Proteomics and aging

Studying the influence of aging on endothelial cells and human plasma

Proteomics en veroudering

Het bestuderen van de invloed van veroudering op endotheelcellen en humaan plasma

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 21 november 2007 des middags te 12.45 uur

door

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geboren op 9 maart 1974 te Zeist

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Printing of this thesis was financially supported by GE Healthcare (Amersham Biosciences) and NOTOX BV. The research described in this thesis was financially supported by a Technological co-operation program from Senter, an agency from the Dutch Ministry of Economic Affairs

ISBN
978-90-393-4701-0

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Printing
Gildeprint, Enschede

Contents

Abbreviations

ANOVA	Analysis of variance
B-Tf	Bovine transferrin
CVD	Cardiovascular diseases
DAPI	4',6-Diamidino-2-phenylindole
DTT	Dithiothreitol
EM	Electron microscopy
GSH	Glutathione
FPE	Fluid phase endocytosis
FTICRMS	Fourier transform ion cyclotron resonance mass spectrometry
HPLC	High performance liquid chromatography
HSA	Human serum albumin
H-Tf	Human transferrin
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
ICAT	Isotope-coded affinity tag
IgG	Immunoglobulin G
IL	Interleukin
LMNB	Lamin B
MM	Multiple myeloma
NO	Nitric oxide
PECAM	Platelet endothelial cell adhesion molecule
PMF	Peptide mass fingerprint
ROS	Reactive oxygen species
RS	Replicative senescence
SA-Beta-Gal	senescence-associated-ß-galactosidase activity
SILAC	Stable isotope labeling of amino acids in cell culture
SIPS	Stress-induced premature senescence
Tf	Transferrin
TfR	Transferrin receptor
TNFα	Tumor necrosis factor alpha
VHH	Variable heavy chain domain of a heavy chain antibody
VWF	von Willebrand factor
2DGE	Two-dimensional gel electrophoresis
2D-DIGE	Two-dimensional difference in gel electrophoresis

Chapter 1

General introduction

I Aging research

In general, human aging is considered one of the most complex and less-well understood processes in biology. It is known that the aging process is accompanied with a decline in functionality and that the lifespan of an organism is largely determined by its adaptive or repair capacity towards damage-causing agents. So far, aging research on humans embraces mainly descriptive and epidemiologic studies in which populations of humans are screened for a long period during their life [1-3]. Although this has resulted in valuable new knowledge, information about age-related mechanisms at the molecular scale is not provided in these studies. More specific and detailed knowledge is gained from aging research in less complex model organisms like, *Caenorhabditis elegans* [4-7], *Saccharomyces cerevisae* [8,9], *Drosophila melanogaster* [10] and laboratory mice [11]. These model systems have provided new knowledge about several mechanisms, which are supposed to be involved in the aging process [12]. In this thesis, we were interested in the influence of aging on the composition of blood and the vasculature, which is thought to be the basis for the development of cardiovascular diseases. In view of this, we will discuss in the next paragraphs age-related changes in the vasculature and blood circulation.

II Aging of the vasculature

Cardiovascular diseases (CVD) in relation to arterial aging.
Although aging clearly affects all organs in the human body, a part of this thesis is mainly focused on age-related changes in a specific part of the vessel wall. The vessel wall is a multi-layered tissue (see Figure 1) which is in general responsible for blood pressure adaptation and the transport of biomolecules from the blood stream to the underlying tissues.

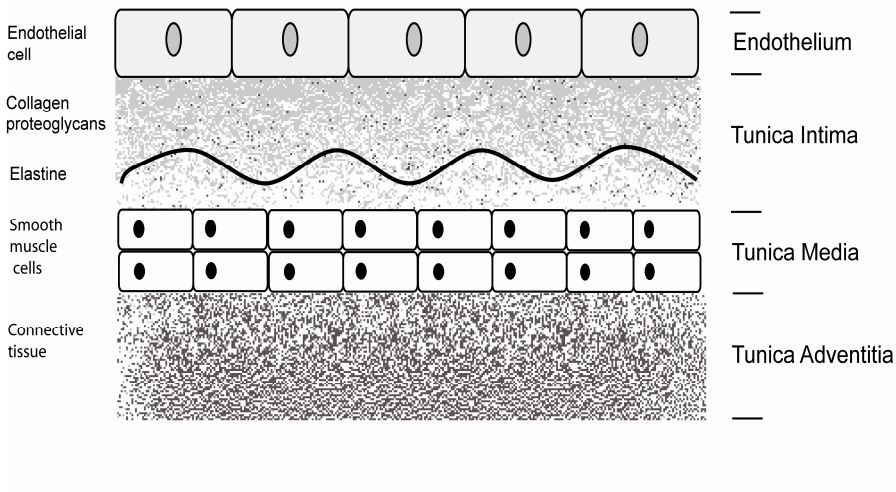

Figure 1. A simplified picture of the vessel wall. *Tunica intima:* The inner layer is covered by the endothelium, which is responsible for the regulation of vasorelaxation, angiogenesis and fluid dynamics. *Tunica media:* This part is formed by vascular smooth muscle cells and a layer of elastic fibers, which is positioned beneath the smooth muscle cells and separates the media from the tunica adventitia. *Tunica adventitia:* The tunica adventitia consists of connective tissue fibers and is tightly connected with the surrounding tissue, which makes the distinction between this layer and the underlying tissue more difficult.

Functional impairment of the vessel wall has major consequences for tissue functionality and will increase the vulnerability for (age-related) diseases. Cardiovascular diseases (CVD) are leading causes of death in industrialized countries and will become the number one disease in developing countries within the next ten years (WHO, 2005). Although thorough research has proven that classical parameters, like hypercholesterolemia or hypertension correlate well with the development of such diseases, it becomes more and more obvious that age is an important denominator as well. CVD's like hypertension and atherosclerosis are accompanied with arterial stiffness, thickened vessel walls, endothelial

dysfunctionality and reduced vasodilatation [13,14], which are determined in aged arteries as well [15,16]. Clearly, upon aging the arterial wall becomes less adaptive and in human as well as in animal models the arterial wall becomes thickened substantially towards aging [14,17]. Healthy males exhibited age-related intima thickening in for instance the carotid arteries [18,19] and even there are indications that vessel wall thickening actually even starts already in childhood [20]. Thickening of the intima is supposed to be induced by age-related changes in the elastin-collagen ratio [16] or crosslinking by advanced glycated endproducts (AGEs) [21,22]. An age-associated increase in thickness is accompanied also with an increase in arterial stiffness [23,24]. An important vessel wall function is vasodilation or vasorelaxation via nitric oxide (NO) signalling. This radical is produced by endothelial nitric oxide synthase (eNOS) and is secreted to the vascular smooth muscle cells. In these cells NO activates soluble guanylate cyclase (sGC), which results in relaxation of the vessel wall [25]. Although NO has not been determined directly in human, infusion experiments in humans with NO synthase inhibitors revealed that with advancing age less sensitivity towards these vasoconstrictors could be detected [26,27]. Next to this, an age-related decrease in NO bioavailability has been found in studies performed with animals [16,28]. One possibility of this decrease might be the formation of peroxynitrite, which is a result of a reaction between NO and superoxide anion [29,30].

It is obvious that, next to CVD specific changes such as atherosclerotic lesions, the pathology of arterial structure in these diseases exhibit a remarkable resemblance. Furthermore, subjection of old rabbits to a CVD-enhancing diet (cholesterol enriched diet) resulted in increased monoctye adhesion, more diffused intimal thickening and decreased eNOS expression compared to younger animals [31]. Therefore, it is convincible to state that aging will contribute significantly to the progression or even the start of a variety of cardiovascular diseases [16,32]. Typically, the age-related phenotypic alterations in the vasculature can be linked to changes in the abundance of vasodilators, vasoconstrictors and stimulators, like nitric oxide, angiotensin II and TGF-beta, which are predominantly produced by the endothelium [33,34]. This specific layer is responsible for vessel wall homeostasis and functions as a selective barrier for transport of bio-molecules from the blood to the underlying tissues. So proper vessel functionality is directly proportional to endothelial cell functionality and with respect to vascular aging, a part of this thesis is mainly focused on age-related processes in the endothelium.

III Aging of the endothelium

Endothelial senescence in vivo

Although endothelial cell turn-over under homeostatic conditions is considered as extremely low, it can be expected that with increasing age replacement of individual cells will take place. Furthermore, extensive studies in animal model systems have revealed that the rate of endothelial replication is increased at specific parts in the vasculature, for instance at branching points in arteries [35-37]. Also is the endothelium chronically exposed to a variety of stressors, like radicals from phagocytes, modified lipoproteins and even by reactive oxygen species (ROS) generated by the endothelial cells themselves (discussed later in this chapter). Altogether, these damage-inducing agents or processes might accelerate the process of replicative senescence (i.e. cellular aging) in the endothelium. Hayflick and co-workers [38] have stated cellular replicative senescence as the final state in which an irreversible loss of replicative capacity occurs in cell culture. Senescent endothelial cells in culture show distinct changes in morphology, like increased cell size, polymorphic nuclei, flattening and increase in lysosomes. A common feature of the senescent phenotype is the positive staining for senescence-associated beta- galactosidase activity (SA-beta-Gal) [39]. This activity at pH 6 is thought to reflect the increase in lysosomal body mass in senescent cells [40,41].

Some investigators have determined the presence of senescent endothelial cells in human atherosclerotic plaques by using SA-beta-Gal activity in combination with cell morphology characteristics [42,43]. Thus it has been shown that cellular senescence possibly plays an important role in the development of an age-related disease like atherosclerosis. This emphasizes the need for more knowledge about the role and effect of endothelial replicative senescence on the vessel wall.

Senescence affects vascular tissue function and integrity

Studies on other cell types have shown that senescent cells are capable of influencing neighboring tissue, thereby negatively influencing its functionality. Senescent fibroblasts are affecting epithelial cells by secretion of vascular endothelial growth factor [44,45] and senescent keratinocytes seem to increase the metalloprotease activity for adequate corneal wound healing [46]. Therefore, it is very likely that senescent endothelial cells will have an impact on endothelium and subsequently vessel wall functionality. The upregulation of ICAM in senescent

endothelial cells will increase the adhesion of leucocytes and monocytes to the endothelium [47], which is an interesting aspect, since migration of adherent monocytes to the sub-endothelial space is one of the initial events in atherosclerosis [48]. Senescent endothelial cells also produce regulators of the extracellular matrix like, fibronectin, collagen III and plasminogen activator inhibitor (PA-1) [33,34]. Upregulation of interleukins have been detected as well in senescent endothelial cells, implicating a possible role in inflammation control [49,50]. The earlier mentioned age-related reduction in NO bioavailability might also be caused by old or senescent endothelial cells since the NO production in these cells is strongly reduced [51,52]. Furthermore, endothelial cells of different passages or age react differently on stimuli or changes in the environment. *In vitro* experiments have shown that late-passage cells adapt differently to shear stress [53]. To fully asses the role of endothelial senescence in the vasculature and vascular disease development, more knowledge about the regulation of endothelial cell lifespan and mechanisms leading to senescence is required.

IV Senescence mechanisms

Replicative senescence (RS)

There are at least two common mechanisms that control the senescent growth arrest. In the first mechanism, senescence as a result of extended cell proliferation is often referred to as replicative senescence and is a consequence of progressive telomere erosion or telomere dysfunction [38,54]. Telomeres are specialized DNA-protein complexes located at both ends of eukaryotic chromosomes and consist of simple DNA repeat sequences. The major function of telomeres is the protection of the ends from degradation or chromosomal fusion [55] and without proper telomere functionality the genome integrity and stability becomes negatively influenced [56]. The majority of the telomere erosion in eukaryotes can be prevented by the ribonucleoprotein telomerase. This enzyme lengthens the terminal regions of telomeric DNA by addition of tandemly repeated sequences [57]. Obviously, the balance between this lengthening and shortening of telomeres determines the average length of the telomere. Overexpression of this enzyme tremendously increased the lifespan of somatic cells in culture [58,59]. However, the abundance of telomerase differs per cell, germline cells and cancer cells are capable of inducing telomerase activity whereas somatic tissue cells hardly have any telomerase [60]. This tight balance is not only strongly influenced by telomerase abundance and activity, but also by environmental [61], genetic and physiological factors. Although cell dependent-telomerase activity in combination with the

multiple telomere eroding factors complicates the use of telomere length as definite age biomarker, there is evidence that a decrease in telomere length strongly correlates with advancing age [62,63]. The open telomeres are suggested to serve as a signal to p53, which induces cyclin kinase inhibitor (CKI) $p21^{cip/waf1}$ to terminate cell division [64].

Stress induced premature senescence (SIPS)

Looking more closely to aging in general, it becomes obvious that the lifetime of an organism, tissue or cell largely depends on the accumulation of damage, due to inefficient repair mechanisms [12]. Important mediators in damage accumulation are Reactive Oxygen Species (ROS), which are free radicals derived from oxygen containing one or more unpaired electrons in their highest electron orbital (See Figure 2).

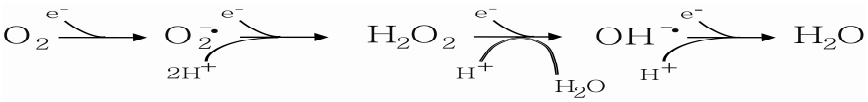

Figure 2. The formation of ROS, caused by sequential electron induced reduction of oxygen to water.

