Chapter 1

General introduction

1. Research on aging

Aging is a degenerative process that occurs with time, often after the age of reproductive ability. Although aging is an inevitable and universal phenomenon, research on age-associated diseases, such as Alzheimer disease (AD), cancer and atherosclerosis, has by far exceeded advances in our understanding of the underlying mechanisms of the aging process itself. An explanation for this observation is that in the minds of many people, we do not die from aging, but from the diseases associated with aging. However, age-associated diseases are caused by the increase of molecular disorder, which is inevitable in any biological system (Hayflick, 2000). Understanding the mechanisms of aging could therefore aid in understanding and prevention of these age-associated diseases. This recognition has led to a growth in depth, breadth and molecular detail in this exciting research field. In the future the interest in aging will probably increase even further as the number of aged people worldwide continues to grow.

In addition to mammalian cellular and organismal aging models, the use of lower eukaryotic organisms, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*, has provided many new insights in aging mechanisms (Gami and Wolkow, 2006; Helfand and Rogina, 2003; Katic and Kahn, 2005). These model organisms share surprisingly many characteristics with higher eukaryotes with respect to pathways that are linked to longevity, and which influence the molecular mechanisms involved in aging that will be described below. Furthermore, these organisms have a reduced complexity, are relatively easy to manipulate, and have a shorter lifespan, which offers great advantages for research over higher eukaryotic systems.

Research on both higher and lower eukaryotic model systems have revealed that numerous aspects are involved in the course of the aging process. In the following paragraphs, the main theories of aging that have evolved over the years will be described, starting at the changes that occur on the molecular level, followed by the consequences of these alterations at the cellular and tissue level.

2 Molecular mechanisms of aging

2.1 Endogenous and environmental challenges

All living organisms have a unique property called homeostasis that is maintained by many defense and repair mechanisms. During life, homeostasis is continuously challenged by environmental and endogenous factors. The severity of these factors and the effectiveness of the organism to counteract these challenges, ultimately determine the organism's lifespan.

One of the most important theories of aging, called the free radical theory of aging, emerged almost 50 years ago and was initiated by Denham Harman (Harman, 1956). Harman based his theory on parallels between effects of aging and effects of ionizing radiation. He suggested that free radicals produced during life cause cumulative oxidative damage, resulting in aging and ultimately death. Through many decades this theory has gained much support by many individual groups and has evolved to a theory with many different facets.

It has become clear that reactive oxygen species (ROS) are produced by internal and external systems, and can oxidize cellular components such as proteins, carbohydrates, lipids and DNA (Davies, 1995; Halliwell and Gutteridge, 1990; Rice-Evans and Burdon, 1993), which can lead to loss of function of these cellular molecules. One of the main sources of ROS are the mitochondria during normal aerobic respiration (Chance et al., 1979), because of the incomplete reduction of oxygen to water. It is estimated that about 0.1% (Fridovich, 2004) or more (Chance et al., 1979) of the oxygen consumed by mitochondria is converted to superoxide anion radicals ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2). The latter can be further reduced to the highly reactive hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 1990) (Figure 1). The conversion of hydrogen peroxide to the hydroxyl radical is unlikely to occur spontaneously in an aqueous solution. However, various transition metals, such as Fe^{2+}, Ti^{3+} and Cu^{+}, are capable of reducing hydrogen peroxide to the hydroxyl radical (Halliwell and Gutteridge, 1990). Hydrogen peroxide itself is relatively neutral and can freely cross membranes. As a result, the catalytic availability of the transition metals determines the cellular localization of hydrogen peroxide transition to the hydroxyl radical. Therefore, the damaging effects of H_2O_2 are not limited to the place of origin.

Several sites in the respiratory chain are involved in ROS production, such as nicotinamide adenine dehydrogenase, ubisemiquinone and flavosemiquinone (Boveris and Chance, 1973; Finkel and Holbrook, 2000; Turrens and Boveris, 1980; Wei et al., 1981). The importance of mitochondrially produced ROS in aging is exemplified by the correlation of metabolic rate to the longevity of an organism. It was found that organisms with a higher respiratory rate had a shorter average lifespan (Livingstone and Kuehn, 1979). Moreover, food or caloric restriction has been shown to have beneficial effects on lifespan extension, which is among other things, thought to be mediated by a lowered respiratory rate (Koubova and Guarente, 2003).
Nevertheless, different species with a similar respiratory rate can have enormous differences in their average lifespan. An example of this is the little brown bat of North America, which is about one-half the size of a mouse with a high metabolic rate, but which can reach 30 years of age (Austad, 1997). Apparently, some organisms have evolved mechanisms that allow the organism to survive longer than would be expected based on their respiratory rate. This can depend on several factors, such as beneficial environmental circumstances or differences in the ability of an organism to deal with the produced damaging agents, as will be discussed later.

Although the mitochondria are the main site of ROS production, there are also other internal sources (Table 1), e.g. peroxisomes produce H_2O_2 as a byproduct during oxidative metabolism of long chain fatty acids (Adams et al., 1982). Furthermore, during the activation of the immune response, ROS can be locally produced in high concentrations by specific immune cells (Adams et al., 1982; Mackaness, 1970). Although this reaction is meant to have a protective function to annihilate the foreign entity, this also causes damage to molecules of neighboring cells.
This illustrates the importance of a well-regulated defense system for the life expectancy of an individual. If an infection results in a delayed or under-induced response, the organism is vulnerable for diseases that can lead to early death. However, an over-induced response can lead to needless cellular damage and a shortened lifespan. Consequently, a well-regulated immune response to different infections would therefore be of critical importance to obtain a long lifespan.

Strikingly, there is compelling evidence that with increasing age major changes arise of the adaptive as well as the innate immune system (Grubeck-Loebenstein and Wick, 2002), which induce a more pro-inflammatory immune state, called inflamm-aging. One of the changes concerns the alteration of important functions of members of the adaptive immune response, such as T-cells. The expression of some very important cytokines needed for T cell clonal expansion, including interleukin (IL)-2, are decreased with aging (Gillis et al., 1981), which contributes to the dysfunction of the adaptive immune response and the increased incidence of infections and several age-related diseases, such as auto-immune disorders, cancers and atherosclerosis (Castle, 2000a; Castle, 2000b; Fulop et al., 2005). Also some specific functions of the innate immune response are altered (Fulop et al., 2004; Plackett et al., 2004), possibly caused by the imbalance in the adaptive immune response. One of these changes is the production of pro-inflammatory cytokines TNFα, IL-1 and IL-6. Indeed, the increased presence of these primary pro-inflammatory cytokines in plasma has been described (Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Pedersen et al., 2000). This pro-inflammatory immune state upon aging might result in an increase in basal ROS production by the immune system.

