH activity in the serum. The results of the LDH activity assay in liver tissue of middle-aged and old rats before and at four points in time after heat stress are shown in Figure 32. In the liver tissue, the heat-stressed middle-aged rats present 2 h after heat stress a peak increase of LDH activities followed by a mild decrease until 24 h after heat stress. In the old heat-stressed rats the LDH activities rise directly after

heat stress (0 h) in comparison to untreated animals and remain at this higher level for the following points in time. The total increase of LDH compared to basal levels of untreated animals is higher in both age groups after the heat stress. However, these findings are only tendencies and no significant differences could be calculated using statistical analysis.

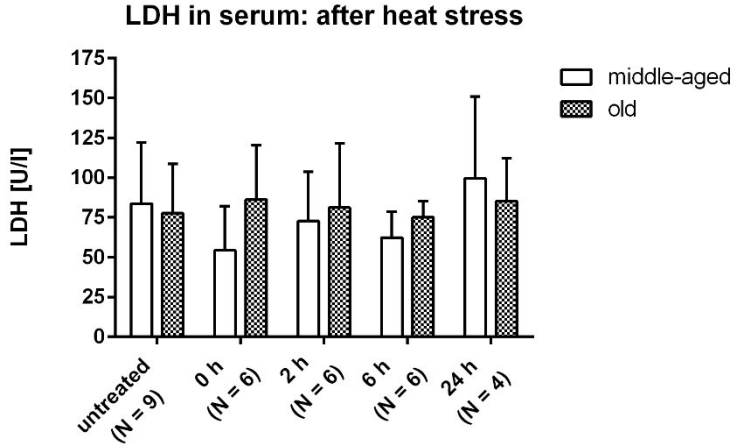


Figure 31: LDH activities [U/l] in serum of untreated and heat-stressed middle-aged and old rats. LDH activities are higher in old rats compared to middle-aged rats in untreated control group as well as after heat stress except for middle-aged at 24 h. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

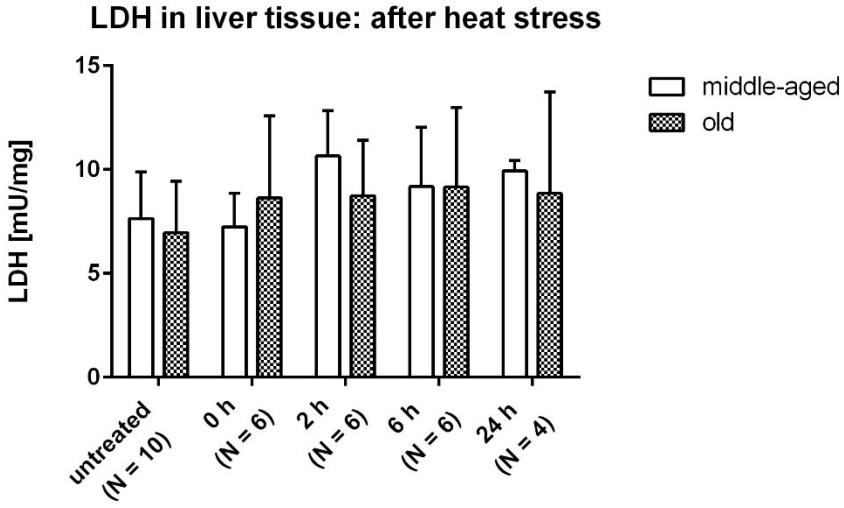


Figure 32: LDH activities [mU/mg] in liver tissue of untreated and heat-stressed middle-aged and old rats. Increase of LDH activities compared to basal levels of untreated animals is higher in both age groups after the heat stress with a peak 2 h after heat stress in middle-aged rats. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

ALT activity assay

The results of the ALT activity assay measured in serum of middle-aged and old heat-stressed rats are shown in Figure 33. In accordance with the serum ALT activities of untreated rats, the ALT activities after application of heat stress are at all points in time higher in old individuals compared to the middle-aged. In the old heat-stressed rats ALT activities stay nearly the same until a drop between 6 h and 24 h after heat stress. The middle-aged group seems to be less affected of the heat stress with nearly equal amounts over time in the heat stress experiment.

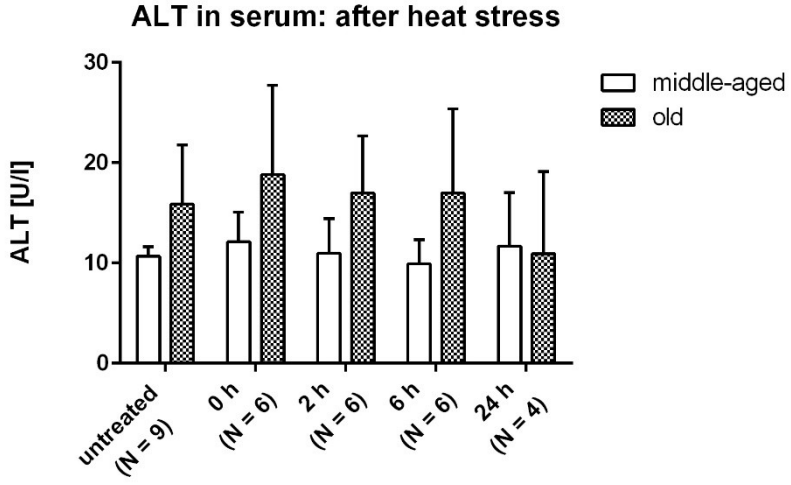


Figure 33: ALT activities [U/l] in the serum of untreated and heat-stressed middle-aged and old rats. ALT activities remain higher in old animals at all points in time compared to their middle-aged counterparts. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

3.4.3. Lipid peroxidation marker

MDA assay

The results of the MDA assay in serum of heat-stressed middle-aged and old rats are shown in Figure 34. In accordance with the serum LDH activities after heat stress, the MDA concentration in old heat-stressed rats is at all points in time higher compared to the middle-aged group and nearly stays at the same level for old rats. Middle-aged rats after heat stress show a decrease directly after heat stress (0 h) with lowest level of MDA 2 h post heating that increases again 6 h and 24 h after heat stress. There are high standard deviations in the old group and no significant difference could be calculated using statistical analysis.