Although not being a radical itself, also hydrogen peroxide is considered to belong to the ROS family as it can access easily into cells or tissues where it is converted into the highly reactive hydroxyl radical in the presence of various transition metals, like iron [65,66] (See Figure 2). There are various exogenous as well as endogenous sources of ROS as depicted in Table 1 [67]. Typical external sources capable of damage induction are ultraviolet and ionizing radiation [68], pollutants, transition metal salts [69] and natural phenolic compounds present in food [70]. Major intracellular sources are the mitochondria, which produce ROS because of leakage of electrons out of the respiratory chain [54].

Endogenous	Exogenous
Mitochondrial respiration	UV radiation
Immune cells	Ionizing radiation
Peroxisomal β-oxidation	Transition metal salts
Prostaglandin synthesis	Pollutants
Cytochrome P450	

Table 1 Multiple endogenous and exogenous sources of ROS [67].

The biological character of ROS demands a cellular mechanism to maintain a proper ROS equilibrium, which is strongly cell dependent. Therefore, to control this delicate balance, an extensive anti-oxidant network is maintained which consists out of a variety of enzymes, like superoxide dismutase, catalase, glutathione peroxidases and thioredoxins as depicted in figure 3. Next to this, cells contain endogenous so-called anti-oxidant molecules, like urate, glutathion and thioredoxin, and use exogenous sources like antioxidants ß-carotene (precursor of vitamin A), α-tocopherol (vitamin E) and ascorbic acid (vitamin C), because they are able to regenerate the radical reducing capacity of the cell. It is obvious that an imbalance between ROS generation and ROS defense will eventually lead to oxidative damage to intracellular biomolecules and organelles.

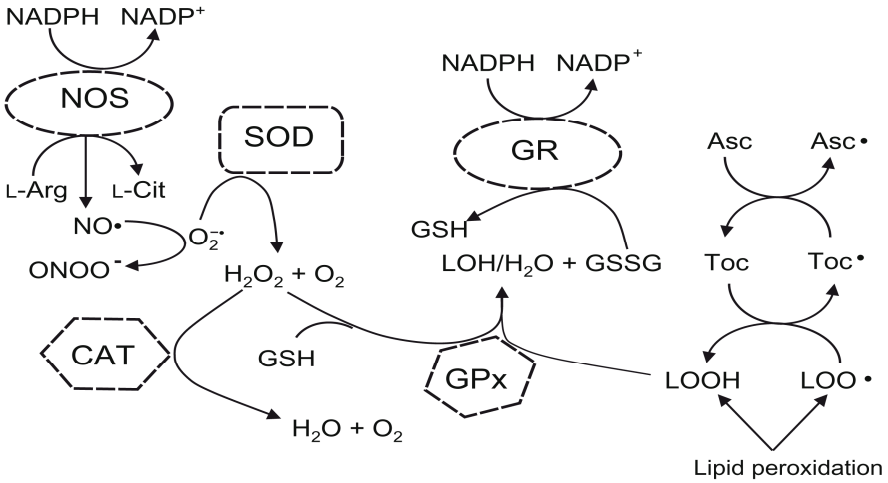

Figure 3. A schematic overview of the intracellular anti-oxidant system. This ROS defense system embraces the enzymes, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and catalase (CAT). The superoxide anion is converted to oxygen by superoxide dismutase (SOD), present in the cytosol and mitochondria. The produced hydrogen peroxide can be converted by catalase, abundant in the peroxisomes or glutathione peroxidase (GPx), present in the cytosol and mitochondria. A specific isoform of GPx is capable of reducing lipid peroxides (LOOH). The subsequently produced glutathione (GSSG) is reduced back to GSH by glutathione reductase (GR) at the expense of NADPH. Small anti-oxidants like ascorbate (Asc) and Tocopherol (Toc) serve as a redox sink. Additionally, in some cells (e.g. endothelial cells), the vasodilator NO is produced by nitric oxide synthase (NOS). Upon reaction with the superoxide anion the highly reactive peroxynitrite is formed, which is able to react with proteins. There are suggestions that nitrated proteins interfere with important signalling pathways.

Is there a relation between ROS and cellular senescence? Incubation of cells with hydrogen peroxide [71,72], high concentrations of oxygen [73] or *tert*-butyl hydrogen peroxide [74-76] induce premature senescence in human fibroblasts. Inhibition of proteins involved in the oxidative stress response or defense, like peroxiredoxins, also trigger a premature senescent state in mouse embryonic fibroblasts [77,78]. There is also evidence that ROS triggers the accumulation of p16, an inhibitor of CDK4 and CDK6. The increase of p16 is independent of telomere shortening and only high levels of this CDK inhibitor will induce the cell cycle arrest through the retinoblastoma (pRB) pathway [79]. Retinoblastoma will be activated, which blocks gene regulatory protein E2F and subsequently inhibits cell cycle progression [80,81]. Next to ROS, the p16/pRb pathway is thought to be induced also by overexpresssion of the oncogene RAS [82] and non-optimal tissue conditions [83,84]. However, telomeric DNA is also subjective to oxidative damage and telomere erosion is accelerated under elevated oxidative stress conditions [85,86], whereas proper maintenance is achieved under low stress conditions [87]. This suggests that telomere-driven replicative senescence might also be a result of oxidative stress [73]. Although the p53/p21 and the p16/Rb pathway regulate the senescence process in different ways, there is evidence that both pathways might interact with each other. Telomere-dependent replicative senescence is thought to induce the p53/p21 pathway [64], but others have found that extensive telomere uncapping also induce p16 in human fibroblasts [88]. In addition to this, upon hydrogen peroxide stress, not only p16 expression was observed in human fibroblasts [71], but also upregulation of p53 and p21. Although exact information about the link between these two pathways is still unknown, suggestions are made that some cells only need p53/p21 to reach full senescence, whereas other cell types need additional p16/pRb activation [89].

Senescence and protein turn-over

Reactive oxygen species are capable of modifying proteins to a certain extend, which can influence negatively protein functionality. Protein carbonylation is known to be mediated by metal-catalyzed oxidation [90] and increased levels of carbonylated or oxidized proteins have been detected in aged tissues [91]. In line with this, stress-induced premature senescent fibroblasts also showed accumulation of oxidized proteins [92,93]. Accumulation of carbonylated proteins has been detected also in aging yeast cells [94]. In order to maintain proper cell functionality adequate protein turnover or removal of damaged proteins is essential and cells have several mechanisms to do this.

In the regulation of protein turn-over and degradation of carbonylated e.g. oxidized proteins in the cell, the proteasome plays an important role. The proteasome is a multi-enzymatic complex present in the cytosol and nucleus of the cell [95-97]. Interestingly, towards senescence cells show a decline in proteasome activity which directly correlates with an increase in the amount of carbonylated proteins [92,98-100]. In mouse model systems it has been shown that during aging the activity of the mitochondrial Lon protease becomes negatively affected [101]. This would lead to an accumulation of oxidized proteins in the mitochondria, since this protease is responsible for removal of damaged proteins [102].

Another organelle involved in protein degradation is the lysosomal compartment. Lysosomes are specifically designed for degradation of extracellular biomolecules, e.g. proteins, but also cell organelles like mitochondria (auto-phagy) [103,104]. During the growth towards replicative senescence, the lysosome functionality becomes impaired due to various changes, like a decline in hydrolase activity, impaired regulation of lysosomal pH and at the same time there is an increase in lysosomal body mass [40]. As a result of this the lysosome cannot degrade all endocytosed or phagocytosed molecules or organelles, which results in the accumulation of lipofuscin. Lipofuscin is an undegradable intralysosmal polymeric material, primarily composed of oxidatively modified proteins, lipid degradation products as well as metals and carbohydrates [105]. The strong correlation between lipofuscin and aging as well as cellular senescence has assigned this lysosomal material as a hallmark of aging [92,93,106]. Although the effects of lipofuscin on cell functionality are hardly known, it has been shown that lipofuscin further diminishes the cellular autophagic capacity. There are suggestions that this will lead to an impaired turn-over of defective mitochondria, which will cause an elevated production of ROS [107]. Obviously, functional impairment of mechanisms like the proteasome and the lysosome will hamper the degradation of oxidized protein. This will result in an accumulation and aggregation of proteins [108], which has been indeed observed towards aging [109,110].

V Aging and blood circulation.

As discussed previously, aging of endothelial cells is involved in vascular aging and the development of the age-related disease atherosclerosis and for that reason, it is one of the focuses of this thesis. Moreover, emphasis will be placed also on the composition of blood plasma during aging, because alterations in plasma components might reflect cellular changes during the aging process and certain age-related alterations in plasma might be involved in age-related diseases.

In relation to the development of age-related atherosclerosis, the blood plasma is the provider of specific components, which play a role in the development of this disease. Disturbances in blood constituents, like lipoprotein balance or cytokine concentrations are considered as risk factors in the development of atherosclerosis [48]. The influence of aging on the initiation of atherosclerosis is illustrated by the results found by others in which accelerated oxidation in human plasma is favored upon aging [111,112], which in turn seem to correlate with early signs of atherosclerosis [113]. Other major constituents of the blood circulation are members of the immune system, which have an important role in the development of diseases like atherosclerosis [114,115] and Alzheimer [116]. In relation to this, it has been shown that age-related changes in these members of the immune system might accelerate or initiate atherosclerosis [3,117]. In conclusion, since blood plasma composition most likely reflects an individual health status in combination with the easy accessibility of this body fluid, it is an excellent source to look for possible age-related changes, which might play a role in the development of a disease.

VI Proteomics.

Actually, to fully understand a complex mechanism like aging or cellular senescence a multidisciplinary approach has to be performed, in which the genome, the proteome and the metabolome are analyzed for the presence of age-related changes. As a part of such a multidisciplinary approach we have analyzed blood plasma and endothelial cells on age-related differences in protein composition. In general, biological processes are considered as extremely complex and the genomics and the transcriptomics research fields have provided new knowledge and tools to gain more fundamental understanding of biological processes in general. Logically, this has increased the interest in proteins as a group of biomolecules, which are actually responsible for many processes in

biological life. The total collection of proteins resulting from genome transcription is referred to as the proteome and the dynamic nature of the proteome is considered to be indicative for the functional status of a cell and finally the organism.

In parallel with genomics, proteomics aims for a full systematic analysis of the total proteome under specified biological conditions, for instance in the development of cancer or during aging. Deciphering protein expression in a particular (e.g. functional) context is one of the most challenging tasks today. Although the human genome is fully sequenced, only a part of it can be linked to proteins with a known function, whereas the other part contains information with unknown functionality. Moreover, the annotated part does not encode for protein isoforms and post-translational modifications, which often strongly influence the biological function of a protein. Also challenging is the huge dynamic range of protein expression in cells or tissues. Analyzing a whole proteome at once demands a technology, which should be able to cover a dynamic range of 10^6 for cells [118] and 10^9 in case of human serum [119]. Therefore, proper proteome analysis requires specific enrichment procedures, which enable the visualization and characterization of protein regulation pathways in a biological context.

Two-dimensional electrophoresis (2DGE)

Since the introduction in 1975 by O'farrell [120] two dimensional gel electrophoresis (2DGE) is widely considered as the technology of preference to separate complex protein mixtures. In 2DGE, proteins are separated on the basis of two physical-chemical properties, the pI and the molecular weight. In the first dimension, isoelectric focusing is performed in immobilised pH gradient strips (IPG). These small strips typically consist of a plastic strip covered with a low percentage of crosslinked poly-acrylamide gel in which a pH gradient is created. Upon application of an electrical field between the ends of the strip, proteins migrate until their net charge is zero, which is at the pI of the protein. In the second, orthogonal dimension, the focused proteins are subjected to an SDS-PAGE gel, where proteins are unfolded and equally negatively charged due to the presence of SDS. Under influence of an electrical field, the negatively charged proteins migrate to the positively charged anode. The migration distance is in relation to the logarithm of the molecular weight of the protein, as depicted in figure 4. This technology enables the simultaneous separation of hundreds of proteins and provides the highest resolution of all protein separation technologies available.

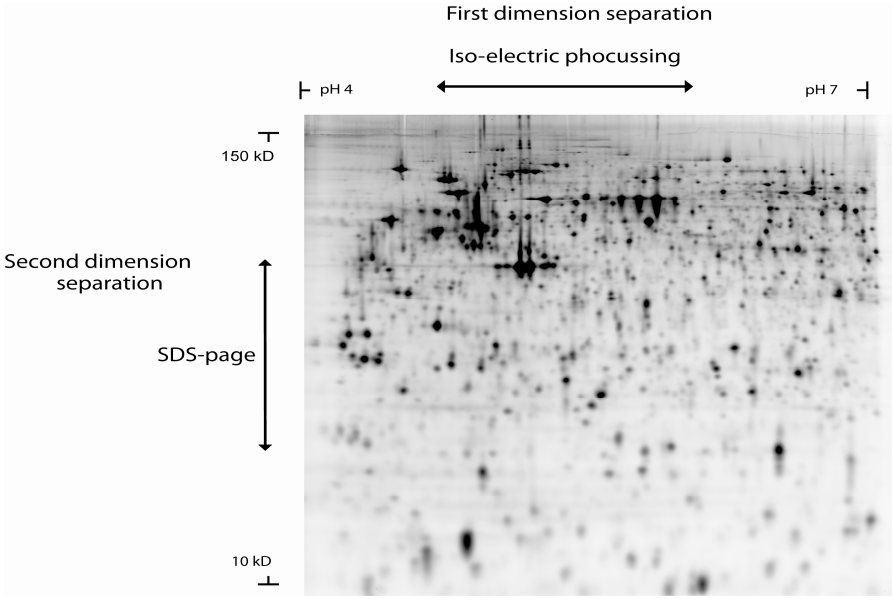

Figure 4. Typical two-dimensional gel, in which proteins are separated according to their pI and molecular weight.