Internal	External
Mitochondrial respiratory chain	UV radiation
Immune cells	Ionizing radiation
Peroxisomal β-oxidation	Transition metal salts
Prostaglandin synthesis	Pollutants
Cytochrome P450	

Table 1: Internal and external sources of ROS. Adapted from Boonstra and Post, 2004.

Next to the internal sources of ROS, there are also external sources (Boonstra and Post, 2004) that can induce damage, such as ultraviolet and ionizing radiation (Cerutti, 1985; Pollycove and Feinendegen, 2003), pollutants (Cosgrove et al., 1985; Stone and Pryor, 1994), transition metal salts (Samson and Nelson, 2000), and natural phenolic compounds present in plant food (Gold et al., 1992) (Table 1).

All these damaging circumstances that an organism faces during life challenge the survival of the organism. To maintain homeostasis, organisms have

evolved two levels of intervention at the molecular level. The first line of defense is focused on neutralization of the damaging agents. If this line of defense is insufficient or fails and damage is induced, there is a second line of defense that can repair the induced damage or ultimately removes the damaged compound.

2.2 Protection against ROS

With regard to the first line of defense, aging research has mainly been focused on systems that deal with ROS. Cells contain an elaborate network of mechanisms to deal with these agents, corroborating the importance of ROS defense for proper functioning of cells. The enzyme superoxide dismutase (SOD) (Stevens et al., 1975) was one of the first findings that gained credibility for the free radical theory of aging. This enzyme reduces two super oxide molecules to oxygen (O_2) and hydrogen peroxide (Figure 1). Other enzymes, catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GRD), peroxireductase (PRD), thioredoxin peroxidase (TPX) and thioredoxin reductase (TRD) were also discovered to be part of an elaborate enzymatic antioxidant defense network (Figure 1).

Support for the free radical theory of aging and the role of antioxidant enzymes herein came from over-expression experiments of enzymes, such as SOD and CAT, in model organisms like *D. melanogaster*, or SOD/catalase mimetics in *C. elegans*, which resulted in an increased lifespan (Melov et al., 2000; Sohal and Weindruch, 1996). This demonstrated that increased protection against oxidative damage could elongate organismal survival. Increased protection has also been described in caloric restricted animals in addition to the earlier described decrease in respiratory rate. Caloric restriction can lead to an increased expression of anti-oxidant enzymes, such as SOD and catalase in various organisms. This is mediated by decreased circulating levels of insulin and insulin growth factor (IGF)-1 in caloric restricted animals, which regulate the expression of these anti-oxidant genes via the insulin/IGF-1 signaling pathway (Heilbronn and Ravussin, 2003).
Interestingly, the level of protection against ROS can differ upon cellular localization. An organelle that is likely to be more sensitive to oxidative stress is the endoplasmic reticulum (ER), which is involved in proper folding of proteins that enter the cellular secretory pathway. In the ER, glutathione

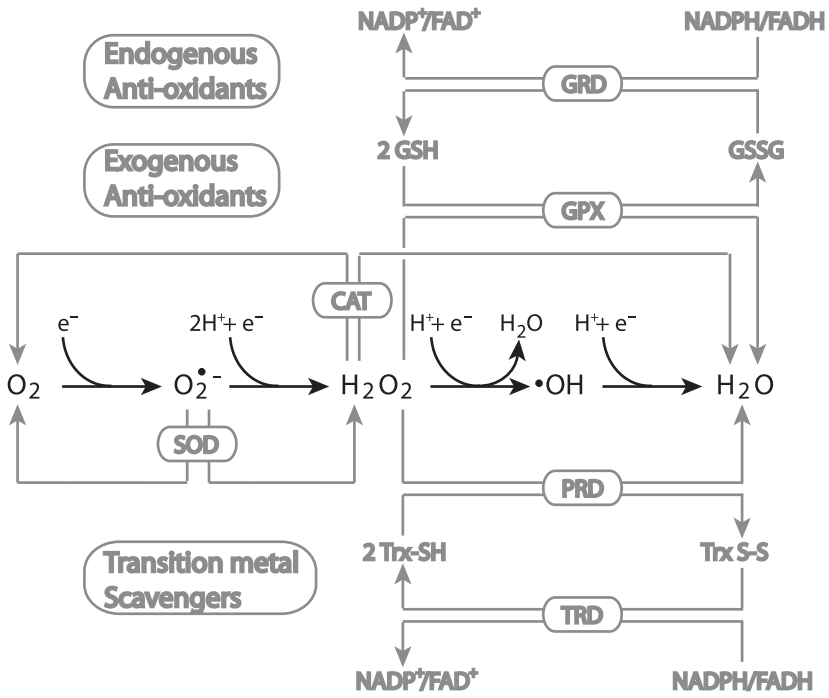

Figure 1: Formation of the different ROS, superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), during the successive one-electron reductions of oxygen to water (black) and the cellular anti-oxidant defense systems that protect the cell from oxidative damage (grey). The defense mechanisms include the anti-oxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GRD), peroxireductase (PRD), thioredoxin peroxidase (TPX), and thioredoxin reductase (TRD). SOD converts superoxide to oxygen and hydrogen peroxide. The latter is subsequently converted to oxygen and water by catalase, or to water by the joint efforts of the enzymes GPX, GRD, PRD, TPX and TRD via the oxidation of NADPH/FADH. Furthermore, a cell contains endogenous and exogenous anti-oxidants and transition metal scavengers. The anti-oxidants can serve as sink for radicals, and the transition metal scavengers mainly inhibit the conversion of hydrogen peroxide to the hydroxyl radical. Scheme modified from Katic and Kahn, 2005.

(GSH), the major redox buffer of cells, is in a more oxidized state compared to the cytoplasm (Hwang et al., 1992). This creates an environment that stimulates proper disulphide bond formation, which is essential for correct folding of the proteins that enter the secretory pathway. As a result, the ER is more vulnerable for oxidative stress compared to the cytoplasm. Indeed, upon high levels of oxidative stress, the highly abundant ER resident proteins that assist in protein folding are primarily oxidized (van der Vlies et al., 2002).

In addition to anti-oxidant enzymes, an organism contains proteins that sequester transition metals (Figure 1) and thereby prevent their catalytic availability for reducing hydrogen peroxide to the hydroxyl radical. One of the most important transition metals is iron. In the circulation, iron is primarily sequestered by transferrin, but after cellular uptake, iron is stored by the intracellular protein ferritin. The importance of iron metabolism in aging is illustrated by the association of iron accumulation in brain regions that are affected in the age-associated neuro-degenerative disorders Alzheimer's and Parkinson's disease (Zecca et al., 2004).

Next to specific enzymes and proteins, cells also contain anti-oxidant molecules or scavengers that either have endogenous origin, such as urate and thioredoxin, or have exogenous origin, such as β-carotene (precursor of vitamin A), α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Figure 1). The exogenous sources of anti-oxidant molecules are primarily determined by food intake. This exemplifies the possible beneficial effects of diet intake on the survival of an organism.

