Summary

Understanding the aging process

Research on both higher and lower eukaryotic model systems has revealed that numerous aspects are involved in the course of the aging process. During life, an organism 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 damaging factors that has received much attention in aging research is reactive oxygen species (ROS) that can have both an internal and external cellular origin. A cell contains many defence mechanisms that attempt to neutralize these damaging agents. However, when this level of defence is insufficient or fails and damage is induced, a cell contains additional molecular mechanisms that attempt to repair or degrade the damaged cellular molecules.

Despite these molecular defence mechanisms, damage accumulates in time, which can affect cellular functioning. There are unique cellular mechanisms that are influenced by the molecular changes that occur in time. When a certain threshold of damage is reached, a cell can die or go into a state of irreversible cell cycle arrest, called senescence. Ultimately, these cellular mechanisms can hamper the regenerative capacity of tissues, and could therefore have an effect on organismal functioning.

Although strides have been made to understand the role of the above described molecular and cellular processes in the aging process, a general consensus has not been reached on the relative importance of each of them. Mapping the changes that occur in the course of the aging process, and thereby define so called biomarkers of aging, could provide the information necessary to understand the aging process and the contributions of the different mechanisms herein. This approach was started with genomics that provides information about genetic expression differences, and is now extending to the field of proteomics that provides information about protein expression and modification differences, which is the focus of this thesis.

Antibodies in proteomics

The large interest in proteomics in the last few years 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. Affinity ligands, such as antibodies, are a useful tool to be applied

in this field, as they can be used for purification and detection of proteins. The successful application of antibodies in proteomics requires reliable affinity molecules in respect to sensitivity as well as specificity. The traditional method of obtaining antibodies is by immunization of an animal with the target antigen, followed by the isolation of the required antibodies from the blood of the immunized animal. This results in either polyclonal or monoclonal antibodies. A polyclonal antibody obtained from the serum of the immunized animal is a pool of different antibodies, each with their own specificity and sensitivity for the target antigen. Because of the heterogeneity of polyclonal antibodies, they are usually less specific than monoclonal antibodies and are therefore less suitable for use in proteomics. Monoclonal antibodies are obtained by isolation of the B-cell repertoire of an immunized animal and subsequent screening of this repertoire for cells that produce antibodies with the required characteristics. Fusion of the obtained B-cells with myeloma cells gives rise to an immortal hybridoma cell line, which results in an indefinite source of antibody of which each has the same specificity and sensitivity for the respective antigen. This makes monoclonal antibodies highly suitable for use in proteomics. Conversely, generation of these antibodies is relatively expensive.

Application of recombinant antibodies offers a less expensive alternative as they allow the construction of highly diverse antibody display libraries from the B-cell repertoire of immunized or non-immunized animals. These libraries offer an *in vitro* selection system that can be used to obtain antibodies with predefined characteristics. As this process can be fully automated, production costs of a monoclonal antibody can be reduced significantly.

Recombinant antibodies derived from animals belonging to the species of *Camelidae* have additional advantages over other recombinant antibodies. Besides classical antibodies that contain two light and two heavy chains, these species possess antibodies that lack the light chain. As a result, the antigen-binding domain of these antibodies (VHH) consists of only one domain, which makes them easy to clone. Furthermore, they represent the smallest antigen binding domains derived from antibodies, they are more stable than conventional antibodies or their derivatives, and they can be produced efficiently in *Escherichia coli* and *Saccharomyces cerevisiae*.

Depletion of bulk proteins from human plasma

Changes that occur during human aging, such as increased cellular death and decreased tissue and organ functioning, could be reflected in the blood plasma protein composition. However, proteomic analysis of blood plasma is hampered by the presence of several highly abundant bulk proteins. In chapter 2 of this thesis, the selection of highly specific VHHs against the abundant plasma proteins human serum albumin (HSA) and immunoglobulin G (IgG) is described for affinity chromatography purposes. These affinity ligands were used to remove these abundant proteins from plasma. This removal resulted in the visualization of previously masked protein spots and an increased visibility of previously non-detectable protein spots on 2D-gel. Furthermore, the potential clinical application of the VHH based anti-IgG affinity ligand was demonstrated in a study using plasma of Good Pasture (GP) and Systemic Lupus Erythematosus (SLE) auto-immune disease patients. In SLE patients, an auto-immune disease with a prevalence of IgG3 subtype specific antibodies, the VHH based anti-IgG column performed better than a proteinA based affinity column. It was demonstrated that this was caused by the IgG subclass specificity of proteinA. Protein A was not able to deplete IgG3 from plasma, whereas the VHH based anti-IgG affinity ligand was able to deplete all four IgG subclasses.

Changes in protein expression and processing in plasma during aging

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. 89 protein spots were considered as differentially expressed during aging of which 48 were positively identified with mass spectrometry. The differential expression of several of the identified proteins was in line with data described by others, and suggested that upon aging a slightly increased pro-coagulant and pro-inflammatory state is induced. Furthermore, remarkable differential expression of protein isoforms, originating from single gene products, were detected upon aging, which demonstrated the importance of studying levels of protein isoforms next to total protein expression levels.

Among the differences, the differential expression of several complement C3 (CO3) fragments was the most striking, with the highest difference in the old population (ten times up-regulation). These results suggested that upon aging this important regulator of both the innate and the adaptive immune response

is activated. From literature, there are conflicting data concerning the expression levels of CO3 during aging, which were all obtained with immunological assays. CO3 levels were either reported to be unchanged or slightly increased during aging. As these assays are not suitable for detecting differences in protein fragments or post-translational modified forms of CO3, this further demonstrates the importance of analyzing levels of protein isoforms, as important information might be missed by analyzing only total protein expression levels.