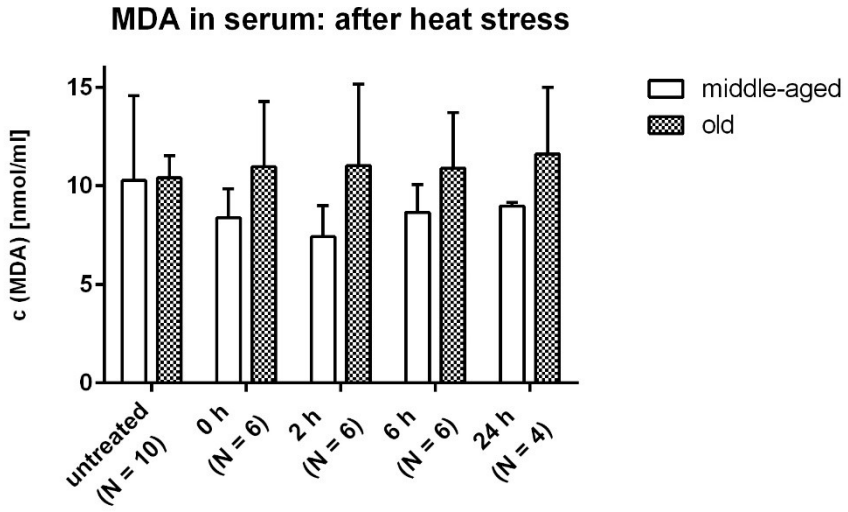


Figure 34: c(MDA) [nmol/ml] in serum of untreated and heat-stressed middle-aged and old rats. Old rats have higher levels of MDA in untreated and all heat-stressed groups. Middle-aged rats show a slight decline until 2 h after heat stress and MDA level rises again 6 h and 24 h after heat stress. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

The results of the MDA assay in liver tissue of heat-stressed middle-aged and old rats are shown in Figure 35. The MDA concentration in liver tissue shows a mild decline in both age groups from untreated rats to groups directly after heat stress (0 h). During heat stress the level of MDA in liver tissue in old rats rises up to the highest amount at 6 h after heating and falls again 24 h after heat stress reaching the level of untreated rats. The MDA concentration in middle-aged rats shows no tendency to increase after heat stress and stays at similar levels in untreated and heat-stressed groups at all points in time after heat stress.

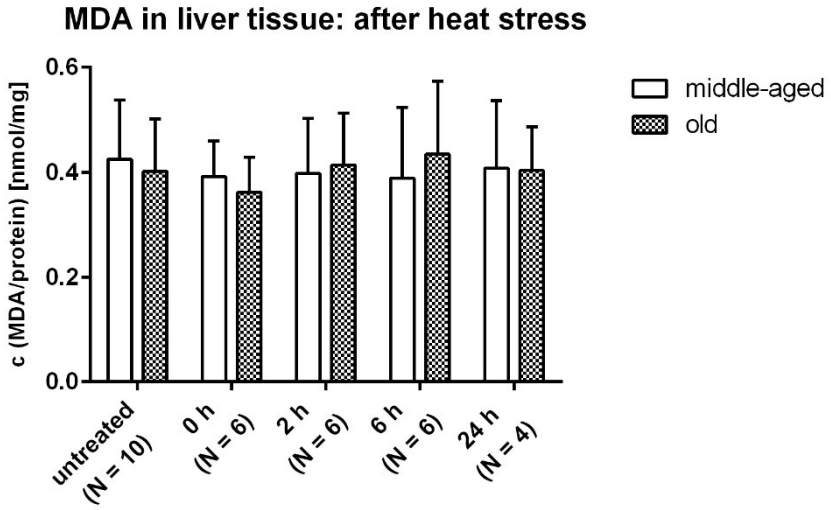


Figure 35: c(MDA) [nmol/mg] in liver tissue of untreated and heat-stressed middle-aged and old rats. The level of MDA in liver tissue before and at different points in time after heat stress (0 h, 2 h, 6 h, 24 h) stays nearly the same in both age groups with a peak 6 h after heat stress in old rats. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

4-HNE ELISA

The results of the 4-HNE ELISA in liver tissue for untreated and heat-stressed middle-aged and old rats are shown in Figure 36. The levels of 4-HNE in both age groups are slightly higher after heat stress compared to the untreated group. Middle-aged rats have higher levels of 4-HNE in every group and there is a constant increase of 4-HNE up to 6 h after heat stress that remains at this higher level 24 h after heat stress. Also 4-HNE levels in old rats show this trend to increase directly after heat stress with a peak after 2 h and a following decrease until nearly reaching the base level of untreated animals 24 h after heat stress. Although a tendency becomes visible no significant difference could be calculated using statistical analysis due to high standard deviation. The serum levels of 4-HNE after heat stress could not be measured due to high interference of serum proteins that could not be removed using common methods.

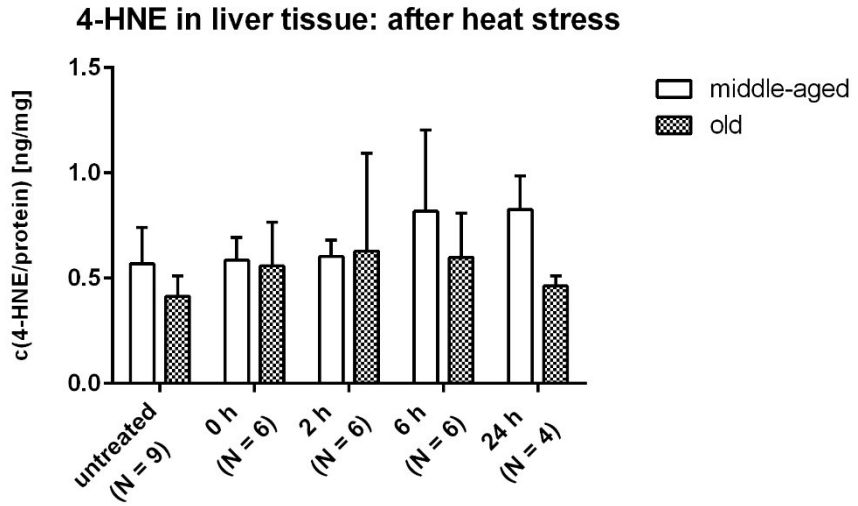


Figure 36: c(4-HNE) [ng/mg] in liver tissue of untreated and heat-stressed middle-aged and old rats. Both age groups indicate increased levels of 4-HNE after heat stress with a peak at 6h for middle-aged and 2 h for old rats. Middle-aged rats remain at higher 4-HNE levels 24 h post-heating compared to old rats that nearly reach level of untreated group. There is no significant difference calculated between the age groups and points in time using statistical analysis. Values are represented as mean ± SD.