Proper differential protein expression analysis requires adequate visualization of the protein spots in the 2D gel. Today a variety of post-electrophoretic staining methodologies are available, like Coomassie (CBB) staining, silver staining or staining by several fluorescent dyes. Coomassie staining is used frequently, because it is an easy-to-use endpoint staining method (i.e. the amount of protein is directly proportional to the CBB color intensity). However, a major drawback of CBB staining is the low sensitivity (200-500 ng protein/spot) [118]. Therefore silver staining is widely used as well. In silver staining, silver ions are reduced to silver with a strong reducing agent, like glutaraldehyde or formaldehyde, which results in black colored protein spots [121]. This method is not an endpoint staining like CBB, but is much more sensitive since it is able to detect abundant proteins in the femtomole range (0.1 ng protein/spot) [118,122]. However, a major drawback of silver staining is the low dynamic range, which is at maximum two orders of magnitude. Visualizing low abundant proteins requires prolonged staining which causes extensive over-staining of high and medium abundant proteins. Besides, extensive exposure of proteins to reducing agents, like formaldehyde will lead to protein cross-linking, which will frustrate proper protein identification with mass spectrometry [123]. Next to this, the staining procedure is a multistep process, which makes adequate reproducible silver images rather laborious and difficult. All

together proper image analysis is highly frustrated when using post-staining methods, like silver and CBB.

The limitations of the silver and CBB staining procedures can partially be overcome by the application of ruthenium-based fluorescent dyes such as Sypro Ruby [124,125]. The typical linearity of at least of three orders of magnitude with these types of dyes [126] together with the non-covalent nature of the interaction with a protein make these dyes extremely suitable for 2DGE analysis and protein spot identification with mass spectrometry [127-129]. Subsequently, other fluorescent dyes have been developed, like Deep purple (Amersham Biosciences) or Lightning Fast, which are based on a natural fluorophore extracted from a fungus [130]. These stains have similar sensitivity and linearity as ruthenium-based fluorophores [131]. Furthermore, by use of specific fluorescent stains it is possible to visualize phosphorylated and glycosylated proteins [132-134]. Clearly, the introduction of fluorescent stains has improved the detection linearity and reproducibility of 2DGE analysis. Despite great efforts and various innovations such as the fluorescent post-staining, the 2DGE technology is difficult to automate and highly laborious, which has a negative influence on the reproducibility. Moreover, protein quantification based on spot intensities and consequently, the analysis of up and downregulation of particular proteins under different conditions (i.e. differential proteomics) is difficult. Small differences in gel homogeneity, protein streaking or smearing and air-bubbles are just some examples, which can complicate proper image analysis. Therefore, multiple spot detection and matching as well as difference in-gel electrophoresis have been developed to cope with this problem, resulting in new dedicated hardware and a variety of software packages [135].

Difference In-Gel electrophoresis

Analyzing and quantifying proteome alterations successfully demands the implementation of analytical as well as biological replicates [136]. To be able to meet the statistical criteria in combination with the superior protein resolution of 2DGE, Unlu and co-workers have developed a method called Difference In-Gel Electrophoresis (DIGE) [137]. In this method, proteins are labeled with fluorescent labels prior to the first dimension separation. The fluorescent cyanine dyes, adapted from DNA array technology, are chemically attached to the ε-amino group of lysine via NHS esterification. Upon this labeling the initial pI of the protein will not change, since the fluorophore will replace the positive charge of the lysine side chain by its own positive charge. Upon labeling a mass of 450 Da will be added to the protein mass, which will hardly influence the separation characteristics, since only 3-4% of the protein will be labeled [138]. Initially, this system used two

spectrally different, but physically similar CyDye labels, Cy3 and Cy5 respectively. Due to their equal match in mass and pI, simultaneous analysis of two samples in a single gel was allowed. More recently, a third spectrally different cyanin dye has been introduced, which allows simultaneous analysis of three samples in one gel, as shown in figure 5. Individual images of each sample are obtained by detection of each fluorophore at its specific excitation-emission wavelength. Due to the similarity in mass and charge of each dye, the migration position of each sample is the same. This enables direct comparison and provides protein abundance differences between the three samples of interest.

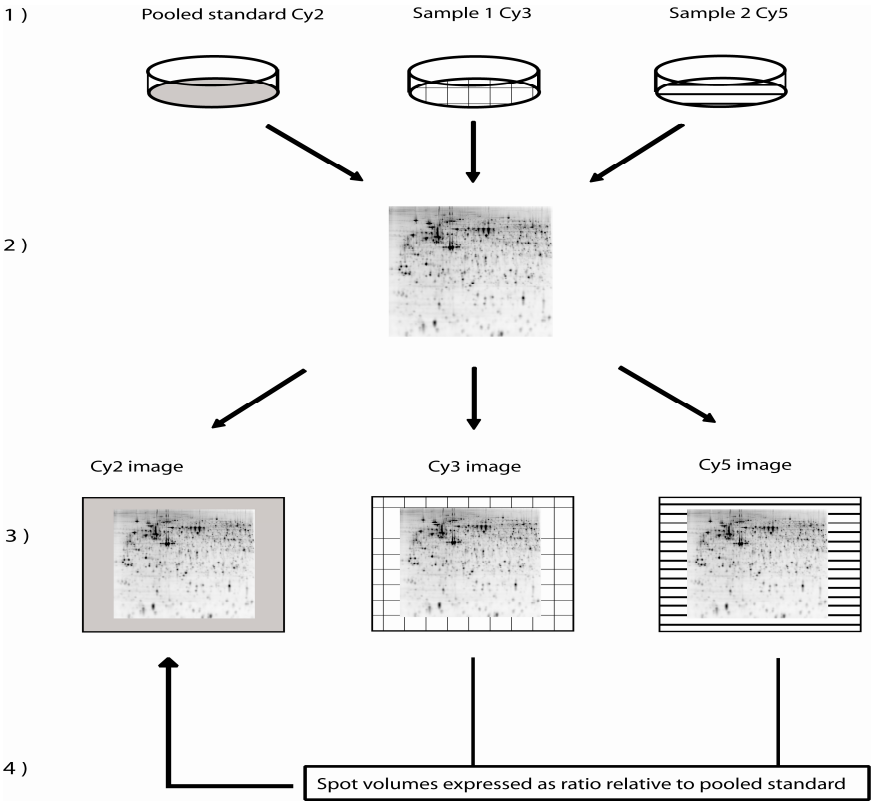

Figure 5. Workflow of the DIGE procedure. 1) Samples are labeled separately with Cy3, Cy5. The pooled standard, consists out of equal protein amounts of each sample included in the experiment. 2) All three labeled protein samples are mixed and subjected to the first and second dimension separation. Gel is scanned with Dye specific excitation and emission wavelength resulting in three images. 3) In each image spot detection and normalization is performed subsequently followed by 4) normalization relatively to the pooled standard.

Typically, the third dye (Cy2) is used to label a pooled standard, which consists out of equal amounts of all the samples included in the experiment. This internal standard is incorporated in all gels of interest and is used for normalization of expression data between gels thereby minimizing experimental variation, which is one of the major bottlenecks in 2DGE [139]. Summarizing, by the combination of three spectrally different protein dyes and the pooled standard principle, the number of gels can be substantially decreased. Furthermore, proper statistical analysis is possible due to the reduction of experimental variation (See figure 5).

In many cases, the amount of protein sample available is limited, which makes proper DIGE analysis difficult. To obtain maximum sensitivity with the DIGE fluorophores, saturation labeling was introduced by Amersham Biosciences [140]. This methodology involves labeling of all cystein residues present in the protein and maximize the fluorescent intensity [141]. A disadvantage is that only proteins are labeled which contain cystein residues.

Mass spectrometry based identification

Differential protein expression analysis will result in a subset of up-or downregulated protein spots. Usually these protein spots are excised and subjected to proteolytic digestion, before identification. Typically, trypsin is used for its highly reproducible proteolytic cleavage of the peptide bond C-terminal after lysine or arginine residues. The resulting peptide mixtures are sequenced with mass spectrometry, which will eventually lead to the identification of the protein spot of interest [142,143]. Prior to sequencing with the mass spectrometer ionization of the peptides is a prerequisite. In proteomics research two ionization techniques are frequently used, matrix assisted laser desorption ionization (MALDI) and electrospray –ionisation (ESI). Both techniques are suitable for the ionisation of biomolecules such as proteins and peptides.

Matrix Assisted Laser Desorption Ionisation (MALDI)

In MALDI, peptides are mixed with an aromatic acidic compound (matrix) and spotted onto a target plate [144]. During drying the peptides become enclosed in matrix crystals. A variety of matrices is available and the type of biomolecule to be analyzed determines the choice of matrix. Frequently used matrices in proteomics research are α-cyano-4- hydroycinnamic acid, 2,5-dihydroxybenzoic acid and sinapinic acid. Ionization energy is provided by the laser, which will be absorbed by the aromatic matrix molecules. Through energy transfer from the matrix molecules to the peptides, gas-phase peptide-ions will be generated. This ionization technique is used in combination with a time-of flight (TOF) mass analyzer [145]. In

TOF instruments, ions are accelerated with the same kinetic energy (Uk) into a flight tube and the time that the accelerated ions need to reach the detector is measured. Due to the equal kinetic energy of the individual ions, the velocity is inversely proportional to the square root of their masses.

Electrospray Ionisation (ESI)
ESI involves analyte ionisation directly from a solution and was originally developed by Fenn and co-workers [146]. In ESI, liquid is sprayed from a small capillary needle by applying a voltage of 1-5kV. This results in a fine spray of charged droplets, due to solvent evaporation the droplet size will reduce further in time. The decrease in droplet size results in an increased charge density at the droplet's surface. When coulombic repulsion overcomes the surface tension of the liquid, droplet fission will occur and smaller droplets are formed. Finally, gas-phase ions are formed via the charge residue model in which small droplets contain only one molecule, or through emission of the analyte from the droplet surface, by the ion evaporation model [147]. ESI can be performed in the positive as well as the negative ionisation mode, which enables the ionisation of a wide variety of (bio)molecules. Preferably, peptides are ionized in the positive ion-mode where the charge is located mostly on the N-terminus of a peptide bond or the amino side chain of histidine, arginine or lysine. Also generation of multiply charged peptides occurs in ESI and allows the identification of large bio-molecules. In proteome research, ESI is widely applied in its miniaturized "nanospray" format [148]. Typically, smaller capillary spray tips are used, which increases the spray efficiency and consequently the sensitivity. The ability of ionizing analytes directly out of a liquid makes ESI highly suitable for application in directly coupled LC-MS systems [149]. Nowadays, ESI is a versatile probe and is routinely coupled to a variety of mass analyzers like quadrupole TOF, ion trap and quadrupole ion trap instruments [150,151].

Identification of proteins

Peptide mass fingerprint.
Analyzing a trypsin-digested protein with MALDI-TOF MS results in a m/z pattern of accurate monoisotopic masses, which are representative for the molecular weight of the present tryptic peptides. These subsets of masses are protein-specific and referred to as a Peptide Mass Fingerprint (PMF). This technique is highly suitable for identification of relatively simple peptide mixtures containing one or two proteins. After retrieving a peptide mass fingerprint or after sequencing of a subset of peptides (discussed in next paragraph), the information obtained need to be

searched against a protein database (e.g. Swissprot, IPI) in order to determine the identity of the protein. This is done by using particular search algorithms [152], which compare the individual peptide masses and their relative intensities of a PMF with a database containing theoretical peptide masses. The success of the identification strongly depends on the peptide tolerance of the search. Next to this, information like digestion enzyme, Cys modification (carbamidomethylation) and additional modifications e.g. oxidation of methionine will increase the reliability of the protein identification. Typically, a sequence coverage of 10-20% is sufficient for a confident protein identification.

Peptide sequencing

Although a peptide mass fingerprint is unique for a protein, it is possible that no proper protein identification can be found e.g. in case of impurities or the abundance of multiple proteins in a peptide mixture. Another option is then to apply tandem mass spectrometry for peptide sequence analysis. Amino acid sequence information is gained via collision-induced dissociation, in which a selected peptide ion collides with an inert gas, like argon. As a result of this the collision energy is absorbed within the peptide and causes fragmentation of internal peptide bonds. The peptide fragmentation products can be divided in two main classes: fragments, which retain the charge on the N-terminal side and those who retain their charge on the C-terminal side. The mass differences found between the generated fragment ions of these two classes represent the residue masses of an amino acid. Figure 6 illustrates the specific fragments obtained after collision induced fragmentation [153,154].

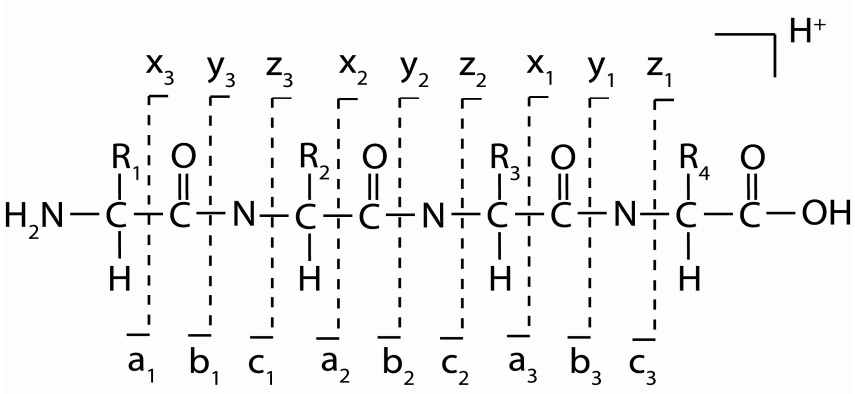

Figure 6. Nomenclature of peptide fragmentation according to Roepstorff and Fohlman [154]. Picture kindly provided by Dr. E.H.C. Dirksen.