Application of common epitope specific antibodies
Another strategy for using antibodies in proteomics is selecting antibodies that recognize epitopes present in several different proteins, so called common epitopes. Such an epitope would allow the use of a single antibody to visualize or purify several different proteins simultaneously. Common epitopes comprise post-translational modifications, such as ubiquitination and phosphorylation, but also conserved amino acid sequences that are used for specific cellular functions, such as cellular localization.

In chapter 4, the successful selection is described of VHHs that recognize a common epitope present on several endoplasmic reticulum (ER)-resident proteins. This C-terminal KDEL amino acid sequence determines the ER localization of these proteins. The application of the KDEL specific VHHs for Western blot analysis of cellular lysates, resulted in a band pattern of eight different proteins.

In an ER stress model, the value of these VHHs was demonstrated. Upon induction of ER stress with hydrogen peroxide, tunicamycin or dithiothreitol, clear upregulation of two ER-resident proteins was revealed, while the other proteins showed no, or a slight decrease in expression.

The existence of several other biological mechanisms that utilize conserved epitopes for a specific function, such as the nulear localization sequence (NLS) or the peroxisomal targeting sequence, could offer a target for similar selection experiments. This could offer a straightforward procedure to simultaneously analyze multiple protein expression differences in a biological sample.

Differential protein expression in senescent endothelial cells

Endothelial cells (EC) have an important barrier function on the interface of the blood and the rest of the vasculature. Damaged ECs are either replaced by division of surrounding endothelial cells or by bone marrow-derived endothelial progenitor cells (EPC) that circulate in the blood. As many other cell types, ECs cannot divide indefinitely and eventually they will enter a state of irreversible growth arrest called senescence. Although these senescent cells are viable and metabolically active, they have altered gene expression, which is reflected by altered protein expression. As these changes suggest altered cellular functioning, accumulation of these cells at specific sites could lead to loss of endothelial function and consequently a disturbed barrier function. This could sensitize these sites for development of pathological processes, such as atherosclerosis. However, the significance of this phenomenon *in vivo* still remains a matter of debate. Mapping the differences that occur upon induction of senescence is needed to further unravel the mechanisms and consequences of EC senescence.

In chapter 5, the expression of several different proteins is analyzed upon induction of senescence based on two hypotheses. (i) During aging, an increase in oxidative stress is observed. As the ER seems especially vulnerable for oxidative stress and expression of ER-resident proteins has been shown to occur during such an event, changes in expression of these proteins can be expected. With one of the VHH anti-KDEL antibodies, the expression of several ER-resident proteins was analyzed. Intriguingly, clear changes were observed. However, these changes did not resemble the expression differences observed during severe ER stress in chapter 4. They do suggest that ER functionality changes upon induction of senescence.

(ii) As senescent cells lose the ability to replicate, the expression of proteins involved in the control of endothelial cell proliferation might change. Therefore, the expression of the membrane protein endoglin, which is needed for cell proliferation, was analyzed during induction of senescence. The expression was clearly decreased in the senescent population, although this decreased expression was only observed in part of the cells in the senescent population. This decreased expression could have an enormous impact on endothelial functioning, as endoglin regulates the expression of the fibrinolysis inhibitor plasminogen activator inhibitor type 1 (PAI-I) and the tight junction component claudin 5.

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These data clearly show that during replicative senescence changes occur at the protein level that could influence cellular and consequently tissue functionality, which could provide an environment for the development of age-associated diseases like atherosclerosis.

The future of affinity ligands in proteomics

Although differences in protein expression have been described in this thesis, these data do not represent the full nature of the differences that occur during aging. Most of the identified differentially expressed proteins in chapter 3 are members of the top 22 abundant proteins present in blood. Therefore, data on the differential expression of proteins that are less abundant are missing. In the proteomics field, this recognition has led to further development of affinity chromatography based techniques that reduce the complexity of proteomes and enable the analysis of low abundant proteins. Strategies are, the removal of high abundant proteins, the enrichment of minor abundant proteins or fractionation of protein samples based on charge, hydrophobicity, solubilization or size of the proteins. However, it should be noted that results obtained with all of these proteomic studies, should be verified with an additional technique, as observed differences can be caused by the variety of different steps that are applied during sample preparation. Furthermore, sample handling is another issue that should be treated with care as it has been shown that prolonged storage of samples may result in modification of some proteins, which can lead to misleading results. This further necessitates the confirmation of the obtained results in additional experiments with preferably fresh biological samples.

A proteomics technique that could enable the expression analysis of low abundant proteins without the need for high abundant protein depletion or sample fractionation, is the application of antibody micro-arrays. In antibody micro-arrays antibodies are immobilized on a surface, each antibody in a separate spot, to capture their respective antigen.

However, there are several obstacles that still have to be overcome. This approach requires the generation of numerous monoclonal antibodies that have to be tested extensively on antigen specificity and sensitivity, which makes it a far from straightforward approach. Furthermore, antibody activity has to be maintained after immobilization on the array surface.

Despite these challenges, the application of antibody micro-arrays offers great potentials for analyzing protein expression differences because of the high specificity and sensitivity that can be obtained. As it is unlikely that the first generation of antibody arrays will contain antibodies that recognize all the proteins from higher eukaryotic organisms, arrays should be constructed to study a predefined set of proteins in a biologically relevant setting. Arrays containing antibodies directed against some of the proteins described in chapter 3 and 5, could be exploited to develop an antibody micro-array for aging research. However, this would require the selection of antibodies that specifically recognize unique epitopes on a specific isoform of a single gene product, which is one of the challenges that affinity ligand based proteomics is confronted with.