4. Discussion

Every organism has a stress level tolerance and former research indicates a higher vulnerability of older individuals to attacks of reactive oxygen species. Different theories and methods have been designed to measure *in vivo* oxidative stress levels and their impact on cellular aging. The aim was to prevent or slow down the aging process leading to a longer and healthier life. Nevertheless, there is not yet a verifiable method established to inevitably prove that accumulated oxidative damage is responsible for the aging process. Numerous former studies have shown that macromolecules including proteins, membrane lipids and DNA are susceptible to oxidative damage after having been exposed to higher levels of oxidative stress with impact on the metabolic pathways of the organism resulting in cellular aging (Stadtman, 2001, Holmes et al., 1992, Catalá, 2009). This thesis was designed to show age-related differences in oxidative damage measured as enzyme activity and lipid peroxidation products in serum and liver tissue of different aged male Wistar rats before and after heat stress. The question was whether aged individuals have higher levels of oxidative damage after heat stress indicating that they are more affected by the heat.

Summing up, our studies could not prove earlier findings since age-related differences in untreated control groups as well as in heat-stressed groups were not statistically significant concerning cell damage, liver injury and lipid peroxidation. Young and old rats might have the same amount of oxidative stress levels and effective protective methods to buffer ROS attacks or the oxidative damage could not be visualized in this series of experiments and further modified studies are necessary to evaluate the level of age-related oxidative stress.

4.1. Review of current studies

The concept of this study was based on the findings of Zhang et al. that proposed young rats have an effective protecting system that buffers oxidative damage in stressful conditions such as heat stress (Zhang et al., 2003). The previous findings suggested that aging in combination with heat stress produces higher levels of ROS and induces hepatic injury that increases *in vivo* oxidative damage to lipids and DNA (Zhang et al., 2003). Additionally, the GSH redox system is

affected and ROS strengthen the redox balance towards the pro-oxidant side leading to increased oxidative stress levels. The authors of recent studies revealed controversial results for lipid peroxidation products as evaluation of the amount of oxidative stress that accumulates during the aging process (Cini and Moretti, 1995, Barja de Quiroga et al., 1990, Castro et al., 2012).

On the one hand in accordance to the findings of Zhang et al., studies in heat-stressed broiler chickens presented elevated levels of plasma and mitochondrial MDA measured via TBA-method that indicate a higher level of lipid peroxidation and increased oxidative damage to macromolecules after heat stress (Mujahid et al., 2007). Increased 4-HNE-adducts were also measured in rat brains using immunohistochemistry after ischemic-reperfusion injury that is related to higher ROS formation (Yoshino et al., 1997). The clinical relevance of increased MDA as a marker for lipid peroxidation was investigated in various oxidative stress-related diseases that show higher incidence in aged individuals. Elevated MDA levels in serum were found in various forms of cancer such as lung and breast cancer (Gonenc et al., 2001) as well as gastric cancer (Bakan et al., 2002). Patients suffering from Alzheimer's disease showed higher MDA levels in brain tissue compared to healthy individuals (Lovell et al., 1995). In brain tissue of patients suffering from Parkinson's disease accumulation of 4-HNE-protein adducts were measured in immunochemical studies (Yoritaka et al., 1996). Arteriosclerosis has been found to correlate with increased serum MDA levels and decreased antioxidants (Tamer et al., 2002). Also patients suffering from congestive heart failure had higher MDA levels in serum compared to the healthy control group (Belch et al., 1991, Ide et al., 1999).

On the other hand, Tian et al. presented results showing that the amount of lipid peroxidation measured as MDA content in liver and brain tissue did not differ significantly in aged rats (Tian et al., 1998). An explanation might be that oxidative damaged lipids *in vivo* are fast degraded due to efficient repair mechanisms of the cell preventing the disturbance of membrane integrity. MDA *in vivo* was found to be oxidized by aldehyde dehydrogenase enzymes into metabolites such as malone semialdehyde and acetate and finally into CO_2 and H_2O (Marnett et al., 1985, Siu and Draper, 1982). Therefore, the measured MDA levels in our setup

might not display the exact *in vivo* oxidative stress level of animals as MDA is further metabolized and escapes the detection. Also Oberley et al. presented no significant differences after a two-phase heat stress experiment performed in young and old rats in the amount of 4-HNE-protein adducts measured by immunohistochemistry after 90 minutes of recovery (Oberley et al., 2008). They came to the conclusion that less 4-HNE protein adducts were formed due to increased interfering protection mechanisms.

Studies analyzing lipid peroxidation levels of different strains and sexes of rats including male and female Fischer 344, Wistar and Sprague-Dawley rats indicated that the amount of lipid peroxidation might differ species- and sex-dependent. Rikans et al. showed an age-related increase of MDA in liver homogenates of male Fischer 344 rats but in female Fischer 344 rats MDA decreased during aging (Rikans et al., 1991). In former study of Zhang et al. male Fischer 344 rats were examined whereas in this study male Wistar rats were used since no old rats were available from various distributors in Europe. Therefore, the gender as well as strain of rats in animal models need to be considered when comparing controversial results. In addition to that tissues from different organs such as heart, brain, liver as well as muscle tissue presented varying results for lipid peroxidation. Female Sprague-Dawley rats exposed to exercise stress that was found to increase oxidative stress levels showed elevated MDA levels in liver, heart and muscle tissue after acute exercise whereas chronic exercise reduces MDA content in brain tissue (Liu et al., 2000). Perez et al. examined lung tissue of young (8 months) and old (27 months) male Wistar rats using TBA-Method with no significant change in MDA level in the old group (Péréz et al., 1991). Further studies performed by Barja et al. in liver and brain tissue of young (8 months) and old male (27 months) Wistar rats also showed no age-dependent difference in old rats (Barja de Quiroga et al., 1990). Further studies are necessary to establish an animal model that evaluates the oxidative stress level identifying differences in strains, genders and tissues of rats.