The results of database searching with peptide sequencing derived data are analogous to those of the PMF procedure with one exception. In the former case the peptide sequence is partially resolved, which increases the confidence of the proteins identification tremendously. Furthermore, the occurrence of peptide sequence information enables the detection of specific oxidation, nitration or phosphorylation sites more easily. Additionally, the sequence coverage is less important, because a partial amino acid sequence of ≥ 20 residues is usually sufficient to uniquely identify a protein.

VII Outline of this thesis

The scope of this thesis is to gain more knowledge about aging on cellular and organismal level. Although all kind of biomolecules are involved in the aging process, this thesis describes mainly the involvement of the proteome during the aging process. Cultured Human Umbilical Vein Endothelial Cells (HUVECs) were cultured to replicative senescence (i.e. cellular aging) since this process is supposed to be involved in the progression of the age-related disease atherosclerosis. HUVECs from three independently isolated umbilical cords were subjected to Differential In-Gel Electrophoresis (DIGE). The results of this experiment are described in ***Chapter 2***, which illustrates that DIGE enables accurate quantitation of protein expression levels in biological replicates.

Results from chapter 2 revealed amongst others, an interesting increase of bovine transferrin in senescent HUVECs. This protein is responsible for iron transport from the blood plasma into the endothelial cells and is essential for proper cell functioning. However, iron can also be involved in reactive oxygen species (ROS) generation, which contributes to intracellular oxidative stress formation and, subsequently, in the progression of the senescent process. The results, shown in ***Chapter 3***, revealed age-related changes in the transferrin mediated iron transport, which will provide new knowledge about this mechanism during the aging process of endothelial cell systems.

The other focus of this thesis was to search for aging biomarkers in blood plasma, which is one of the most important and accessible human biological fluids. Plasma samples of individuals from three different healthy age populations were depleted from serum albumin and immunoglobulin G. Thereafter extracts were screened with DIGE on the presence of age-related biomarkers. The results are described in

Chapter 4, which demonstrates that interesting processes occur during healthy aging.

The dataset obtained from Chapter 4 showed relevant expression changes between healthy young and old individuals. Next to this, we were interested also in markers which enables the distinction between healthy and unhealthy aging. Therefore, we have conducted in ***Chapter 5*** a proteomics experiment in which we determine protein abundance differences between healthy and unhealthy aging. Clearly, some proteins involved in healthy aging revealed also remarkable differences, between healthy and unhealthy aging. In ***Chapter 6*** we have summarized the results as described in this thesis and discussed the future prospects of proteomics research in the aging research field.

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Chapter 2

Protein expression dynamics during replicative senescence of endothelial cells studied by two-dimensional difference in-gel electrophoresis

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Based on: Electrophoresis, 2006, 27 (8), 1669-1682

Abstract

Endothelial senescence contributes to endothelium dysfunctionality and is thereby linked to vascular aging. A dynamic proteomic study on Human Umbilical Vein Endothelial Cells, isolated from three umbilical cords, was performed. The cells were cultured towards replicative senescence and whole cell lysates were subjected to two-dimensional Difference Gel Electrophoresis (DIGE). Despite the biological variability of the three independent isolations, a set of proteins was found that showed senescence dependent expression patterns in all isolations. We focussed on those proteins that showed significant changes, with a paired analysis of variance (RM-ANOVA) p-value ≤ 0.05. Thirty-five proteins could be identified with LC-FTMS and functional annotation revealed that endothelial replicative senescence is accompanied with increased cellular stress, protein biosynthesis and reduction in DNA repair and maintenance. Nuclear integrity becomes affected and cytoskeletal structure is changed as well. Such important changes in the cell infrastructure might accelerate endothelium dysfunctionality. This study provides biological information which will initiate studies to further unravel endothelial senescence and gain more knowledge about the consequences of this process in the *in vivo* situation.

Introduction

Aging of the vasculature is accompanied by a series of structural and functional changes. The vascular wall becomes less adaptive and exhibits enhanced reactivity towards mechanical and chronic stimuli [1].This decline in functionality leads to an increased vulnerability for age related diseases such as atherosclerosis [2]. Many of the age-related alterations in the vascular wall are correlated with endothelium dysfunctionality. The endothelium is formed by a monolayer of endothelial cells that covers the interior of the vascular wall and the role of the endothelium in aging is illustrated by the age-associated changes in vascular tone regulation, which are caused by an alteration in activity of endothelial-derived NO synthase (eNOS) [3].

Under normal conditions endothelial cells divide with a turn-over number of approximately 3 years. Proliferation is initiated by endothelial injury, wound healing and angiogenesis [3]. However, endothelial cells have a limited ability to proliferate and the cells enter a state of irreversible growth arrest, known as replicative senescence [4,5]. Cells in this condition are metabolically active, have altered gene expression and are unable to divide after addition of growth factors, which distinguish them from quiescent cells [6].The senescent endothelial cell may partially be responsible for impaired vessel wall relaxation, since eNOS activity is reduced in senescent human endothelial cells [7]. Senescence of vascular endothelial cells is not an artifact of continued cell culturing, since senescent endothelial cells have also been observed in *in vivo* situations, namely in atherosclerotic plaques [8-12]. Since cellular senescence has been proposed as an underlying cause of aging in general, research has been done with several tissue culture systems, especially human fibroblasts [13,14]. These studies revealed that senescence can be triggered or accelerated by various stresses like telomere shortening, DNA damage, oxidative stress, glycation, or overexpression of certain oncogenes [12,15-21]. It is known that the effect of senescence on cell function and the surrounding tissues is strongly cell type dependent [22,23]. To fully understand the influence of senescence on endothelium dysfunction and its role in vascular aging it is therefore essential to unravel the senescence process specifically in endothelial cell systems.

Proteomics has opened the possibility to gain more fundamental knowledge of biological processes by exploring expression profiles globally and provides the possibility to detect new biological pathways. Two-dimensional gel electrophoresis is an important proteomic tool with an impressive spot resolution, which enables the visualization of thousands of proteins. Accordingly it is the most frequently used method despite some well documented disadvantages towards membrane proteins, proteins with extreme pI and gel-to-gel reproducibility [1,24]. Recently, advanced 2D-gel technology (2DGE) has become available which makes it possible to detect small expression differences with high confidence. Two-dimensional Difference In-Gel Electrophoresis (2D-DIGE) reduces the amount of experimental variation due to a combination of multiple sample analysis in a single gel and internal standard correction [25-27].

Despite the importance of proteomics, there is only one proteomics study available that focuses on unraveling the relationship between endothelium dysfunction and senescence. That study showed a small subset of eight proteins differentially expressed when comparing young and senescent endothelial cells using conventional 2D analysis [28]. Applying the 2D-DIGE technique more information might be obtained when analyzing the endothelial proteome during growth towards senescence at different moments in time. In view of this, we explored the growth towards senescence of human umbilical vein endothelial cells (HUVECs) at three different moments in time with 2D-DIGE . To ensure the biological relevance of the observed trends in protein expression and to exclude genetic variations, cell cultures from three different umbilical cords were used. This has led to a subset of thirty-five proteins showing statistically significant expression profiles with regard to replicative senescence.

Material and methods

HUVEC isolation and culturing

Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from umbilical veins according to the method of Jaffe [29]. Culturing of HUVECs was performed in fibronectin-coated culture flasks in EGM-2 (Cambrex, New Jersey, USA) at 37 ^{0}C in 95% rel. humidity and 5% CO_2. Medium was changed every two or three days and cells were passaged at 90% confluency. Cells were counted at the moment of seeding and passaging, to allow calculation of Population Doubling (PD= (ln[number of cells seeded]-ln[number of cells harvested])/ln2) after each passage. When the cells ceased proliferation they were kept in culture for two weeks with continued medium replenishment. In addition, senescence-associated-ß-galactosidase activity (SA-beta-Gal) (Cell Signalling Technology, Beverly, MA, USA) at each passage was detected to determine the presence of senescent cells, together with microscopical inspection of cell morphology.

Protein extraction

HUVECs were scraped in buffer containing 7M urea, 2M thiourea, 4% CHAPS, Bromophenol Blue and Complete^{TM} protease inhibitors (tablet) (Roche, Mannheim, Germany) at 4 ^{0}C and subjected to two freeze/thaw cycles in liquid nitrogen to ensure full cell lysis. Thereafter extracts were centrifuged for 30 min at 14,000 g at 4 ^{0}C to remove cell debris. Supernatants were stored at – 80 ^{0}C until further use. Prior to labeling the protein concentration was determined with the 2D Quant kit (Amersham Biosciences, Uppsala, Sweden). The pooled standard was prepared according to the instructions of the manufacturer (Amersham Biosciences). Briefly, from each protein extract 50 µg of protein was taken and pooled.

Protein labeling

In order to obtain the optimal reaction conditions, 50 µg of each protein sample was purified with the Clean-Up kit (Amersham Biosciences) and transferred in 15 µl lysis buffer (30 mM Tris-HCl, 7 M urea, 4% CHAPS, pH 8.5). Cy5, Cy3 and Cy2 labelling was performed according to the manufacturer (Amersham Biosciences). The labelled samples and the pooled standard were mixed and diluted up to 70 µl with buffer (7 M urea, 2 M thiourea, 4% CHAPS, Bromophenol Blue, 2.8 % v/v IPG buffer pH 4-7 (Amersham Bioscience), 2.8% DTT).

Two Dimensional gel electrophoresis

Immobiline DryStrips pH 4-7 of 24 cm (Amersham Biosciences) were rehydrated overnight with 450 µl rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, Bromophenol Blue, 0.5 % v/v IPG buffer pH 4-7, 10 mM DeStreak (Amersham Biosciences)) and overlaid with 2 ml Coverfluid in an Immobiline Drystrip reswelling tray (Amersham Biosciences). First dimension separation was performed with an IPGphor system (Amersham Biosciences). Anodic cuploading was used to load the protein samples and optimal protein transfer into the IPG strip was achieved by application of 300 V for 4 hrs. Subsequently, the voltage was increased to 1000V within 6 hrs, followed by a gradual increase to 8000 V within 6 hrs. Thereafter, a constant voltage of 8000 V was applied until a total of 48 kV. After focusing, the strips were equilibrated in equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS), first in 65 mM DTT and subsequently in 135 mM iodoacetamide. The second dimension was performed on lab-cast 12% polyacrylamide gels (BioRad, Hercules, CA, USA). IEF strips were loaded on the 12% SDS-PAGE gel and sealed with a solution of 1% (w/v) agarose containing a trace of Bromophenol Blue. The gels were run on the Ettan DALTtwelve system (Amersham Biosciences) at 1W/gel overnight, followed by 10W/gel both at 20^0C until the Bromophenol Blue dye front reached the bottom of the gel.

Gel imaging

All gels were scanned in between low fluorescent glass plates at 100 µm pixel resolution with the Typhoon Image scanner 9400 (Amersham Biosciences). The Cy5 images were scanned using a 633 nm laser and a 670nm-BP30 emission filter. Cy3 images were scanned with a 532 nm laser in combination a 580 nm BP 30 emission filter. Cy2 images were scanned using a 488 nm laser and an emission filter of 520 nm BP40. The photomultiplier tube was set to achieve maximum sensitivity without pixel saturation. Silver-stained images were analysed with a GS-710 Calibrated Imaging Densitometer (BioRad, Hercules)

Differential analysis

Prior to differential analysis, images were cropped with ImageQuant (Amersham Biosciences) to remove insignificant parts of the gel. Subsequently, images were subjected to median filtering in Image Quant Tools (Amersham Biosciences) to remove dust-related pixels. Differential analysis was performed with the Decyder V 5.01 (Amersham Biosciences) software package. Paired (RM)ANOVA was applied to extract relevant expression profiles.

Spot picking and in-gel tryptic digestion

Spot picking was performed with post-stained silver images of CyDye labeled gels to minimize the risk of mismatched spot picking. Silver staining was performed according to literature [30]. For improved sequence coverage, preparative gels were made and stained with SyproRuby according to the manual (BioRad). These preparative gels were matched visually to the CyDye gels. Spots of interest were subjected to in-gel tryptic digestion as described previously [30].

Nano LC-MS/MS and protein identification

Nano-LC-MS/MS was performed with an Agilent 1100 series LC system (Agilent, Palo Alto, USA) coupled with a Thermo Finnigan LTQ-FTICRMS (Thermo electron Company, Waltham, MA, USA) as described previously in literature [30]. Briefly, peptide extracts were acidified with 0.1 M acetic acid and injected on a trap column (Aqua^{TM} C18 RP (Phenomenex, Torrance, USA), 20 mm x 100 µm ID) at 5 µl/min. Subsequently, the peptides were transferred with a split-reduced flow rate of 150 nl/min on the analytical column (Aqua^{TM} C18 RP (Phenomenex), 20 cm x 50 µm ID). Elution of the peptides was achieved with a linear gradient from 0-40% B (0.1 M acetic acid in 80% (v/v) actonitrile) in 60 minutes. The column effluent was directly introduced into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives, Woburn, MA, USA). The mass spectrometer was operated in the positive ion mode and parent ions were selected for fragmentation in data-dependent mode. Proteins were identified using a Mascot search engine with a confidence limit set at < 0.05, where P is the probability that the observed factor is a random event. Searches with LTQ-FT obtained data were performed with a peptide tolerance window of 10 ppm. Subsequently, all searches were performed by using the SwissProt database with two miss-cleavages, carbamidomethylaton and methionine oxidation as variable modifications. Peptide sequences with an ionscore > 20 were regarded as positively identified.