Several studies have revealed the beneficial effects of endogenous and exogenous agents on the attenuation of oxidative stress or damage, and on the attenuation of functional deterioration associated with aging (McDonald et al., 2005; Seidman, 2000; Socci et al., 1995).

2.3 Molecular repair mechanisms and their implications in aging

When the first line of defense is inadequate, cellular molecules such as DNA, lipids and proteins are damaged, which challenges cellular functionality. Therefore, a cell is equipped with several mechanisms that repair or degrade the affected molecules. Below, the importance of these repair mechanisms and their implications in aging will be described.

2.3.1 Chromosomal DNA damage

Many different DNA repair mechanisms have been described, which underlines the important role of DNA in cellular integrity. Damage or mutations cannot only be inflicted by ROS, as described above, but can also be caused by chemicals and radiation or during DNA replication prior to cell division. For oxidative damage alone, more than 100 different oxidative DNA lesions have been described (Hoeijmakers, 2001), which exemplifies the complex nature of DNA repair mechanisms. These repair mechanisms are well-regulated and

repair is executed by large protein complexes that each recognize and repair a different class of DNA damage (Hoeijmakers, 2001). Like all biological systems, DNA repair systems are not perfect, which ultimately leads to accumulation of genetic errors. A classic theory of aging, called the somatic mutation theory, is based on this aspect of life. This theory states that the rate of accumulation of genetic mutations, and thus accumulation of illegal gene products, ultimately determines an organism's lifespan. Indeed, there appears to be a correlation between DNA repair rates and lifespan among mammals (Promislow, 1994). Furthermore, dysfunctioning DNA repair mechanisms are associated with pathologies that show early signs of aging and a decreased lifespan, as is found in Werner's syndrome and Cockayne syndrome patients (Bender et al., 2003; Chen et al., 2003a; Chen et al., 2003b), or an increased susceptibility for developing age-related diseases, such as cancer (de Vries et al., 1995). Interestingly, in mice it has been shown that mutation of genes involved in the functioning of a limited set of DNA repair mechanisms induce symptoms of accelerated aging (Hasty et al., 2003), while mutations concerning other DNA repair mechanisms have no obvious or an embryonic lethal effect (Engelward et al., 1997; Tebbs et al., 1999). These different effects probably depend on the frequency of certain DNA damages and the availability of other DNA repair mechanisms that can replace the dysfunctioning DNA repair mechanism. These examples clearly demonstrate the important role of several DNA repair mechanisms in maintaining chromosomal DNA integrity and thus in determining lifespan and protection against diseases (Figure 2).

2.3.2 Telomere shortening

Next to chromosomal DNA mutations, telomere shortening has been implicated in the organismal aging process (Figure 2). Telomeres are simple DNA repeat sequences at the chromosome ends that form complexes with proteins to protect the chromosomes from degradation, end-to-end fusions and activation of the p53-dependent DNA damage response pathway involved in induction of apoptosis (van Steensel et al., 1998). Shortening of the telomeres can lead to genomic instability and loss of proliferative ability, called senescence (von Zglinicki, 1998; von Zglinicki, 2000), which will be discussed in more detail later. The loss of proliferative ability is thought to be caused by disruption of the telomere structure when telomere DNA reaches a critical

length. Indeed, it has been shown that disruption of the protein structure forming the telomere complex induces premature senescence mediated via the p53 or the p16/pRB pathway (Smogorzewska and de Lange, 2002).

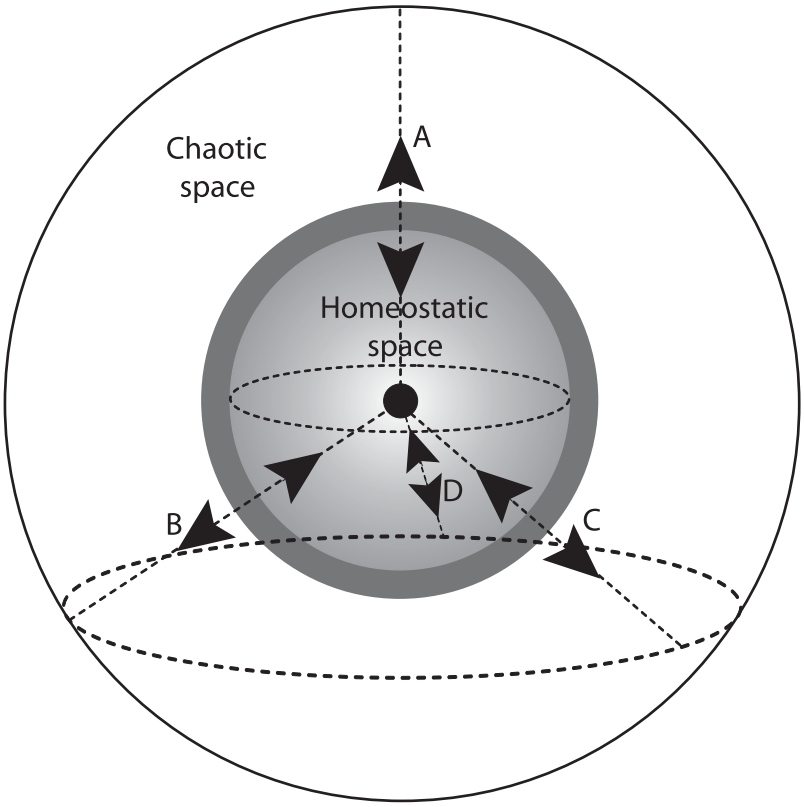

Figure 2: Model of the molecular processes that influence cellular fate during aging. Several molecular protection and repair mechanisms, such as ROS protection, DNA repair, protein quality control and others (strings A-D), are involved in maintaining cellular homeostasis (homeostatic space). Upon loss of efficiency of one or more of these molecular mechanisms during aging or upon stress, the cell will shift from the center of homeostatic space. The molecular protection and repair mechanisms will attempt to regain cellular homeostasis. These processes are also dependent on the energy levels of the cell, which decrease with increasing age. Consequently, aged cells experience larger shifts from the center of homeostatic space when challenged compared to young cells. When the molecular disorder reaches a critical level (the boundary between homeostatic and chaotic space) the cell will go into a state of replicative arrest, or go into apoptosis or necrosis.

Telomere erosion is caused by the inability of DNA polymerases to replicate the 3'-ends of chromosomes to their very end during DNA replication prior to every cell division. This is called the end-replication problem. Telomere shortening can be significantly accelerated by oxidative damage to telomeric DNA. It seems that DNA repair mechanisms are less able to repair this damage because of the tight nature of the protein complexes that form the telomeres. These damages cause stalling of DNA polymerase activity, which can lead to substantial telomere erosion (Cordeiro-Stone et al., 1999; von Zglinicki, 2000).