The histological evaluation of liver section in accordance to the increased ALT activities indicates a higher grade of hepatocellular damage in older individuals. Such morphological changes are often accompanied by increased oxidative

stress markers including lipid peroxidation products and oxidized DNA. MDA is known to be mutagenic and reacts with DNA forming MDA-DNA adducts via cross-links that hinder DNA replication and damage DNA in form of DNA strand breaks and mutations (Niedernhofer et al., 2003). Former studies of Draper et al. showed that higher concentrations of MDA-DNA adducts occur in liver and kidney tissue but not in testes of old rats (25 months) compared to young (4 months) rats (Draper et al., 1995). This theory is underlined by findings that DNA repair mechanisms might slow down during aging leading to the accumulation of DNA mutations with more dysfunctional cells. Intano et al. presented that in hepatocytes of old mice DNA base excision repair mechanisms decrease up to 50% compared to young mice (Intano et al., 2003). The accumulation of oxidative DNA damage was observed by Hamilton et al. that reported significant increased oxidative DNA damage in liver tissue of aged mice as well as senescent Fischer 344 rats (Hamilton et al., 2001). They compared several tissues of Fischer 344 rats with different mice strains leading to the conclusion that the amount of age-related accumulated oxidative DNA damage is tissue- as well as strain-dependent. In general, more DNA damage was found in heart and brain tissue compared to liver tissue and Fischer 344 rats showed overall a higher increase. However, in contrast to the findings of Intano et al. they suggested that a higher sensitivity towards ROS attacks is responsible for the accumulation of oxidized DNA during aging and to a lesser degree deficient repair mechanisms.

Another possible explanation for not significantly age-related increased levels of liver damage and oxidative stress markers in this setting might be the ability of the liver to adapt to increased stress levels as well as efficient protection mechanisms that prevent cell damage. The liver is known for its high ability to regenerate after injury which is reduced during aging of an organism. A loss of regenerative capacity up to 30% in old rats was observed in rodents after removing two-thirds of liver tissue also called partial hepatectomy (Timchenko, 2009). Nevertheless, numerous studies showed protective effects in form of preconditioning in the liver. Preconditioning is defined as preadaptation to mild levels of stress that partly reduce the grade of severe injury when confronted to high and continuous levels of stress. Two forms of liver preconditioning are

distinguished: early preconditioning within minutes after stimulus and late preconditioning after 12-24 h (Carini and Albano, 2003). In our setting, the liver might be preconditioned for increased temperatures after exposure to the first heat stress and therefore the second heat impulse 24 h later is weakened. Beneficial effects of liver preconditioning such as reduced ischemic-reperfusion injury and improved liver regeneration were shown in animal models after partial hepatectomy and liver transplantation (Fernandez et al., 2003, Bedirli et al., 2005). In general, liver preconditioning is observed after short phases of mild ischemia or hyperthermia prior to actual liver surgery (Yamada et al., 2001, Oba et al., 2010). The ischemic preconditioning (IPC) is defined as a short period of ischemia followed by slight reperfusion of the organ prior to the unavoidable long ischemia during liver surgery (Wang et al., 2013). Peralta et al. presented that aminotransaminase levels (AST and ALT) in rat serum were reduced after 10 minutes of liver ischemia followed by 10 minutes of reperfusion prior to continuous ischemia (Peralta et al., 1999, Tsuyama et al., 2000). The first study that demonstrates these protective effects of IPC in the human liver was designed by Clavien et al. (Clavien et al., 2000). The ischemic-reperfusion injury is clinically relevant as it often occurs during liver surgery when the common Pringle maneuver is applied including the temporary occlusion of portal vein and hepatic artery to prevent major bleeding for resection of liver tissue (Selzner et al., 1999). Different studies presented that decreased levels of aminotransferases (AST and ALT) as well as reduced caspase activities displaying fewer apoptosis are found after IPC of the liver (Clavien et al., 2000, Glanemann et al., 2004). To understand the underlying processes of IPC two different mechanisms are considered. First the short period of ischemia at the beginning might directly interfere with signaling pathways induced by cell damage and prevent more severe damage. Second the sublethal stress during ischemia is essential for the adaptation of the liver to build up a defense mechanism that enables the liver later to be more effective against high levels of stress (Rüdiger et al., 2003). It is further hypothesized that the protection generated by IPC is a form of positive oxidative stress mediated by a short sublethal release of oxygen radicals that activate protection mechanisms in the cell (Sindram et al., 2002).

Another form of liver preconditioning is the heat stress preconditioning (HPC). It was performed as sublethal heating before ischemia followed by reperfusion in rat livers (Saad et al., 1995, Yamagami et al., 1998). Recent studies showed positive effects of heat shock pretreatment on different organs such as reduced ischemic-reperfusion injury in rabbit hearts and rat kidneys (Currie et al., 1993, Stokes et al., 1996), reduced damage of rat retina after light injury (Barbe et al., 1988) and protection against sepsis-induced injury in humans (Bruemmer-Smith et al., 2001). Different mechanisms take place in an organism when confronted to elevated temperatures. Among these is the ability to upregulate the production of heat shock proteins (HSP) that serve as protection in hyperthermic conditions by decreasing irreversible cell damage (Li et al., 2013). It is proposed that cells with the ability to highly upregulate HSP production react more efficiently to oxidative stress in form of heating. Early studies about HSP *in vitro* showed species-dependent differences in temperatures that are needed to trigger the induction of HSP varying from 45°C in sheep to rodents and humans where temperatures between 41°C and 43°C suffice to elevate HSP synthesis (Polla, 1988). Saad et al. and Kume et al. observed that heat shock prior to ischemic liver injury in rats increases gene expression and synthesis of HSP72 and reduces severe hepatocellular damage indicated by decreasing ALT and LDH activities in serum (Saad et al., 1995, Kume et al., 1996). Further Yamamoto et al. investigated the effect of heat shock preconditioning on HSP72 induction and liver injury focusing on lipid peroxidation and presented increased levels of MDA measured via TBA-method in preconditioned rats but declined amounts of 4-HNE-protein adducts (Yamamoto et al., 2000). They concluded that lipid peroxidation seems to remain unaffected from increased levels of HSP72 but the denaturation of proteins induced by LPO product 4-HNE is reduced leading to decreased oxidative damage rather than prevention of the LPO process. HSP might interfere with the specific antibody making it unable to detect 4-HNE in the ELISA. In accordance to these findings, Yamagami et al. confirmed the upregulation of HSP72 production in liver tissue of heat shock preconditioned rats as well as decreased formation of 4-HNE-protein adducts and reduced ALT and LDH activities after ischemic-reperfusion injury (Yamagami et al., 1998). Also

decreased ALT and LDH activities as well as higher survival rates were observed after heat shock prior to warm ischemia in form of Pringle maneuver and correlated to higher amounts of HSP in comparison to the control group (Saad et al., 1995). Another approach presented by Oba et al. combined exposure to heat shock with application of mild electric current that leaded to higher HSP72 production, decreased aminotransaminase activities (AST and ALT) and less histologically evaluated hepatic injury (Oba et al., 2010). These results support the theory that there is a strong protective function of HSP induced by heat stress preconditioning of the liver against ischemic hepatic injury finally reducing the amount of oxidative liver injury.