Immunofluorescence analysis

Cells grown on fibronectin-coated coverslips were fixed in 4% paraformaldehyde for 20 min followed by permeabilisation in paraformaldehyde with 0.1 % Triton X-100 for 10 min and a wash in PBS (1 x PBS, 50 mM Glycine). Blocking was performed with blocking buffer (2% BSA, 0.1 % PBST). Coverslips were incubated with primary antibody (1:15 anti-PECAM, CD31 antibody (Abcam, Cambridge, UK); 1:100 anti-endoglin, CD105 (BD Biosciences San Jose, CA, USA) for 1 hour at RT. After washing, cells were incubated with secondary antibody (1:250, Alexa Fluor 488 goat anti-mouse IgM, (Molecular Probes, Eugene, OR, USA) in blocking buffer for 1 hr at RT. For actin staining, cells were incubate with phalloidin-TRITC

(Sigma, St Louis, MO, USA) for one hour at RT. Subsequently, cells were washed in blocking buffer and incubated with 0.2 µg DAPI in PBS at 37^0 C for 5 min. After washing, coverslips were mounted with Mowiol-PPD (Molecular Probes). Cells were examined with a Leitz Orthoplan Fluorescence Microscope, equipped with a Leica DC350F camera (Leica, Wetzlar, Germany).

Western blot analysis

Cells were lysed in ice-cold buffer (25 mM Tris/HCl, pH8, 1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 1 mM Na_3VO_4, Complete protease inhibitor cocktail (tablet) (Roche)). Extracts were clarified by centrifugation (20,000 g x 10 min) and supernatant was subjected to a BCA Protein Assay reagent kit (Pierce, Rockford, IL, USA) for protein determination. Proteins (10 µg) were loaded on 10% SDS-PAGE gels of 8 cm by using a mini-Protean gel system (BioRad). Separation was achieved with a constant voltage of 200 mV until bromophenol blue dye reached the end of the gel. Proteins were transferred semi-dry onto a PVDF membrane in a Trans-Blot SD cell (BioRad). Subsequently, detection of p21 was performed using a mouse monoclonal anti p21 (Zymed, Carlsbad, CA, USA) in combination with ECL staining.

Results and discussion

Inhibition of cell proliferation

Cellular replicative senescence entails an irreversible arrest in proliferation. Besides the loss of cellular proliferation, senescence also leads to a change in cell functionality. In order to map properly the dynamics of proteome regulation in endothelial cells during replicative senescence, HUVECs were isolated and cultured towards senescence for an extended period of time. Culturing of cells from three different umbilical cords resulted in distinct differences in the time to induction of senescence for the three isolations (Figure 1). The protein expression profile of each isolation was examined at three time points; half lifetime, the prior-senescent state and the senescent state as indicated in figure 1.

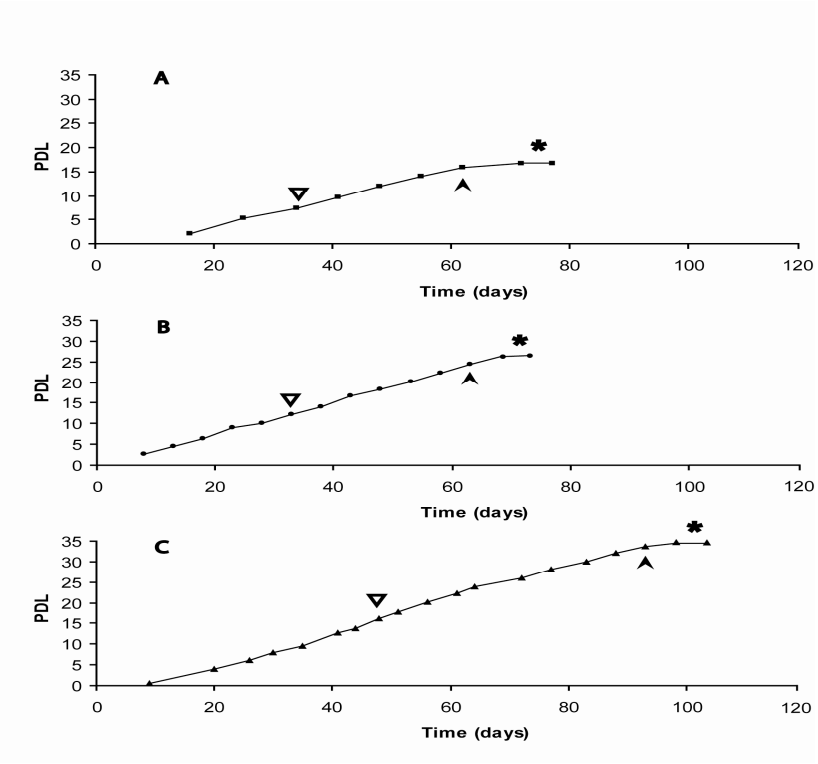

Figure 1. Growth profiles of endothelial cells Endothelial cells were cultured from three independently isolated umbilical vein preparations (A, B and C) and were maintained in culture until they reached senescence. Population doubling level (PDL) was plotted against time. Differences in senescence endpoints can be observed. Protein extracts were made from cells harvested at half lifetime (▽), prior-senescent state (▲) and the senescent state (✱).

The prior-senescent state displayed a slowing down of proliferation and an increase in enlarged flattened cell morphology and formed a sub-confluent cell layer. When these cells were trypsinised and replated, hardly any proliferation occurred and sub-confluency was not reached, an indication that during this culture passage the cells ceased cell division. Indeed after changing the culture medium and thereby replenishing nutrients and growth factors, no further cell division was detected, which demonstrates that the cells reached the senescent state. To further demonstrate that the cells were in a replicative senescent state, lysosomal senescence–associated-ß-galactosidase (SA-ß-Gal) activity was determined (Figure 2). Microscopic evaluation of the SA-ß-Gal activity showed that about 40% of the cells in the prior-senescent passage were active. The amount of positively stained cells increased to 50% at the senescent state and confirmed the accumulation of SA-β-Gal active cells during senescence as found by others [15,17,31,32].

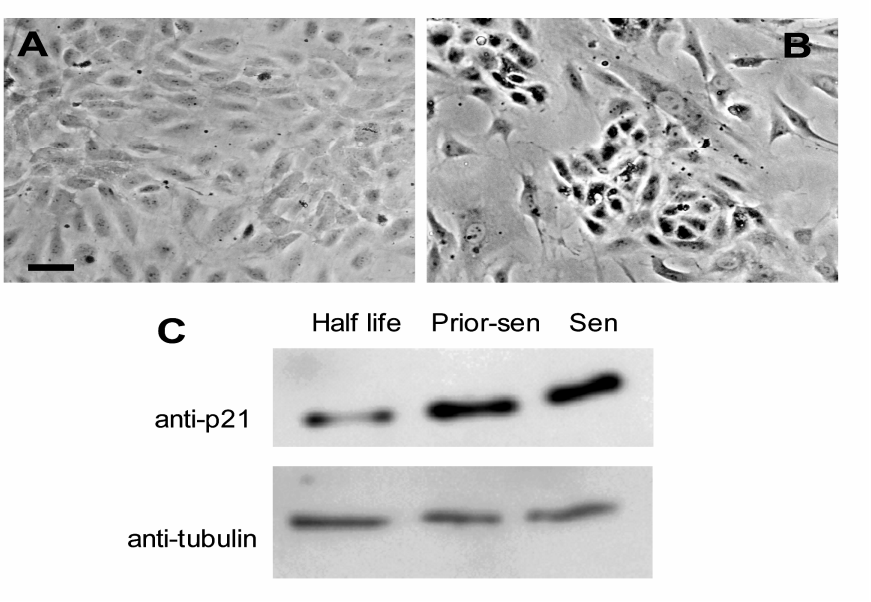

Figure 2. Senescence-associated-ß-Galactosidase activity and p21 expression
Staining for SA-β-GAL-activity at pH 6 was used to monitor accumulation of senescent cells in time. Cell monolayer of HUVECs at 90% confluency after 12 population doublings exhibit a low SA-β-GAL activity (A). This is in contrast with cells in the senescent state which show no confluency and a high SA-β-GAL staining (B). Enlarged flattened cell morphology can also be observed. Induction of p21 was detected with Western blot by using 10 µg of total protein per sample with tubulin expression as loading control (C). Cyclin kinase inhibitor p21 becomes upregulated towards senescence.

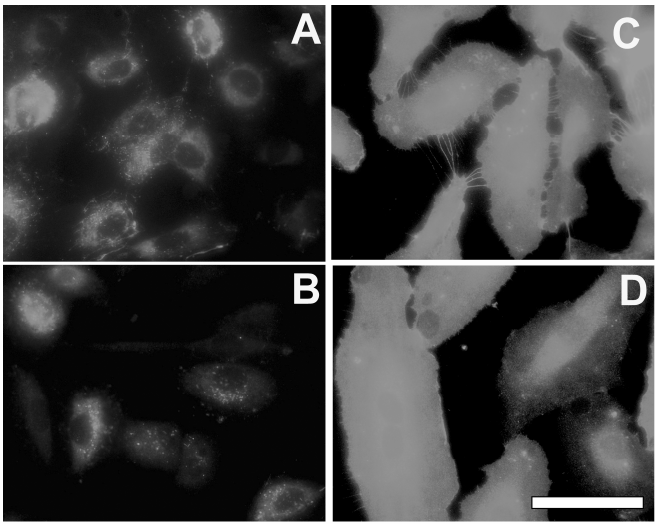

Figure 3. Endothelial characteristics of the cells in the senescent state
Young (A/C) and senescent (B/D) HUVECs were labelled for vWF (A/B) and PECAM (C/D) and examined by fluorescence microscopy. A clear labelling of all the cells was observed, indicating that the cells do express these endothelial markers at both stages. Omission of the primary antibody resulted in a complete loss of the signal (not shown). The bar represents 25 µm.

A strong induction of cell cycle protein p21 was observed in the cells at the prior-senescent state and the senescent state (Figure 2). This cyclin kinase inhibitor (CKI) is part of the DNA damage p53 response pathway which is induced when telomere integrity is lost. The distinct upregulation of p21 in this study confirms the inhibition of cell proliferation [23,33,34]. Finally, to ensure that the cells in the senescent state are still endothelial cells, it was tested whether they exhibit endothelial characteristics, such as the expression of the Von Willebrand factor and PECAM. Figure 3 clearly illustrates that the senescent cells do express these endothelial cell markers.

Protein expression dynamics analysis
Protein extracts were subjected to 2D-DIGE analysis. The application of intra-gel co-detection with inter-gel internal standard correction generated a HUVEC 2D gel proteome of about 1500 properly matched protein spots. Analysis of variance (ANOVA) was used to compare protein expressions from three independent biological sources at three time points. The use of three different donor tissues will result in a so-called donor dependent variation and when using unpaired ANOVA

statistics proteins will be rejected as a result of high variation between the different time groups (Figure 4A). Still, the expression might be significant for each independent cell isolation and can be regarded as biologically relevant. Using paired analysis of variance (RM-ANOVA) this problem is circumvented and expression profiles are corrected for biological variation (Figure 4B, dashed line).

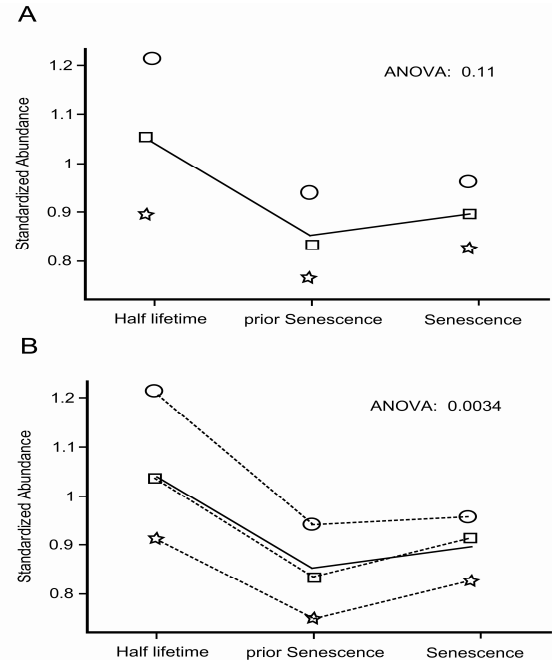

Figure 4. Application of paired Analysis of Variance (RM-ANOVA)
Internal standard-corrected abundances (standardized abundance) of a protein were plotted against relative time (half lifetime, prior-senescence and senescence). Unpaired ANOVA statistics indicates that the expression profile (A) of this protein does not differ significantly in time, as illustrated by the ANOVA p-value of the calculated expression curve (solid line). However, paired ANOVA accounts for samples from the same biological source (B, dashed line) and shows that this protein exhibits an expression profile that significantly changes in time for each of the isolations. This is reflected by the small RM-ANOVA p-value of the calculated expression curve (B, solid line) (Output Decyder 5.01).