In germ line and cancer cells telomere length is maintained by the activity of the ribonucleoprotein telomerase that can elongate telomeric DNA (Greider and Blackburn, 1985). However, most cells normally do not possess telomerase activity. Many studies have shown that with increasing age, telomere length is shortened in a variety of tissues (Allsopp et al., 1992; Coviello-McLaughlin and Prowse, 1997; Frenck et al., 1998; Hastie et al., 1990; Op den Buijs et al., 2004). Furthermore, in birds and mammals telomere shortening has been shown to be correlated with maximum lifespan (Haussmann et al., 2003; Vleck et al., 2003). This suggests that telomere shortening is not only involved in cellular replicative lifespan, but also in organismal lifespan (Katic and Kahn, 2005).

2.3.3 Mitochondrial DNA damage

Another aspect of DNA damage and repair in aging involves mitochondrial DNA, which is included in the mitochondrial free radical theory of aging. This theory states that the ROS species produced by the mitochondria during normal aerobic respiration primarily subject these same mitochondria to a high degree of oxidative stress. Mitochondria contain double stranded closed circular DNA (mtDNA) that encodes for 13 mitochondrial proteins, of which most are involved in the electron transport chain. The induced oxidative stress can damage these genes, which could lead to dysfunctional electron transport proteins and an increased incomplete reduction of oxygen to water, resulting in increased oxidative stress (Beckman and Ames, 1998; Cottrell and Turnbull, 2000; Ozawa, 1998; Wei, 1998). Indeed, an age-related decline in mitochondrial respiration has been shown in several tissues of different species (Hsieh et al., 1994; Sugiyama et al., 1993; Yen et al., 1989) as well as an increased production of ROS (Sohal et al., 1994). This theory explains, at

least in part, the observed increased oxidative stress with increasing age and the reduced mitochondrial coupling found in aging tissues (Marcinek et al., 2005; Ritz and Berrut, 2005). Especially the reduced mitochondrial coupling could be very important for the ongoing deterioration of an aging individual, as all maintenance systems that protect and repair damaged cellular molecules require a lot of energy. A decrease in energy levels will greatly influence the efficiency of these maintenance systems and predispose a cell to accumulation of damaged molecules (Figure 2).

2.3.4 Protein damage

Next to damage to DNA, proteins are also damaged in the course of aging (Figure 2). At least two theories involving proteins have been implicated in the aging process, the altered proteins theory and the waste accumulation theory (Kirkwood, 2005). In normal functioning cells with an adequate energy supply, there is a relatively high turnover of proteins, even of undamaged proteins. The regulation of protein synthesis and degradation rates allows the cell to rapidly modify intracellular protein levels to adapt efficiently to intra- and extracellular environmental changes (Martinez-Vicente et al., 2005).
Over time, proteins are subjected to numerous damaging circumstances that can alter the protein and could lead to impairment or loss of protein function. This includes heat-induced denaturation, but especially oxidative modification, which can lead to increased levels of protein carbonyls (Levine, 2002), oxidized methionines and cysteines (Davies, 2000; Stadtman, 2001; Stadtman et al., 2003), glycation (Baynes, 2001), protein cross-linking (Squier, 2001) and aggregate formation. A cell has evolved mechanisms that either create an environment that enables the protein to regain its original composition, or target it for degradation. Both mechanisms require energy for proper functioning.

The first protein regulation mechanism is carried out by chaperones that sequester and assist in folding of denatured or newly synthesized proteins. Chaperones reside both in the ER, called ER chaperones, and in the cytoplasm, called heat-shock proteins (HSP) and ultimately determine the fate of the damaged protein. Chaperone binding of a denatured protein prevents the protein to aggregate and creates an environment for the denatured protein to refold. When a cell is exposed to high levels of stress and thus massive protein

denaturation occurs, the maximum refolding capacity of the chaperone system can be reached. This triggers a stress response, which results in the specific up-regulation of chaperone and protein degradation genes and thus in an increased refolding and degradation capacity (Riezman, 2004; Schroder and Kaufman, 2005). For the ER and the cytoplasmic protein refolding machinery, these responses are referred to as the unfolded protein response (UPR) and the heat-shock response.

The second protein regulation mechanism involves the recognition of the altered proteins and their subsequent degradation. There are several systems that are involved in protein degradation, such as the ubiquitin-proteasome system, calpains and lysosomes. Of these systems the proteasome system and the lysosomal system are responsible for most of the intracellular protein turnover.
The proteasome system has been shown to be responsible for the selective degradation of oxidized proteins in mammalian cells, via ubiquitination of the damaged protein prior to degradation (Sitte et al., 1998).
The lysosome is a cellular organelle that contains a large assortment of hydrolases capable of degrading a wide variety of macromolecules. Lysosomes do not only degrade extracellular proteins that are internalized via endocytosis, but can also degrade long-lived intracellular macromolecules or even whole organelles, in a process called autophagy, which is activated under different kinds of stress conditions (Cuervo, 2004; Shintani and Klionsky, 2004). These macromolecules and organelles are surrounded by de novo formed membranes, prior to fusion to and degradation by the lysosome (Klionsky, 2005; Shintani and Klionsky, 2004) or are directly engulfed by the lysosome (Farre and Subramani, 2004; Klionsky, 2005).

Despite the existence of these protection and repair mechanisms, oxidative protein damage is still detectable under normal physiological conditions (Agarwal and Sohal, 1994; Smith et al., 1991), which suggests that these systems are insufficient to protect against all oxidative damage even during basal levels of ROS generation. Furthermore, there is evidence of the functional decline of the chaperone and several degradation mechanisms with increasing age (Carrard et al., 2002; Colotti et al., 2005; Cuervo and Dice, 2000; Soti and Csermely, 2003). As the chaperone and degradation pathways

are also composed of proteins, the decreased functioning of these systems can also, at least in part, be caused by damage of members of these pathways, as was proposed for ER chaperones (Rabek et al., 2003). In addition, mildly oxidized proteins have been shown to be good substrates for degradation, but extensive oxidatively modified proteins are more resistant to degradation and are prone to aggregate (Grune et al., 1997). This implies that with increasing oxidative stress upon aging, proteins that are bad substrates for degradation will accumulate. Furthermore, the lysosome also reveals striking changes with increasing age that impair lysosomal function, such as decreased regulation of lysosomal pH, changes in hydrolase activities, increase in lysosomal volume and accumulation of indigestible materials as lipofuscin (Terman and Brunk, 2004). A consequence of the progressive decline of these mechanisms would be the accumulation of oxidized proteins, macromolecules and organelles during aging. Indeed, with age increased levels of oxidatively modified proteins have been found in tissues of several species (Agarwal and Sohal, 1994; Garland et al., 1988; Head et al., 2002; Smith et al., 1991; Starke-Reed and Oliver, 1989). This increase could have deleterious effects on cellular functioning and could thus be involved in the decline of organismal vitality.