The two-phase heat stress was utilized in this study to simulate heat stress conditions of elderly humans during heat waves with multiple periods of heating (Bloomer et al., 2014). Especially in heat wave conditions the body is exposed to multiple following periods of heat stress and there is a significantly increased rate of mortality in people older than 65 years emphasizing the importance of heat stress in aging (Kenney et al., 2014, Conti et al., 2005). Heat stress shifts the redox balance towards oxidative stress by enhancing ROS production and weakening the mitochondrial antioxidant defense system (Slimen et al., 2014). This leads to the assumption that heat stress preconditioning might reduce hepatocellular damage and therefore in this study the expected increase of oxidative damage in the liver of aging rats after heat stress was weakened by the first heat stress and could not be measured.

By evaluating possible pathways for liver damage increased activation of caspases was found to increase apoptosis leading to a loss of liver tissue. Cursio et al. observed that inhibition of caspase activity protects rats from lethal liver damage normally occurring 12-24 h after surgery (Cursio et al., 1999). Other studies showed that apoptosis measured via caspase activity is modulated by induction of HSP during chronic heat stress in mice and there is correlation between HSP levels and heat-induced liver injury (Li et al., 2013).

Although the possibility of preconditioning after the first heat stress could not be excluded as discussed above, different studies indicated that heat stress models with two heat shocks induce higher rates of oxidative damage and reduce the

tolerance towards heating in old rats compared to single heat stress (Hall et al., 2000, Kregel et al., 1990). The use of animal models instead of human heating experiments is reasonable due to higher target temperatures in animals up to life threatening high levels. In animals core temperatures could be raised up to 41 °C with control of surrounding conditions such as level of exercise and loss of fluid whereas *in vivo* human heating experiments achieved only mild hyperthermia (37-38°C) (Armstrong and Kenney, 1993, Pandolf et al., 1988).

4.2. Limitations of methods

Restrictions of the methods used in our study need to be mentioned for the correct interpretation of the results that lay the basis for further research in this field. The amount of lipid peroxidation product MDA was measured applying the TBA-method that is based on the reaction of MDA with thiobarbituric acid (TBA) forming MDA-TBA adducts. These MDA-TBA adducts could be measured photometrically but the results display all substances that are reactive with TBA (TBARS). Therefore, the actual amount of MDA in the sample is higher when measured via TBA-method due to other TBA-adducts (Dalle-Donne et al., 2006). In addition to that, the heating and acidic conditions *in vitro* during the assay are prone to produce additional oxidative products (Del Rio et al., 2005, Seljeskog et al., 2006). The results of the MDA assay using TBA-method should be analyzed carefully because the results are often overestimated not displaying the actual amount of MDA in the sample. Other detection methods of MDA in serum and tissue samples were discussed in former studies. The combination of high performance liquid chromatography (HPLC) with the TBA-method was applied to receive more exact values for MDA-TBA adducts in samples without further generating *in vitro* oxidative products (Janero, 1990, Fukunaga et al., 1995, Mateos et al., 2005). The HPLC method increased the specificity of MDA measurements but components in serum were found to interfere and they had to be removed beforehand finally artificially reducing the MDA levels (Agarwal and Chase, 2002). Newer methods presented the quantification of MDA by using gas chromatography (GC) coupled with mass spectrometry (MS) that could be performed at room temperature with a two to six times higher sensitivity

compared to the common TBA-method (Liu et al., 1997). Although the derivatization of samples is needed prior to GC-MS that alters the structure of molecules, the newer methods have a higher specificity compared to the common TBA-Method but their use is still limited as they are more expensive, time-consuming and require experts for performing the procedure (Del Rio et al., 2005). Therefore, the TBA based test is still widely used and offers a facile sensitive method to compare MDA amounts when results are carefully interpreted (Carbonneau et al., 1991). As MDA reacts with DNA molecules forming MDA-DNA adducts, the measurement of oxidized DNA molecules in serum and tissue indicating increased oxidative stress will more closely replicate the *in vivo* accumulated oxidative damage.

For the detection of 4-HNE, an aldehyde discovered after MDA as stable end-product of lipid peroxidation, different methods were presented in former studies. Immunoassays such as the sandwich ELISA method use the high reactivity of 4-HNE to macromolecules to measure the amount of 4-HNE in tissue samples by adding specific antibodies that recognize and trap 4-HNE (Borovic et al., 2006). Other methods such as HPLC or GC-MS directly detect 4-HNE but due to its high reactivity the major amount of free 4-HNE is bound in 4-HNE-adducts and escapes direct measuring (Spickett, 2013). For the evaluation of 4-HNE in liver tissue in addition to the ELISA assay, the immunohistochemical staining of 4-HNE based on the theory of 4-HNE accumulation in hepatocytes might visualize more clearly higher levels of lipid peroxidation in older rats. Haak et al. presented that the amount of 4-HNE in mitochondria of liver tissue measured via immunoblotting technique significantly increased after a two-phase heat stress experiment in young and old male Fischer 344 rats at 24 h after the second heating leading to the conclusion that oxidative damage appears to a greater extend (Haak et al., 2009).

The determination of enzyme activity in serum and tissue samples might be affected by repeated freeze-thaw cycles that could reduce enzyme activities. Therefore, the results of LDH and ALT activity assays performed in this study might be affected as enzymes are inactivated and therefore no significant difference could be displayed. Finally, the possibility of preconditioning after the

first heat stress needs to be further examined by comparing different heat stress experiments. The following questions for researchers include how the heat stress upregulates age-depending protection mechanisms and whether HSP play a role for protecting tissues in heat stress conditions.