Next to this, the use of an internal pooled standard enabled us to correct for inter-gel variation and to look for expression differences ≥ 10%. Expression profiles with an RM-ANOVA p-value ≤ 0.05 were regarded as significant and resulted in a senescence-associated proteome map of sixty spots. Thirty-five spots were positively identified and characterized with respect to their molecular weight, pI and expression dynamics (Table 1) (Figure 5). Of the identified proteins, nineteen were

down-regulated and seventeen were up-regulated towards senescence. The majority of these proteins showed a gradual expression profile towards senescence. Protein identification of this subset of proteins indicated that they belong to various biological classes, i.e. proteins involved in oxidative stress, DNA repair and maintenance, protein biosynthesis and nucleoplasmic transport, and cell structure. Some of these proteins will be discussed in more detail in the next paragraph.

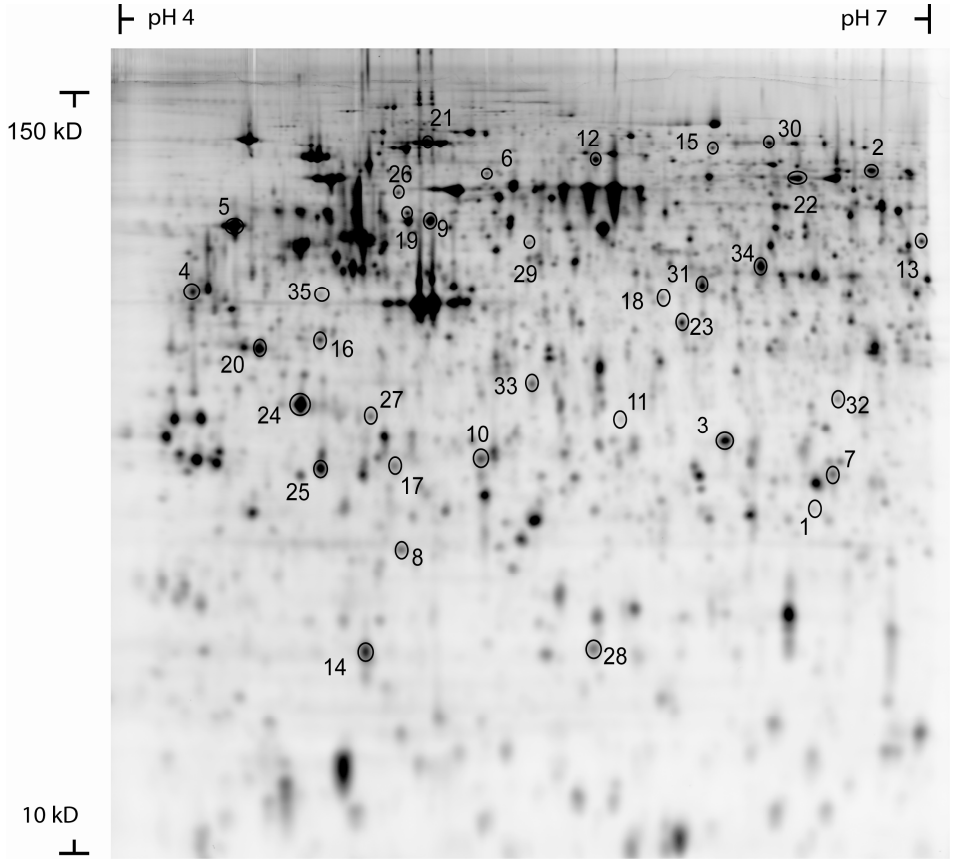

Figure 5. Representative 2D gel image of 50 µg HUVEC cell lysate.
Representative 2D gel image labelled with Cy5, scanned with a pixel size of 100 µm. Proteins that exhibit a senescence-associated expression in time are numbered and encircled. Corresponding protein ID and description can be found in Table 1.

Spot No.	Name	Protein Description	Molecular function	Acc.no.	RM-ANOVA p-value	Exp. Sen/HF	Score	Pep.	SC
		Stress							
1	HSP27	Heat shock protein 27	Chaperone	P04792	0.022	2.7	193	5	23
2	TRFE	Serotransferrin precursor (Bovine)	Iron transport	Q29443	0.026	2.2	1919	27	52
3	GSTO1	Glutathione transferase omega 1	Glutathione/ascorbate metabolism	P78417	0.009	1.5	623	9	38
4	RCN1	Reticulocalbin I	Calcium binding	P08670	0.005	1.5	685	9	33
5	PDIA1	Protein disulphide isomerase A1	Oxidative folding	P07237	0.010	1.5	4148	20	54
6	NUAM	NADH-ubiquinone oxidoreductase subunit	Mitochondrial electron transport	P28331	0.028	1.3	1854	21	36
7	PSMA6	Proteasome subunit alpha type 6	Endopeptidase/ proteasome	P60900	0.040	0.9	721	6	28
8	UCH-L1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Ubiquitin thiolesterase activitty	P09936	0.026	0.9	462	5	30
9	HSP60	Heat shock protein 60	Folding chaperone	P10809	0.041	0.7	9565	28	66
		DNA repair and replication							
10	NNMT	Nicotinamide N-methyltrans	Pyridinium metabolism	P40261	0.039	1.9	268	8	25
11	RFA2	Replication protein A 32 kDa subunit	DNA binding	P15927	0.003	0.9	263	3	11
12	KU80	Ku80-protein	Double-stranded DNA binding	P13010	0.047	0.7	4494	32	44
13	IMDH1	IMP dehydrogenase II	Guanine nucleotide biosynthesis	P20839	0.043	0.6	2452	16	44
		Protein biosynthesis and nucleoplasmic transport							
14	EIF5A	Elongation initiation factor 5A	Translation activity/protein binding	Q7Z4L1	0.014	0.8	617	4	33
15	UNR	N-ras upstream gene protein	RNA-binding	O75534	0.004	0.7	1832	27	34
16	HNRPC	Heterogeneous nuclear ribonucleoprotein C	RNA-binding	P07910	0.012	0.6	1062	15	49
17	RANG	Ran-Binding Protein 1	Signal transduction	P43487	0.033	0.6	501	5	26
18	TADBP	TAR DNA-binding protein-43	Microtubule binding	Q13148	0.019	0.5	646	9	22
19	HNRPK	Heterogeneous nuclear ribonucleoprotein K	RNA-binding	P61978	0.030	0.5	2728	20	44
20	NPM	Nucleophosmin 1	RNA and protein binding	P06748	0.033	0.4	960	13	39
		Cell structure							
21	ACTN1	Alpha actinin	Actin binding	P12814	0.000	1.6	5582	36	44
22	MOES	Moesin	Receptor/cytoskeleton binding	P26038	0.011	1.6	7197	30	62
23	CAPG	Actin-regulatory protein CAP-G	Actin filament capping	P40121	0.012	1.4	1377	11	35
24	ANXA5	Annexin V	Phospholipid binding	P08758	0.049	1.3	6197	29	94
25	GDIR	Rho GDP-dissociation inhibitor 1	GTPase activator activity	P52565	0.040	1.2	2281	8	48
26	LMNB1	Lamin B1	Intermediate filament	P20700	0.007	0.5	5058	31	63
27	MARE1	Microtubule-associated protein EB 1	Microtubule binding	Q15691	0.007	0.4	618	9	38
28	STMN1	Stathmin	Microtubule binding	P16949	0.017	0.3	699	6	53
		Other							
29	VATB2	V-ATPase B2 subunit	Proton transport	P21281	0.042	1.8	423	9	18
30	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	Collagen crosslinking	O00469	0.023	1.6	861	18	23
31	VAT1	Synaptic vesicle membrane protein VAT-1	Oxidoreductase activity	Q99536	0.012	1.6	1231	12	38
32	PtdInsTP	Phosphatidylinositol transfer protein alpha	Phosphatidylcholine transporter	Q00169	0.018	1.1	501	9	34
33	PPase	Inorganic pyrophosphatase	Inorganic diphosphatase activity	Q15181	0.029	0.8	583	8	25
34	RABGDI	Rab GDP dissociation inhibitor	GDP-dissociation inhibitor activity	P31150	0.011	0.7	1224	17	44
35	ADRM1	Adhesion regulating molecule 1	Cell adhesion	Q16186	0.016	0.6	510	5	12

Table 1. Proteins showing senescence-associated expression

The protein identity, (ion)score (>20), unique peptides (Pep) and percentage sequence coverage (SC) were determined with the Mascot search engine in combination with the Swiss-prot dBase. Proteins are classified according to their biological function in relation to senescence. Expression changes (Exp.) were calculated by using the average value of senescent cells (SEN) in comparison with the average values of cells at halflifetime (HF). Significance of expression trends was determined by calculating RM-ANOVA p-values.

Functional classification of identified proteins

Oxidative stress

Stress, more specifically oxidative stress, is closely related to senescence. Incubation of cells with free radical-generating compounds can accelerate the senescence process [15,18,19,35] and reactive oxygen species play a key role in general accepted mechanisms of ageing [36]. In the present study a set of stress-associated proteins was found and their regulation profiles are depicted in figure 6A. Heat shock protein 27 (HSP27), strongly up-regulated in this cell model, belongs to the small HSP family which can modulate the ability of cells to respond to oxidative stress. Previously, it has been shown that induction of senescence with hydrogen peroxide was accompanied with up-regulation of HSP27 when the senescent phenotype developed [18]. This protein exists in a number of dynamic states depending on the physiological state of the cell [37]. In response to oxidative stress large oligomers are formed, followed by phosphorylation-dependent dissociation of these large oligomers into small oligomers [38]. Indications of elevated oxidative stress can also be deduced from the upregulation of NADH ubiquinon reductase (NUAM) and transferrin (TRFE). The NUAM subunit is part of the complex I protein of the respiratory chain in the mitochondria and one of the sources of ROS in the electron transport chain. TRFE is a source for intracellular iron in endothelial cells and is taken up from the blood serum via the transferring receptor pathway. The increased cellular content of bovine serotransferrin suggests an increased uptake towards senescence. Interestingly, the expression of the NUAM subunit is strongly modulated by the intracellular iron content [39] and might thus be coupled to the increased cellular transferrin. The observed upregulation of both proteins in the present study confirms that in the senescent endothelial cell increased iron-signalling takes place, which is accompanied with increased iron levels as shown by others [40]. Excess of intracellular iron can lead to oxidative stress, by triggering the formation of reactive oxygen species via the Fenton mechanism [41,42]. Replicative aged yeast cells exhibited iron accumulation which resulted in increased carbonylated proteins [43]. Increased production of reactive oxygen species (ROS) is in conjunction with other senescent cell models as well [15,18,44]. Another indication for an increased oxidative stress is the distinct upregulation of glutathion-s-transferase omega (GSTO) found in the present study. Interestingly, GSTO has been related to high dehydroascorbate reductase activity, which suggests that GSTO may significantly protect against oxidative stress by recycling ascorbate [45]. GSTO has been suggested to influence also the onset of other age-related diseases, like Alzheimer and Parkinson [46-48]. The expression of GSTO might be a cellular response towards the increased ROS production upon

senescence. Another aspect of cellular stress and senescence relates to the endoplasmic reticulum. ER resident proteins are very sensitive to oxidative stress [49] and in view of the increased ROS production with age [36] it has been proposed that ER functioning might be hampered towards senescence [49]. Indeed we do find changes in the expression levels of several ER resident proteins (PDA1, RCN1 listed in Table 1). Reduced activity of the proteasome has been linked to senescence as well [21,50,51]. Indications of reduced proteasome activity may be involved since several proteasome subunits (PMSA6 and UCHL1 in Table 1) are changed towards senescence. It has been postulated that cells are able to switch from proteasome derived protein degradation to lysosomal protein degradation [52-54]. Supporting evidence for this is possibly reflected by the upregulation of the proton pump V-ATPase (VATB2 Table 1). This protein is abundant in lysomes [55] which are increased during senescence when looking at the lysosomal SA-ß-Gal – activity (Figure 2) and correlates with lysosomal body increase [56].

DNA repair and maintenance

Telomere maintenance is essential for continued proliferation of cells. Erosion of the telomeric DNA is thought to be one of the causes of senescence and affects the DNA damage response mechanisms of the cell [17,31,57]. In this study we found a subset of proteins involved in DNA damage response (Figure 6B). The heterogenous nuclear ribonucleoproteins (Figure 6C), HNRNPC and HNRNPK were downregulated in time and both proteins are proposed to be involved in DNA repair. HNRNPK yeast homologs are suggested to play a role in the structural and functional organization of telomeric chromatin in yeast [58]. A study with hepatocytes clearly showed increased HNRNPK levels during proliferation [59]. HNRNPC is suggested to bind chromatin after DNA damage and is thereby possibly involved in DNA repair [60-62]. Two other DNA repair related proteins, Ku-protein 80 (KU80) and replication protein A (RFA2) are distinctly downregulated in the present study. Ku80 is part of DNA PK, which is essential for DNA repair [63]. Normally, a strong upregulation of Ku80, as well as its partner Ku70, is correlated with induced DNA-damage [62,64]. RFA2 is essential for multiple processes in DNA metabolism, including DNA replication, recombination and repair. The function of RFA2 in the cell is determined by its phoshorylation state. This is tightly controlled by DNA-PK [65-67], which might be down regulated in our study. The possibility of DNA damage accumulation in this endothelial cell system is in agreement with other senescent cell systems [68]. On the other hand, human fibroblasts induce other DNA repair pathways, which could not be determined in our endothelial cell system [69,70].