2.3.5 Lipid damage

As for DNA and proteins, lipids can also be damaged by a wide variety of agents (Figure 2). Although information about these changes is currently not so widespread as for DNA and proteins, there are indications that lipid damage can play an important role in the decline of cellular functioning. Lipid oxidation results in lipid peroxide formation that has been shown to reduce membrane fluidity, can inactivate membrane-bound proteins and can be degraded into cytotoxic aldehydes, such as malondialdehyde and hydroxynonenal (Richter, 1987). Increased levels of these breakdown products have been shown in tissues of *D. melanogaster* and rat with increasing age (Odetti et al., 1994; Zheng et al., 2005). Oxidized lipids are also substrates for degradation. For instance, it has been shown that phospholipase A2 preferentially hydrolyzes fatty acids from oxidized liposomes and reveals increased activity upon oxidative stress (van den Berg et al., 1993; van Rossum et al., 2004).

3 Changes at the cellular and tissue level during aging

The described molecular changes of DNA, proteins and lipids that occur in time, will have an impact on cellular functioning, and therefore on functioning of tissues, organs and the organism itself. However, there are unique cellular mechanisms involved in aging that are influenced by the molecular changes described above.

3.1 Senescence

As mentioned above, normal cells cannot divide indefinitely and can go into a state of replicative arrest, called replicative senescence (Figure 2). This was first demonstrated in tissue culture experiments with primary human fibroblasts by Hayflick and Moorhead (Hayflick and Moorhead, 1961). It was proposed, and later shown, that the erosion of telomeres caused this loss of proliferative capacity (Harley et al., 1990; Olovnikov, 1973). Cells that go into senescence undergo morphological changes, changes in chromatin structure and gene expression and cease to respond to mitogenic stimuli (Narita et al., 2003; Serrano and Blasco, 2001; Shelton et al., 1999). They can survive in this non-dividing state for months and are less sensitive for induction of apoptosis, a process that involves programmed cell death, which will be discussed later. The physiological role of senescence is still not clear, as one would need to know how many cells *in vivo* undergo senescence and how many senescent cells need to accumulate to cause organismal aging.

However, induction of senescence is thought to protect damaged cells from unlimited cell proliferation, which could lead to cancer (Campisi, 2005). Therefore, senescence is thought to have a protective function early in life, but with increasing age accumulation of these cells could have deleterious effects on tissue functionality. In recent years it has become clear that senescence can also be induced by different kinds of stress, such as DNA damage and oxidative stress (Serrano and Blasco, 2001), which occurs within days after stress induction. Although these different signals probably elicit a common cellular response via different pathways, a distinction is made between replicative senescence, which is induced after extensive proliferation, and stress-induced senescence, which is induced by various forms of stress. It is very well possible that in the *in vivo* situation even a combination of the two may occur.

3.2 Cell turnover

Whenever a cell faces severe stress, the cell is dependent on his ability to adapt to or resist the opposed stress and to replace or repair the damaged molecules. The mechanisms that play a part in these processes have been described earlier. However, whenever the stress and consequently the damage are too severe, cellular homeostasis will be lost and the cell will eventually die (Figure 2). There are two distinct forms of cellular death, necrosis and apoptosis. Necrosis is often induced by severe forms of stress in which cellular integrity cannot be maintained, which leads to cell lysis and secretion of cellular proteins in the circulation. This can occur during a local immune response or during obstruction of circulation, which leads to local and relatively large areas of cellular death. Indeed, intracellular proteins have been detected in human blood samples (Pieper et al., 2003a), although the precise origin of these proteins is still obscure.

Apoptosis is a consequence of the induction of an internal suicide program, which can be induced by several signal transduction pathways that detect cellular stress or damage, such as DNA damage via the p53 signaling pathway (Gomez-Lazaro et al., 2004), ER stress via the activation of the UPR (Orrenius et al., 2003) or protein denaturation via the heatshock response (Finkel and Holbrook, 2000), some of which also have been shown to induce senescence. Induction of senescence or apoptosis probably depends on environmental circumstances, the level of cellular damage and on cell cycle status (Boonstra and Post, 2004). Apoptosis distinguishes itself from necrosis, in that the cell retains its membrane integrity and is thus not subjected to cell lysis. During apoptosis, chromatin is segregated in sharply circumscribed masses at the edge of the nuclear envelope. The cytoplasm is condensated and closed membrane vesicles are formed by blebbing of the plasma membrane, which are phagocytized by nearby cells. Therefore, during apoptosis there is no severe leakage of cellular proteins into the circulation.

Cells that are lost by processes as apoptosis or necrosis are normally replaced either by cell division of neighboring cells or by differentiation of stem cells (Op den Buijs et al., 2004), depending on the tissue type and environmental circumstances. As mentioned above, there is a limitation in the number of cell divisions in normal cells, which can be caused by the erosion of telomeres. Stem cells are also subjected to the aging process, which can result in exhaustion of the stem cell pool or a reduced differentiation capacity of stem

cells (Anversa et al., 2005; Kamminga and de Haan, 2006; Quarto et al., 1995). This is also caused by the molecular changes that occur in time. A consequence of these two factors is the decrease in regenerative capacity, and ultimately a decrease in functioning of tissues upon aging. This also illustrates the two-edge sword of processes such as apoptosis in aging. When apoptosis pathways have a low threshold, there is a decreased chance of developing diseases as cancer, as relatively low levels of damage will cause programmed cell death. However, it also predisposes the organism to a high cell turnover rate, which sensitizes the organism for a reduced vitality later in life. This was elegantly demonstrated in a mouse model in which a mutant form of p53 showed constitutive activation. These mice had a lower incidence of cancer, but showed faster aging (Tyner et al., 2002).

4 Understanding the interactions of aging theories

All of the above mentioned changes will, at least in part, play a role in the aging process and the associated decline in organismal survival. Although strides have been made to understand the role of these processes in the development of aging, a general consensus has not been reached on the relative importance of each of them. This has led to initiatives to develop a theory in which the different contributors that are thought to play a role in the aging process are considered together, called the network theory of aging (Kirkwood et al., 2003). This would allow the understanding of the interaction and possible synergistic mechanisms of these processes. Furthermore, understanding these relations is necessary to determine the processes that are involved in the early and the late stages of aging. Recognizing these connections could be very important in our search for optimal mechanisms of intervention in the aging process (Kirkwood, 2005). As every species invests differently in maintenance of damage prevention and repair, differences herein are expected. Even in different tissues of an organism the importance of the various aging mechanisms may differ. For instance, highly proliferative tissues will suffer more from telomere erosion and somatic mutations than post-mitotic tissues because of the requirement of DNA replication prior to every cell division. On the other hand, post-mitotic cells will suffer more from waste accumulation, such as protein aggregates, as in proliferative tissues these aggregates will be divided between daughter and mother cell (Kirkwood, 2005), which will result in dilution of the aggregates in newly synthesized cell material. Furthermore, every tissue is exposed to different environmental circumstances, which requires different levels of protection.