4.3. Conclusion

Our data showed controversial results concerning the heat stress experiment to the earlier findings of Zhang et al. and therefore three main aspects need consideration in following studies: a) role of preconditioning in two-phase heat stress experiments, b) if other organs are affected by oxidative injury, c) role of antioxidative protection. This thesis was a first approach to identify useful biomarkers that display age-related *in vivo* oxidative stress levels by analyzing the effect of heat stress on different aged rats. Further investigations will be necessary to evaluate the level of oxidative damage in heat stress experiments with altered settings including one- and two-phase heat stresses. Studies showed that lipid peroxidation products differ between sexes and strains of animals and therefore might also vary in humans. In addition to that, the identification of protection mechanisms in form of antioxidants is essential to prevent oxidative stress damage. Antioxidative substances including vitamin E and quercetin were investigated in various studies for their protective function. Vitamin E was found to inhibit lipid peroxidation in rat liver microsomes (Pulla Reddy and Lokesh, 1992). Quercetin a plant-derived substance contained in different foods was found to protect rat livers against injury caused by sodium fluoride-induced (Nabavi et al., 2012) as well as alcohol-induced oxidative stress (Liu et al., 2010). An antioxidative substance that efficiently reduces oxidative damage from ROS attacks and still enables proper cell signaling might contribute essentially to the control of oxidative stress and the prevention of cellular aging. As the liver has a high regenerative capacity, it might be not as affected by oxidative tissue injury as e.g., nerve or brain tissue of the central nervous system. In different neurodegenerative diseases, higher levels of lipid peroxidation products were measured that support this hypothesis. Especially age-dependent diseases such as Alzheimer’s disease and Parkinson’s disease clearly indicate a correlation to

oxidative stress injury. Age-related differences in stress response, oxidative damage and protection mechanisms might be seen by comparing brain tissue of healthy elderly with patients suffering from neurodegenerative diseases. Newer approaches for *in vivo* studies of oxidative stress observing the effect of caloric restriction (CR) presented interesting results. Researchers showed extended life spans for different species and reduced oxidative DNA damage in CR animal models. Species-dependent extended life spans after CR were studied in yeast *Saccharomyces cerevisiae*, the fruit fly *Drosophila melanogaster* and mice (Gems and Partridge, 2013). In theory, the caloric restriction leads to decreased metabolic rate and lower body temperature due to less energy metabolism that leads to fewer oxygen processing in mitochondria with decreased formation of ROS and therefore is thought to reduce the oxidative damage (McCarter et al., 1985). Hamilton et al. showed that CR significantly reduces age-related accumulation of oxidative damage DNA in rodents (Hamilton et al., 2001). Sohal et al. discovered that CR alters the age-related production of O_2^-• and H_2O_2 and weakens further accumulation of oxidative damage (Sohal et al., 1994a, Sohal et al., 1994b). CR further reduced the loss of membrane fluidity seen in older rats (Chen and Yu, 1994). Future animal models designed for examination of *in vivo* age-related effects of heat stress should include caloric restriction and further observe whether caloric intake influences accumulation of oxidative damage.

Our study serves as a window for the understanding of oxidative stress-induced damage as well as age-related deterioration caused by accumulated damage over time. On balance, further studies are needed to establish useful animal models that could be transferred to humans. The next steps following are the regulation of cellular aging without increased susceptibility to diseases or even the prevention of aging processes to enable the human being to live a healthy life within its maximum life span.

5. Abstract

Aging of an organism is defined as progressive decline of physiological functions with increased probability for disease and death within the life span. Increased production of reactive oxygen species (ROS) combined with a reduced capability of protection in form of antioxidants represents oxidative stress that is thought to be a major contributor to cellular aging. This study is integrated in the joint research project OXISYS of the Federal Ministry of Education and Research (BMBF) and investigates age-related changes of enzyme activity, liver damage and oxidative stress before and after a two-step heat stress experiment in serum and liver tissue of different aged male Wistar rats. Young (7 weeks, N = 8), middle-aged (6/7 months, N = 10) and old (23 months, N = 10) rats were included in the control group without treatment. The heat-stressed group was divided into middle-aged (7 months, N = 22) and old (23 months, N = 22) rats liquidated at four points in time after the second heat stress (0 h, 2 h, 4 h, and 6 h). Based on former findings of Zhang et al., lactate dehydrogenase (LDH) activity indicating general cellular damage as well as alanine-aminotransferase (ALT) activity specifically monitoring liver damage were measured. As oxidative stress affects macromolecules such as proteins, DNA and lipids, two lipid peroxidation end-products, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were analyzed in the samples. Hematoxylin & Eosin Staining of liver sections was performed to histologically evaluate hepatocellular damage. Our data shows that the expected increase of enzyme activity and lipid peroxidation markers before and after the heat stress could not be significantly verified in this setup. Histological liver damage appeared more severe in older rats whereas the liver morphology in young rats was normal. Controversial results of oxidative stress in animal models after heating lead to the hypothesis that age-related oxidative damage in the liver is less in comparison to other organs. The role of precondition after the first heat stress due to high regenerative capacity of the liver needs to be further investigated. To finally understand the molecular pathways of oxidative stress in aging, the analysis of protection mechanisms in form of enzymatic and non-enzymatic antioxidative protection is necessary. Further research in the field of oxidative stress is essential to finally encode the mystery of cellular aging.