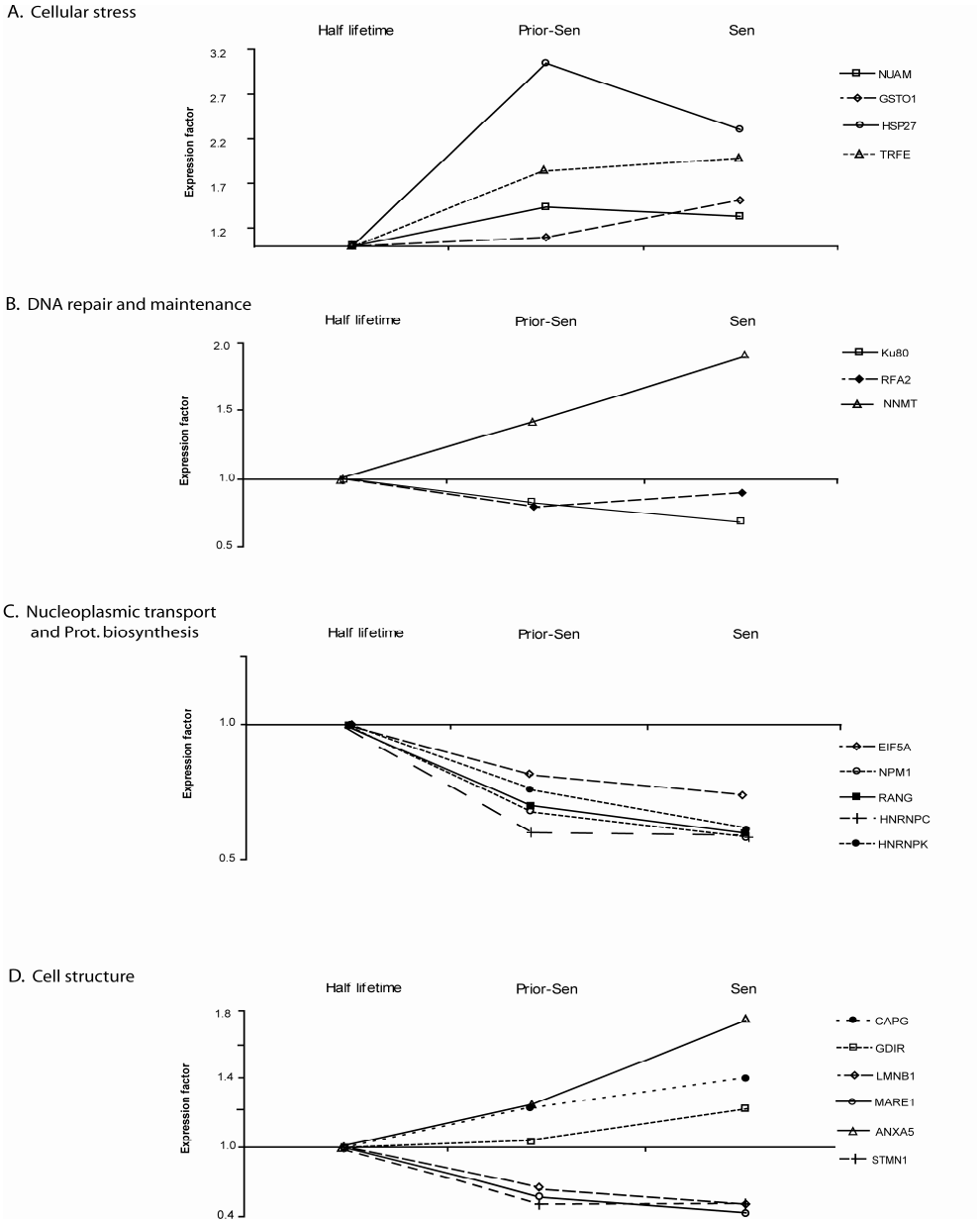

Figure 6. Protein dynamics towards replicative senescence. The expression of various proteins involved in oxidative stress (A), DNA repair and maintenance (B), nucleoplasmic transport & protein biosynthesis (C) and cell structure (D) is influenced towards senescence. Average expression changes are normalised to the expression at half lifetime. For explanation of protein abbreviations see Table 1.

Another interesting observation is the upregulation of nicotinamide methyl transferase (NNMT).This enzyme is involved in the formation of s-adenosyl-homocysteine, which is associated with the hypomethylation status of DNA in HUVECs and elevated levels of this enzyme reduce DNA methylation [71]. Since a normal DNA methylation pattern is essential for the maintenance of genomic integrity it can be speculated that an increased level of NNMT will cause altered DNA methylation towards senescence. In mouse embryonic fibroblasts DNA hypomethylation caused premature senescence via a p53 independent pathway [72].

Nucleoplasmic transport and protein biosynthesis

An inhibition of cell cycle progression is accompanied by a downregulation in protein biosynthesis. This study shows clearly that several proteins involved in protein biosynthesis are downregulated during senescence (Figure 6C). Indications of impaired protein biosynthesis can be extrapolated from the downregulation of nucleophosmin (NPM1). This protein is strongly involved in ribosome biogenesis [73]. Elongation Initiation factor 5A (EIF5A) is downregulated in the present study which agrees with expression data obtained from cells originating from aged skin [74]. This protein is involved in protein biosynthesis and is essential for cell proliferation [75-77]. Also downregulated towards senescence are heterogeneous nuclear RNP C (HNRPC) and K (HNRPK) (Figure 6B). These proteins are crucial in pre-mRNA processing and facilitate translocation of mature RNA strands from the nucleus to the cytoplasm for protein biosynthesis [78]. Besides the reduction in RNA translocation, other forms of nucleoplasmic transport seem to be influenced as well. In this study, a strong inhibition of expression of Ran-binding protein (RANG) is observed, which will impair the import/export function of the Ran GTPase network. The Ran GTPase network is involved in a variety of processes like cell cycle progression, chromosome stability, nuclear organization and nucleoplasmic transport. RANG shuttles Ran back into the nucleus for a new import/export cycle. Mammalian cells have RANG present in a limited window of the cell cycle . No expression is observed in G0 and G1 phases of the cell cycle [79]. Accordingly, the down regulation at replicative senescence is in agreement with the G0/G1 status of the HUVECs and a diminished nucleoplasmic transport. Studies with fibroblasts from different donor age groups revealed also a reduction in nucleoplasmic transport [80].

Cell structure

The cytoskeleton is known for its interaction in a variety of cellular functions such as intracellular traffic of organelles, mitosis, cell motility and mechanical stability. Towards senescence it is clear that the cytoskeleton becomes affected and results in enlarged flattened cells (Figure 2B). In addition to this a change in the actin skeleton, specifically the stress fiber formation, can be distinguished (Figure 7), which is in agreement with other senescent cell systems [81]. Figure 6D clearly shows a subset of proteins involved in cell structure re-organization. Actin-regulatory protein CAPG plays an important role in the adaptation to mechanical forces in endothelial cells. Overexpression of this protein resulted in an increase in motility potential in endothelial cells [82]. Rho-GDI inhibitor (GDIR) is postulated to regulate the activity of small G-proteins of the Rho family. This group plays an important role in various cell functions such as cell shape change, cell motility and cytokinesis, through reorganization of actin filaments. The reorganization of cytoskeleton can be initiated when GDIR interacts with ERM which links transmembrane proteins to the cytoskeleton [83,84]. ANXA5 has lipid-binding properties and is localized at the plasma membrane. Effects on the membrane curvature and cell shape can be expected when this protein becomes upregulated [85].

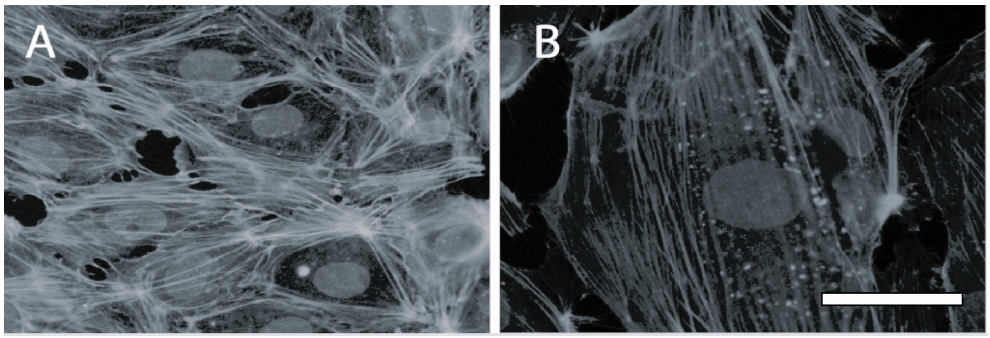

Figure 7. Stress fiber alteration in HUVECs. Actin skeleton in non-senescent (A) and senescent (B) HUVECs, visualized by phalloidin -TRITC. A clear difference can be observed between the two cell populations. Nuclei were stained with DAPI. Bar represents 25 µm.

Stathmin (STMN1) and microtubule-associated protein EB1 (MARE1), both strongly downregulated, regulate microtubule dynamics. The pronounced downregulation of STMN1 is in accordance with another proteomic study, comparing brains of young and old humans, in which STMN1 appeared to be strongly downregulated as well [86]. Low levels of this protein could be correlated

with a reduction in proliferative capacity [87]. Thus it is evident that the cytoskeleton becomes influenced strongly during senescence. Due to the variety of cellular processes in which the cytoskeleton is involved, it is obvious that these changes may accelerate endothelial dysfunctionality. Evidence has been found that actin dynamics will generate ROS and is involved in apoptosis and senescence [88].In the present study lamin B (LMNB1) is strongly downregulated towards replicative senescence. B-type lamins are essential for cell viability and are expressed in all cells during development [89]. Lamin B degradation is primairly related to nuclear envelope breakdown prior to G2/M phase transition in the cell cycle and also in apoptosis. B-type lamin knockout is lethal in mice. However, cultured fibroblast from these mice displayed grossly mishaped nuclei, impaired differentation, increased polyploidy and premature senescence [90]. The reduced nuclear integrity is maybe one of the causes why senescent HUVECs switch earlier to apoptosis as found by others in literature [22].

Concluding remarks

In this study, we have explored the use of 2D-DIGE to unravel replicative senescence in an endothelial cell model system. The application of 2D-DIGE has enabled us to perform a dynamic proteomic study on cell lysates from HUVECs isolated from three different umbilical veins. This has resulted in a subset of proteins which show regulation profiles with a significant RM-ANOVA p-value. The functional classification of this subset of proteins has revealed some interesting biological pathways, which may play a role in endothelial senescence. The replicative senescent process in the endothelial cell is accompanied with increased production of reactive oxygen species (ROS), which is in agreement with other senescent cell models. Results suggest that accumulation of DNA damage might occur since a part of the DNA repair mechanism is down regulated, which is possibly the cause for the observed upregulation of cyclin kinase inhibitor p21. Next to this there are indications that the endothelial senescence is accompanied with a disturbed DNA methylation pattern. A remarkable observation is the regulation of lamin B1 and to our knowledge it has never been shown that this protein becomes strongly down regulated towards endothelial senescence. The protein is highly important for nuclear integrity. Additionally, destabilizing the nuclear integrity may accelerate impaired nucleus function like DNA replication and protein biosynthesis. The pronounced re-organization of the cytoskeleton may accelerate the replicative senescence process in endothelial cells as well. Furthermore, it is very likely that this morphology will influence negatively the

adhesion contacts between endothelial cells and thereby will compromise the endothelium permeability function.

Clearly, 2D-DIGE is an excellent tool to explore the senescent proteome globally. This is illustrated by our finding that part of the differentially expressed proteins are part of biological pathways found to be regulated also in other senescent cell systems and were detected via hypothesis-targeted approaches. It is obvious that the biological pathways discussed embrace a large variety of other proteins, which were not detected in the present study. Next to this, proteins are subjected to a large variety of post-translational modifications, that might partly result in the observed protein expression profiles. Therefore, future studies have to be focused on validating the proposed pathways by identifying more members of the same pathway and deciphering protein modifications. In this way it is possible to address properly the functionality of the biological pathways presented here in relation to replicative endothelial senescence. Nevertheless, this study provides information which will contribute to the initiation of these future studies, which will provide more detailed knowledge about the molecular background of the endothelial senescence process and its consequences in the *in vivo* situation.

Acknowledgements

This research was in part supported by the Netherlands Proteomics Centre. We acknowledge Unilever Research and Development Vlaardingen The Netherlands and The Dutch Ministry of Economic Affairs (SENTER:TSGE1028) for their financial support.

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Addendum Chapter 2

Ultrastructural changes during endothelial senescence

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Introduction

Since the results described in chapter 2 might suggest changes in the cellular architecture we decided to study ultra-structural changes during endothelial replicative senescence. For that purpose cells were subjected to a culturing protocol identical as applied in chapter 2 and subsequently analyzed by Electron Microscopy (EM).

Materials and methods

HUVEC isolation and culturing

Endothelial cells were isolated and cultured as described before [1]. Young cells (PD4) and replicative senescent cells were processed for electron microscopy.

Electron microscopy

For electron microscopy the cells were fixed using half strength Karnovsky fixative [2], supplemented with 50 µM $MgCl_2$. Postfixation was performed with 1% osmiumtetroxide (EMS) and 1.5% potassium ferrocyanide (Merck) in sodium-cacodylate buffer pH 7.4. After several rinses with distilled water fixed cells were scraped in 0.1% low melting agarose (Roche Diagnostics, Mannheim, Germany) in distilled water and centrifuged at 16000g for 30 seconds. Thereafter, the cell pellet was resuspended in 2% low melting agarose (Roche Diagnostics) and centrifuged at 16000g for 3 minutes. The cell pellet was placed on ice for 15 minutes and cut into small pieces. Subsequently, pieces were dehydrated in a ethanol-propyleenoxide mixture and embedded in Epon (containing Fluka Epoxy resin #45345, Araldite M Hardener 964 #10953, MNA Hardener #45347 and DMP-30 # 45348, mixed according to the manufacturer, Fluka, Buchs, Switzerland). Polymerisation was performed at 60^{o}C for 72 hrs. Ultra-thin sections (50nm) were made using a Reichert Ultracut E (Leica Microsystems, Vienna, Austria) and stained with 20% uranylacetate (SPI-Chem, West Chester, USA) in 70% methanol and Reynold's lead citrate [3]. Sections were examined on a Jeol 1010 electron microscope.