Mapping the differences that occur in the course of the aging process, and thereby defining so called biomarkers of aging could provide the information necessary to understand the aging process and the contributions of the different mechanisms herein. Moreover, it would enable the analysis of the effects of aging intervention studies and ultimately, it could be used to determine the biological over the chronological age of an individual. The heterogeneity of the aging process, most likely caused by life style and variation in genetic make-up (Kirkwood et al., 2005), and the difficulty of discriminating between changes caused by normal aging and age related pathologies make it difficult to obtain high accuracy in sensitivity and specificity with single markers. For that reason, multiple analytes in different

tissues, organs or bodily fluids should be investigated to really grasp the importance of aging theories in the onset and progression of aging, as with multiple independent markers one can increase the sensitivity and specificity compared to the use of a single marker (Anderson, 2005).
This approach was started with the genomics era that provided information about genetic expression differences (Lee et al., 1999; Lee et al., 2000; Zou et al., 2000), and is now extending to the field of proteomics that provides information about protein expression and modification differences, which will be the focus of this thesis.

4.1 Antibodies in proteomics

In the last few years, proteomics has become one of the fastest growing research fields. This has led to the development of numerous high throughput assays and improvement of several existing techniques that enable the analysis of several proteins in a single experiment. Despite these developments, it has become clear that this field faces us with even more challenges than the genomics era, because of some unfavorable characteristics of proteins, e.g. the presence of differently modified isoforms of a single protein, differences in protein stability, protein-protein interactions and the presence of highly abundant bulk proteins that hamper the analysis of less abundant proteins.
Affinity ligands, such as antibodies, can provide solutions for several of these problems. Antibodies can be used for detection of a single protein in a biological sample with high specificity and sensitivity. The use of a large set of different antibodies, each with different antigen specificities thus would enable the simultaneous analysis of several proteins. This principle forms the basis of the development of antibody micro-arrays, in which antibodies are immobilized on a surface, each in a separate spot, to capture their respective antigen (Angenendt, 2005). Depending on the specificity and affinity of the used antibodies, this technique provides the expression analysis of several predetermined target proteins simultaneously and with high sensitivity.
Another application for antibodies in proteomics is as an affinity ligand for purification or depletion of target proteins (Pieper et al., 2003b). The purification of a protein from a biological sample provides methods to study protein-protein interactions, as proteins that are co-purified with the target antigen can be analyzed on gel or with mass spectrometric techniques.

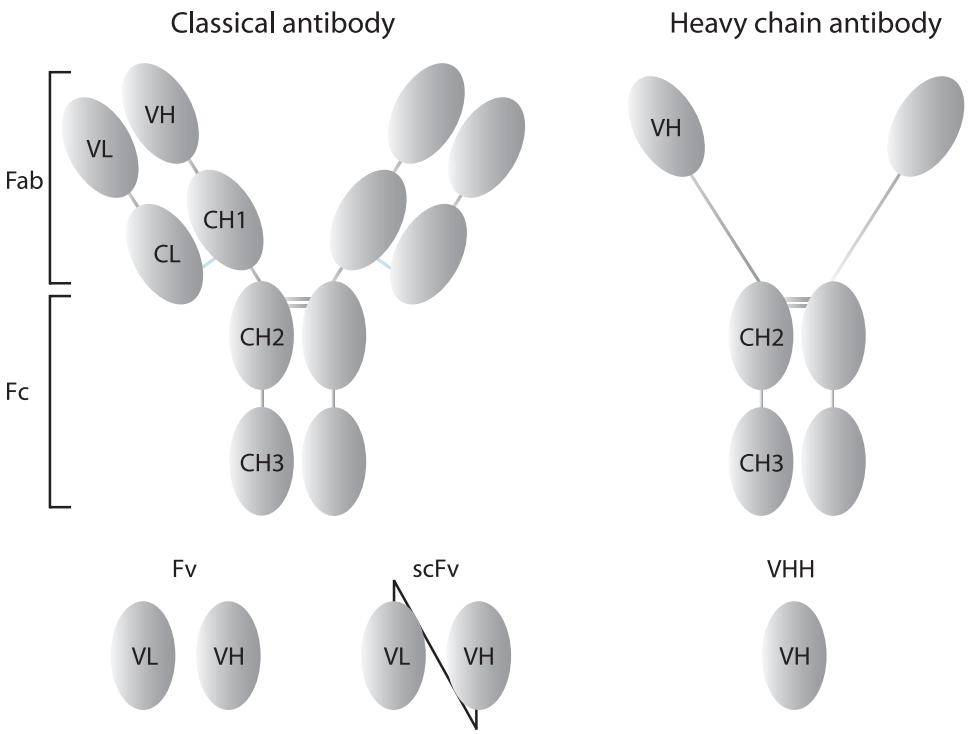

Figure 3: Schematic representation of the differences between classical and heavy chain antibodies, and their antigen binding fragments. A classical antibody consists of two identical light and heavy chains, whereas the heavy chain antibody consists of only two identical heavy chains. The smallest antigen binding domain of a classical antibody (Fv) consists of the combination of the variable light (VL) and variable heavy (VH) chain domain, which can be linked via a synthetic linker to obtain a single chain Fv fragment (scFv). The smallest antigen binding domain of a classical antibody consists of only the variable domain of the heavy chain (VHH).

Furthermore, many biological samples contain highly abundant bulk proteins that interfere with the detection of less abundant proteins. Affinity ligands can be used to deplete these abundant proteins, thereby enabling the analysis of previously non-detectable proteins (Pieper et al., 2003a).

Moreover, antibodies can be used to analyze protein expression patterns by making use of the existence of common epitopes present in several different proteins. Such an epitope enables the use of a single antibody to visualize or purify several different proteins simultaneously. This approach has been successfully applied for post-translational protein modifications, such as phosphorylation and ubiquitination (Gronborg et al., 2002; Maguire et al., 2002; Richter et al., 2005).

4.2 Generation of specific antibodies

The successful application of antibodies in proteomics requires reliable affinity molecules in respect to sensitivity as well as specificity. Antigen specific antibodies can be obtained using several different techniques.

The traditional method of obtaining antibodies is by immunization of an animal with the target antigen, followed by the isolation of the polyclonal serum a few weeks after immunization. Although the generation time for these antibodies is short and the costs are relatively low, there are several disadvantages. The polyclonal antibody pool contains many different antibodies with various affinities for the target antigen. This heterogeneity renders polyclonal antibodies relatively less specific compared to monoclonal antibodies, as each different antibody in this pool could potentially cross-react with proteins other than the target antigen. Polyclonal antibodies are therefore less suitable for use in proteomics. Furthermore, the antibody source is not infinite, requiring new immunizations and new specificity and sensitivity tests, as every new immunization will yield a different antibody pool with their own specificity and sensitivity.