6. Zusammenfassung

Das Altern ist definiert als ein fortschreitender Prozess begleitet von konstanter Abnahme der physiologischen Eigenschaften eines Organismus, der im Laufe der Zeit zu Krankheit und letztendlich unweigerlich zum Tode führt. Als eine der Hauptursachen für den zellulären Alterungsprozess wurde eine erhöhte Zellschädigung durch vermehrten oxidativen Stress entdeckt, die definiert ist als Ungleichgewicht zwischen zunehmender Freisetzung reaktiver Sauerstoffspezies (ROS) und dem Abfall protektiver Antioxidantien. Diese Studie ist ein Bestandteil des über das Bundesministerium für Bildung und Forschung geförderten Verbundprojektes OXISYS und untersucht altersabhängige Zellschäden vor und nach einem zweiphasigen Hitzestressversuch in Serum und Lebergeweben von männlichen Wistar-Ratten in verschiedenen Altersgruppen. Die Kontrollgruppe ohne Hitzestressbehandlung besteht aus jungen (7 Wochen, N = 8), mittelalten (6/7 Monate, N = 10) und alten (23 Monate, N = 10) Ratten. Im Hitzestressversuch wurden mittelalte (7 Monate, N = 22) mit alten (23 Monate, N = 22) Ratten verglichen und die Probenentnahme sowie die Liquidation erfolgte an vier verschiedenen Zeitpunkten (0 h, 2 h, 4 h, 6 h) nach dem zweiten Hitzestress je Altersgruppe. Basierend auf Vorstudien von Zhang et al wurden die generelle Zellschädigung mittels Lactatdehydrogenase (LDH)-Aktivität und die spezifische Leberschädigung zusätzlich anhand der Alaninaminotransferase (ALT)-Aktivität bestimmt. Oxidative Schäden an zellulären Bestandteilen wie Proteinen, DNA und Fetten akkumulieren im Alter und es wurden zwei Endprodukte der Lipidperoxidation, Malondialdehyd (MDA) und 4-Hydroxynonenal (4-HNE) bestimmt, um den Grad des oxidativen Stresses zu verdeutlichen. Ergänzend wurde die Leberzellschädigung durch histologische Beurteilung Hämatoxylin & Eosin eingefärbter Schnitte von Lebergewebe dargestellt. Die Untersuchungen dieser Studie zeigten ein Ausbleiben des erwarteten signifikanten Anstieges der Enzymaktivitäten und Markern der Lipidperoxidation vor und nach Hitzestress in älteren Ratten. Histologisch konnte eine höhere Leberzellschädigung in alten Ratten im Vergleich zu weitgehend normalem Lebergewebe in jungen Ratten gezeigt werden. Kontroverse Ergebnisse im Tiermodell nach Hitzestress könnten eine geringere

Beeinträchtigung der Leber im Vergleich zu anderen Organen nahelegen. Besonders aufgrund der hohen Regenerationsfähigkeit der Leber sollte die Möglichkeit einer Präkonditionierung in dieser Untersuchung nach dem ersten Hitzestress in Betracht gezogen werden. Dazu kommt die mögliche Rolle protektiver Antioxidantien, die in weiteren Studien zusätzlich untersucht und ebenfalls miteinbezogen werden sollte. Eine endgültige Entschlüsselung des menschlichen Alterungsprozesses ist weiterhin grundlegender Bestandteil der molekularen Alterungsforschung und weitere Untersuchungen sind notwendig um effektive Gegenmaßnahmen zu entwickeln und dem Menschen ein langes gesundes Leben zu ermöglichen.

7. References

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8. Appendix

Table 7: Protein content, LDH, ALT and MDA in human serum for preliminary experiments. Values are represented as mean ± SD. ***p<0.01 (young vs. old group), ns (not significant).

	Young people	Old patients
Number	8	8
Mean age [Years]	26.75	71.75
Male [m] or female [w]	4 m + 4 w	4 m + 4 w
Protein mass concentration [mg/ml]	84.89 ± 13.05	71.11 ± 7.16 (ns)
LDH activity serum [U/L]	36.82 ± 14.38	67.74 ± 13.54 (***p<0.01)
ALT activity serum [U/L]	13.72 ± 9.50	26.47 ± 19.17 (ns)
MDA serum [nmol/ml]	4.02 ± 1.04	5.20 ± 1.41 (ns)

Table 8: Protein content, LDH, ALT, MDA and 4-HNE in serum and liver tissue of young, middle-aged and old untreated rats. Values are represented as mean ± SD. *p<0.05 (young vs. old group), ***p<0.01 (young vs. old group), ns (not significant).

	Young	Middle-aged	Old
Protein content serum [mg/ml]	22.09 ± 6.07 (N = 8)	27.13 ± 9.23 (N = 10, ns)	29.56 ± 5.67 (N = 10, ns)
Protein content liver tissue [mg/ml]	31.44 ± 7.61 (N = 8)	30.57 ± 6.52 (N = 10, ns)	30.89 ± 2.60 (N = 10, ns)
LDH activity serum [U/L]	66.13 ± 26.39 (N = 8)	83.60 ± 39.29 (N = 9, ns)	77.71 ± 33.37 (N = 9, ns)
LDH activity liver tissue [mU/mg]	8.58 ± 2.39 (N = 8)	7.64 ± 2.24 (N = 10, ns)	6.96 ± 2.49 (N = 7, *p<0.05)
ALT activity serum [U/L]	6.83 ± 3.89 (N = 8)	10.68 ± 0.93 (N = 9, ns)	15.85 ± 5.92 (N = 9, ***p<0.01)
MDA serum [nmol/ml]	9.39 ± 1.14 (N = 7)	10.28 ± 4.29 (N = 10, ns)	10.41 ± 1.13 (N = 10, ns)
MDA liver tissue [nmol/mg protein]	0.38 ± 0.13 (N = 8)	0.43 ± 0.11 (N = 10, ns)	0.40 ± 0.1 (N = 10, ns)
4-HNE ELISA liver tissue [ng/ml]	0.52 ± 0.07 (N = 8)	0.57 ± 0.17 (N = 9, ns)	0.42 ± 0.1 (N = 8, ns)

Table 9: Protein content, LDH, ALT, MDA and 4-HNE in serum and liver tissue of middle-aged heat-stressed rats at four points in time. Values are represented as mean ± SD. There is no significant difference calculated between the age groups and points in time.

Middle-aged	**0 h** (N = 6)	**2 h** (N = 6)	**6 h** (N = 6)	**24 h** (N = 4)
Protein content [mg/ml] serum	29.51 ± 4.29	27.04 ± 5.93	25.46 ± 2.72	25.07 ± 3.02
Protein content [mg/ml] liver tissue	32.02 ± 3.53	29.47 ± 4.73	31.12 ± 5.22	26.01 ± 2.11
LDH serum [U/L]	54.26 ± 27.71	72.65 ± 31.12	62.33 ± 16.22	99.74 ± 51.19
LDH liver tissue [mU/mg]	7.24 ± 1.62	10.65 ± 2.17	9.18 ± 2.87	9.94 ± 0.49
ALT serum [U/L]	12.11 ± 2.96	10.95 ± 3.44	9.90 ± 2.41	11.67 ± 5.34
MDA serum [nmol/ml]	8.38 ± 1.47	7.42 ± 1.59	8.66 ± 1.41	8.98 ± 0.18
MDA liver tissue [nmol/mg protein]	0.39 ± 0.07	0.40 ± 0.10	0.39 ± 0.14	0.41 ± 0.13
4-HNE liver tissue [ng/ml]	0.59 ± 0.11	0.60 ± 0.08	0.82 ± 0.38	0.83 ± 0.16

Table 10: Protein content, LDH, ALT, MDA and 4-HNE in serum and liver tissue of old heat-stressed rats at four points in time. Values are represented as mean ± SD. There is no significant difference calculated between the age groups and points in time.

Old	**0 h** (N = 6)	**2 h** (N = 6)	**6 h** (N = 6)	**24 h** (N = 4)
Protein content [mg/ml] serum	31.57 ± 6.78	32.41 ± 11.07	30.46 ± 9.57	25.95 ± 5.57
Protein content [mg/ml] liver tissue	31.46 ± 5.48	31.19 ± 6.41	28.93 ± 8.28	28.86 ± 6.08
LDH serum [U/L]	86.31 ± 34.13	81.22 ± 40.26	75.24 ± 10.00	85.28 ± 26.97
LDH liver tissue [mU/mg]	8.63 ± 3.96	8.74 ± 2.66	9.15 ± 3.83	8.86 ± 4.88
ALT serum [U/L]	18.79 ± 8.93	16.97 ± 5.67	16.98 ± 8.37	10.94 ± 8.15
MDA serum [nmol/ml]	10.96 ± 3.33	11.03 ± 4.14	10.91 ± 2.81	11.62 ± 3.37
MDA liver tissue [nmol/mg protein]	0.36 ± 0.07	0.41 ± 0.10	0.43 ± 0.14	0.40 ± 0.08
4-HNE liver tissue [ng/ml]	0.56 ± 0.21	0.63 ± 0.46	0.60 ± 0.21	0.46 ± 0.05

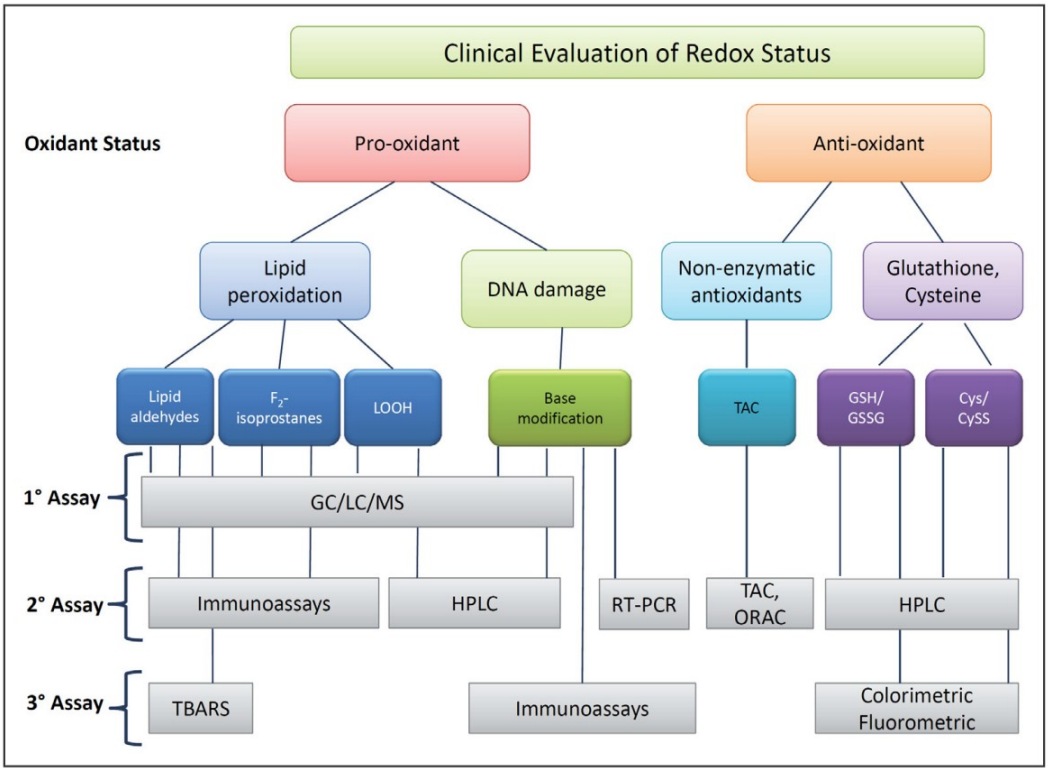

Figure 37: Decision tree for measuring oxidant status in clinical tissue. [adopted from (Griendling et al., 2016)].

9. Erklärung zum Eigenanteil

Die Arbeit wurde im Siegfried Weller Institut für Unfallmedizinische Forschung der Berufsgenossenschaftlichen Unfallklinik Tübingen unter Betreuung von Professor Dr. A.K. Nüssler durchgeführt.
Die Durchführung der Hitzestressexperimente und die Bereitstellung der Seren und Lebergewebe der Ratten erfolgte durch die Pharmacelsus GmbH (Saarbrücken, Deutschland).
Das Anfertigen der Leberschnitte und deren Hämatoxylin- und Eosin-Färbungen erfolgte in Zusammenarbeit mit Nicole Hebel sowie des pathologischen Instituts der Universität Tübingen.
Sämtliche Versuche und die statistischen Auswertungen wurden nach Einarbeitung von Dr. Britta Burkhardt von mir eigenständig durchgeführt.
Ich erkläre hiermit, dass ich diese Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen habe ich als solche gekennzeichnet.
Ich versichere die Richtlinien zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten an der Eberhard Karls Universität beachtet zu haben.
Ich erkläre außerdem, dass die hier vorliegende Dissertation nur in diesem und in keinem anderen Promotionsverfahren eingereicht wurde und dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind.

Tübingen, den_____

10. Publication

Results of this thesis were partially published in the following publication:

Title:

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Authors:

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Journal:

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