Monoclonal antibodies are obtained by screening the B-cell repertoire of an immunized animal for cells that produce antigen specific antibodies. These cells are then fused to myeloma cells giving rise to an immortal hybridoma cell line (Kohler and Milstein, 1975). Once the fabrication of such a cell line is established an indefinite source of antibody is obtained. On the other hand, the generation time of such an antibody is relatively long, because of the elaborate screening protocols and therefore the costs are high compared to polyclonal antibodies.

Recombinant antibodies share all the advantages of monoclonal antibodies and moreover, have additional advantages. Most recombinant antibodies only contain the antigen binding domain of classical antibodies (Figure 3), although recombinant whole classical antibodies have been described. As they are relatively small and have a reduced complexity compared to the classical antibodies, they can be efficiently cloned and produced in microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*. An additional advantage of recombinant antibodies is that they can be genetically modified (Clackson, 1991), which enables the addition of affinity tags or the construction of multi-valent antibodies to increase the avidity of the antibody molecule.

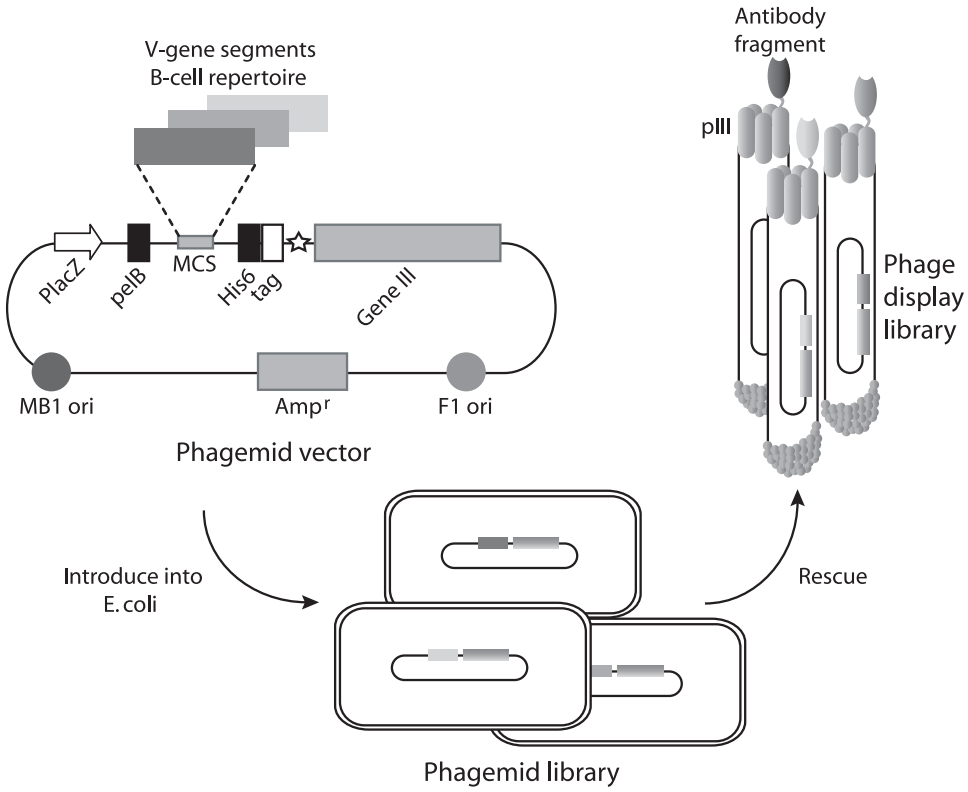

Figure 4: Schematic representation of the construction of a phage display library. V-gene segments, of B-cells from immunized or non-immunized animals, can be amplified by cDNA synthesis and PCR. The obtained genes are inserted into the multiple cloning site (MCS) of a phagemid vector, in frame with a purification (His6) and a detection tag (tag), and gene III, the gene encoding the phage coat protein pIII. A phagemid library is obtained by introduction of this vector into *E. coli*, after which phage particles, displaying the antibody fragments on their surface, can be rescued via infection with helperphage to obtain a phage display library. The lacZ promoter, pelB sequence and the amber stop codon (star) allow the production and transportation of soluble antibody fragments to the periplasmic space. These antibody fragments can be purified by means of their His6 tag.

Most importantly, these characteristics also allow the construction of highly diverse antibody display libraries from the B-cell repertoire of immunized (Clackson et al., 1991) or non-immunized animals (de Haard et al., 1999; Marks et al., 1991). These libraries offer an *in vitro* selection system that can be used to obtain antibodies with predefined characteristics (Dolk et al., 2005a; Verheesen et al., 2003). The methodology of these libraries depends on the linkage of phenotype, the antibody, and genotype, the gene that codes

for the antibody. This linkage is accomplished by the immobilization or display of the antibody on a particle that contains the gene. Display methods using yeast (Boder and Wittrup, 1997; Schreuder et al., 1996), bacteria (Francisco et al., 1993), phage (Smith, 1985; Winter et al., 1994) and even ribosomes (Hanes and Pluckthun, 1997) have been developed. Historically, phage display is the most widely used of the display libraries. The most popular phage display methodology uses phagemid vectors, which are small vectors containing a phage packaging signal and a multiple cloning site that is used to clone the gene coding for the antibody in front of geneIII of the non-lytic filamentous phage fd or M13, which codes for the phage minor coat protein pIII (Figure 4). Expression of this construct in phage infected bacteria results in the display of the antibody fused to the pIII coat protein on the newly synthesized phage particle (Garrard et al., 1991; Hoogenboom et al., 1991). Large phage display libraries can be used to enrich for antibodies that bind a specific antigen. This is achieved by phage binding to the target antigen, washing to remove non-specific phage, followed by elution of the bound phage (Figure 5). Multiple rounds of selection can be performed sequentially.

The strength of phage display is that the selection protocols can be adapted to enrich antibodies suitable for a specific application, which significantly reduces the need for elaborate screening protocols, as is needed for hybridoma technology.

Furthermore, the use of phage display offers an additional advantage. Large parts of the selection and screening protocols can be robotized and provide the basis for high throughput selection of antibodies. This can reduce the production cost of an antibody significantly.

However, it should be noted that the successful selection of specific antibodies depends on the selection strategy used. As phage display drives the selection to clones that have advantages over other clones, the use of a sub-optimal selection strategy can result in selection of antibodies with unwanted characteristics, such as clones with high growth rates or clones that bind to a dominantly exposed epitope. It is therefore essential that much attention must be paid to the selection strategy used and to the analysis of the selection output after every cycle.

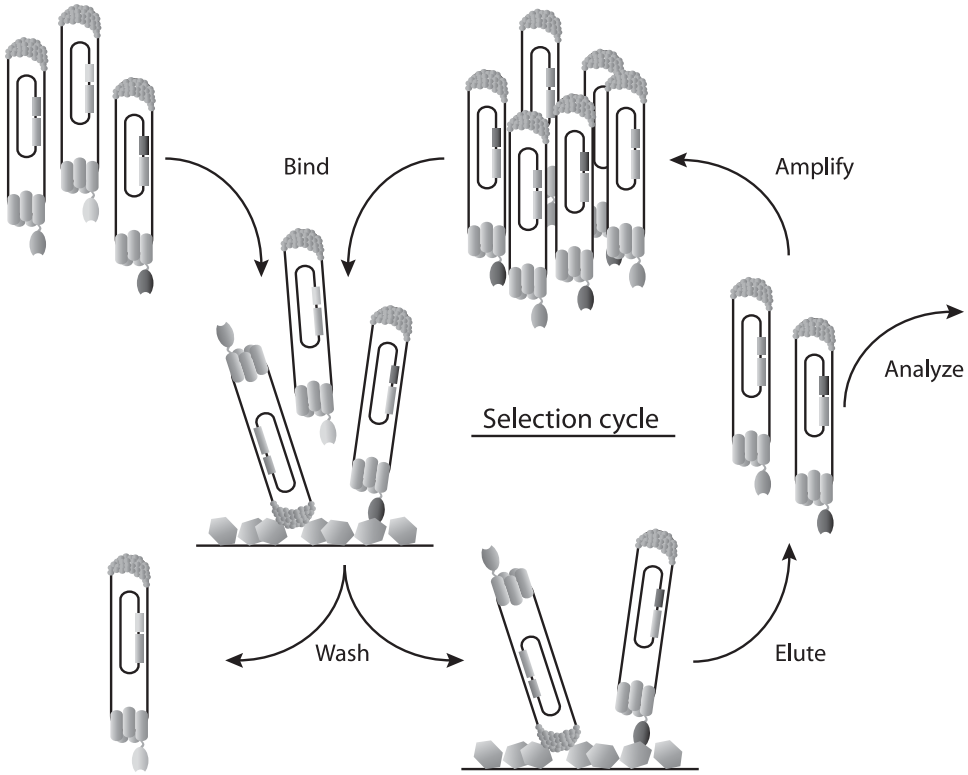

Figure 5: Schematic representation of the *in vitro* phage display selection cycle. Enrichment of antigen specific antibodies from large phage display libraries that can contain billions of different antibodies is accomplished by successive selection cycles. Each cycle consists of a binding step, where the phage display library is incubated with immobilized antigen, followed by a washing step to dispose of non-bound phage, and an elution step to obtain the bound phage. These phage can be amplified to start a new selection cycle, via infection of *E. coli* cells with the eluted phage and subsequent phage rescue using helperphage, or can be analyzed for antigen specificity and sensitivity in several different applications.

Antibodies derived from animals belonging to the species of *Camelidae* have additional advantages over other recombinant antibodies. Besides classical antibodies, these species possess antibodies that lack the light chain (Hamers-Casterman et al., 1993) (Figure 3). As a result, the antigen-binding domain (VHH) of these antibodies consists of only one domain, which offers several advantages over conventional recombinant antibodies. They represent the smallest antigen binding domains derived from antibodies (Muyldermans, 2001), and they are more stable than conventional antibodies or their

derivatives (Dolk et al., 2005b; van der Linden et al., 1999), making them extremely suitable for affinity chromatography (Verheesen et al., 2003). Furthermore, the single domain structure enables easy cloning to make highly diverse libraries and enables high production in *Escherichia coli* and *Saccharomyces cerevisiae*, which makes them economically attractive (Frenken et al., 2000).

5 Outline of this thesis

As brought forward, identification of protein changes that occur in time could provide new insights in the aging process. Moreover, it would enable the analysis of the effects of aging intervention studies and ultimately, it could be used to determine the biological over the chronological age of an individual.
The use of proteomic techniques could provide the identification of multiple changes that occur upon aging. Antibodies are an important tool in the development or improvement of proteomic techniques. As described above VHHs are antibody fragments with several advantages over classical antibodies and their derivatives. In this thesis, the use of these antibodies is explored in the development of new proteomics tools that can be used in the analysis of protein expression differences in aging-related biological samples.

One of the most important and most easy accessible human clinical biological samples is blood plasma, which is typically used for assessment of health status. Changes that occur during aging, such as increased cellular death and decreased tissue and organ functioning, should at least in part be reflected in the blood plasma protein composition. However, proteomic analysis is hampered by the presence of several highly abundant bulk proteins. In chapter 2, the selection of highly specific single domain Llama antibody fragments (VHH) for affinity chromatography purposes is described, which can be used to remove the highly abundant human plasma proteins, human serum albumin (HSA) and immunoglobulin G (IgG). This removal resulted in the visualization of previously masked protein spots and an increased resolution on 2D-gel. Intriguingly, these affinity ligands have superior characteristics compared to presently available affinity ligands, and use of these ligands can be expected for research as well as therapeutic purposes.
In chapter 3, the use of these affinity ligands, combined with two-dimensional difference gel electrophoresis (2D-DIGE), is shown in a human blood plasma proteomics study to reveal protein expression differences between young and old individuals. This revealed the importance of studying not only total protein expression levels, but also the different isoforms of single proteins. Furthermore, this approach demonstrated that upon aging a slightly increased pro-coagulant and pro-inflammatory state is induced. In addition, some protein expression differences indicated increased cellular damage upon aging,

manifested by an up-regulation of proteins involved in scavenging harmful cellular molecules.

The increase of oxidative stress observed during aging can damage various different cellular molecules. An organelle that seems especially vulnerable for oxidative stress is the ER. Increased oxidative stress might interfere with protein folding and lead to differences in expression of the protein involved in protein folding, the ER-resident proteins. These proteins contain a C-terminal signal sequence, which determines their ER localization. In chapter 4, the selection of VHHs specific for the C-terminal KDEL sequence present on several ER resident proteins is described that can be used to study protein expression patterns. These antibodies were used to analyze ER-resident protein expression differences upon different kinds of ER stress, to show their applicability. This clearly demonstrated the feasibility of selecting and using an antibody that recognizes a common amino acid sequence epitope for studying protein expression patterns.

Chapter 5 provides preliminary data on the expression differences of the membrane protein endoglin and several ER resident proteins found in a human umbilical vein endothelial cell (HUVEC) senescence model comparing young and senescent cells. The obtained results are used to discuss the challenges that are encountered when analyzing protein expression differences in this and other aging models.