Results and discussion

Electron microscopy of continuously cultured HUVECs clearly showed some remarkable differences between young and old endothelial cells. At low magnifications a clear difference was observed between de senescent and young endothelial cells, as the senescent cells showed a large number of electron dense structures (see Figure 1A/B), which at higher magnifications (Figure 2) were identified as lysosomal structures. Evaluation with Light Microscopy (LM) showed that the majority of these old cells were SA- β-GAL active (Figure 1C/D), which indicates that those cells reached the replicative senescent state [1,4-6].

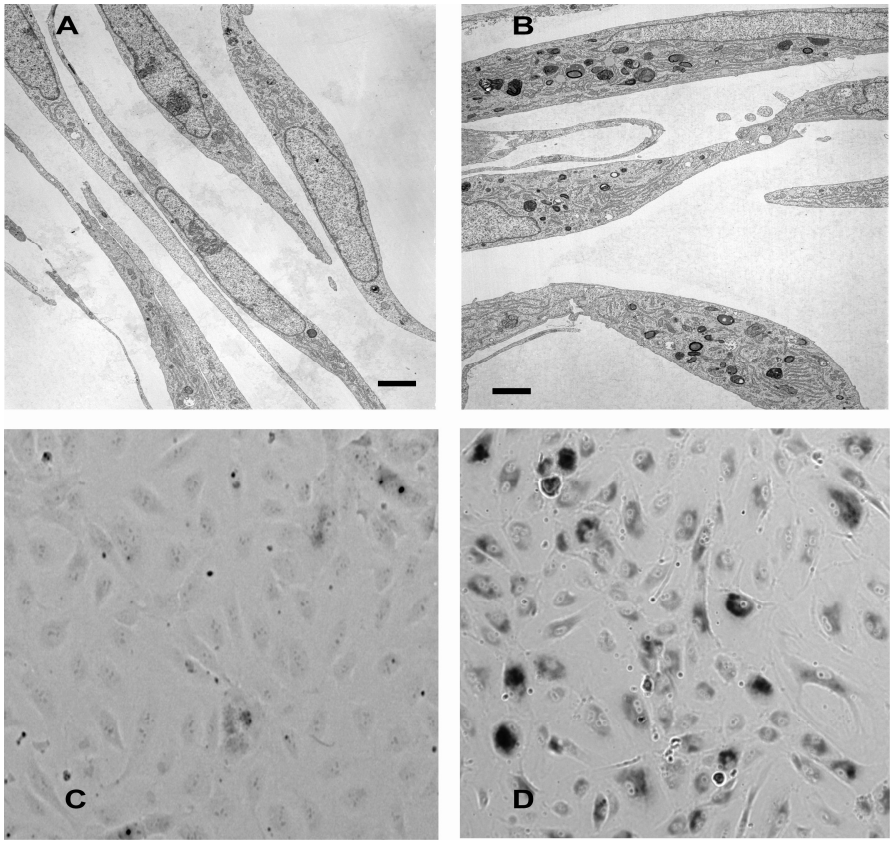

Figure 1. **Overview picture of young and old endothelial cells.** Bar represents 2 µm. Old cells (B) show electron dense structures compared to young cells (A). Young cells (low PD) (C) show hardly any SA- β-GAL activity, which is in contrast with the old cells (high PD) (D). This clearly indicates that the majority of the old cell population has reached the senescent state

In a more detailed view, the differences between the two cell states become more distinct as shown in figure 2. In this figure more lysosomes can be distinguished in old cells than in young cells and the lysosomes can be clearly identified by their multilayered appearance as well as their electron-dense content.

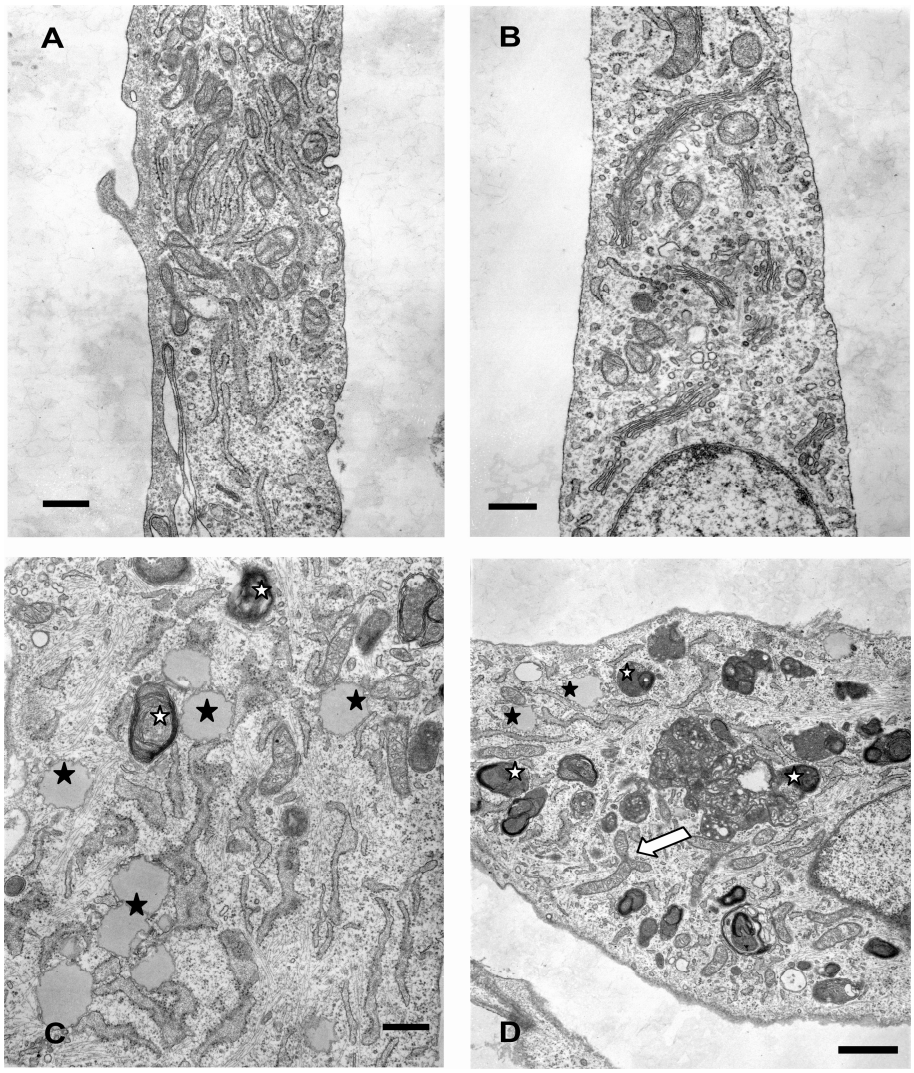

Figure 2. A more detailed view of young (A/B) and senescent endothelial cells (C/D). Bar represents 500 nm. In old cells more lysosomes (open stars) and fat droplets (closed stars) can be distinguished compared to their younger counterparts. Also a branched mitochondrion (open arrow) is present in the old cells.

The increase in the number of lysosomes is in line with the increase in SA- β-GAL activity in old cells, which can be correlated with a lysosomal body increase in the cells [7]. Furthermore, the increase of the lysosomal content can explain the observed increase in V-ATPase B2 subunit (Chapter 2) upon replicative senescence, which is a component of the lysosomal proton. Another interesting observation indicates a change in the metabolism of the endothelial cell. An increase in cytosolic triglyceride droplets is observed, as indicated in figure 2C/D. This might indicate that during the growth towards senescence the endothelial becomes altered, which is supported by NMR experiments, which show an increase in fatty acid metabolism upon stress induced premature senescence (SIPS) (Van Dorsten, personal communication). Next to this, branched or fused mitochondria are observed (figure 2) in senescent cells. In the proteomic study we observed a decrease in NUAM a subunit of the NADH ubiquinone oxidoreductase, a component of the mitochondrial electron transport chain. Although this was the only mitochondrial protein with differential expression observed it is tempting to speculate that this might be involved in or be the cause of altered mitochondrial physiology.

The primary role of mitochondria in eukaryotic cells is the production of energy and to maintain optimal functionality, mitochondria fuse and divide (fusion/fission process) continuously [8]. However, towards aging or replicative senescence mitochondria seem to be less efficient in energy production and even might accelerate the aging process due to elevated radical production [9-11]. There are indications that the continuous fusion and fission of mitochondria becomes hampered towards senescence [12]. The data we obtained indeed indicate that fission might be reduced in senescent cells, resulting in fused mitochondria. Another protein that showed down regulation towards senescence in our previous proteomics study is lamin B1. This protein is of importance for nuclear integrity and intriguingly mutations in lamin A/C has been linked to premature aging and seem to accumulate in endothelial cells [13]. Furthermore, fibroblasts from Lamin B mutated mice embryos displayed grossly misshapen nuclei and premature senescence [14]. However, in the present study no clear changes were observed at the nuclear ultra-structural level and more detailed studies are needed to unravel this possibility in endothelial cells.

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Chapter 3

Evaluation of transferrin recycling in young and old Human Umbilical Vein Endothelial cells.

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Abstract

Replicative senescence is a cellular process in which the cell replication capacity is stopped, even in the presence of growth factors. In a previous proteomics study we have found that bovine transferrin was more abundant in senescent endothelial cells compared to young cells. In general, cells use this protein to take up iron into the cytosol, where it is essential for proper cell functioning. However, iron becomes deleterious to the cell when the intracellular iron balance is disturbed, because iron is involved also in generation of Reactive Oxygen Species (ROS). These radicals are capable of damaging DNA, proteins and lipids, which will lead to impairment of cell functionality. In view of this, we have used cultured human umbilical vein endothelial cells to further characterize the transferrin pathway in relation to the growth to replicative senescence. We have studied cellular transferrin content and uptake to get more insight in the cellular pools and kinetics of this protein. The results showed that in late passage cells transferrin levels are increased in comparison to early passage cells. This increase cannot be explained by an increase in transferrin receptor expression towards senescence. Neither can it be explained by increased recycling rates of transferring upon senescence. Therefore we propose that towards replicative senescence, cells might become more susceptible to non-receptor mediated transferrin uptake. Clearly, more research has to be initiated to get more detailed knowledge about the transferrin pathway in relation to replicative senescence and the implications in the *in vivo* situation.

Introduction

Endothelial cells in culture exhibit a limited lifespan, known as replicative senescence. Senescent endothelial cells are detected *in vivo* also in atherosclerotic lesions. There are suggestions that endothelial senescence might play a role in the initiation and/or progression of this disease [1,2]. Therefore, further insight in this process is important and further elucidation of the role of senescence in atherosclerosis demands more knowledge about biological processes associated with endothelial senescence. In chapter 2 we have performed a proteomic study on endothelial senescence, which revealed some interesting protein regulations [3]. One of the proteins that gained our interest was serum transferrin, which showed an elevated intracellular abundance in senescent cells. Transferrin (Tf), a major plasma iron-binding protein, is synthesized and secreted mainly by the liver. This 80 kD protein is capable of binding two Fe(III) ions under neutral pH conditions. Endocytosis of this iron-containing (holo)-Tf is performed by specific cell membrane transferrin receptors (TfR), of which the expression is tightly iron-regulated [4]. Upon binding to the receptor clathrin–coated endocytic vesicles are formed, which are transported to specific parts of the cell. Due to pH lowering in the endocytotic vesicle the affinity of iron for Tf becomes reduced and Fe (III) ions are transported into in the cytosol. The remaining vesicle with the receptor-(apo)-Tf complexes are recycled back to the plasma membrane, where the (apo)-Tf becomes released from its receptor into the extracellular space [5,6]. Under normal physiological conditions Tf is involved in providing the cell with sufficient amounts of iron, needed for a variety of processes, as it is a ubiquitous element in cells and used by many proteins and enzymes. Enzymes, like prolylylhydroxylase need iron to perform their catalytic function properly [7] and proteins such as cytochrome a, b, and c use this element to function in mitochondrial respiration and ATP synthesis [5]. This illustrates the important role of this element in cell proliferation. However, iron can also be involved in the generation of reactive oxygen species (ROS), which contributes to intracellular oxidative stress formation. In the presence of iron, the relatively harmless intracellular hydrogen peroxide becomes reduced via the Fenton assisted Haber-Weiss reaction into the hydroxyl radical. This radical is highly reactive towards all biomolecules, like proteins, lipids and DNA [8,9]. Furthermore, iron-mediated lipid peroxidation of membranes and lipid carrying proteins (lipoproteins) is proposed to be involved in the progression of age-related diseases, like atherosclerosis and Alzheimer [10,11]. Eventually, increased oxidative stress and radical formation will lead to damaged DNA, proteins and lipids, which will finally result in impaired cell

functionality [12]. Not only can increased levels of iron result in oxidative stress, also cellular oxidative stress can initiate transferrin-mediated iron uptake [13,14]. Others have shown that due to increased oxidative stress or upon glutathione (GSH) depletion, the iron regulatory protein 1(IRP-1) activity and subsequently TfR protein expression becomes upregulated, which will result in elevated iron uptake [15]. A relation between iron and cellular aging has indeed been reported. Increased levels of iron have been detected also in senescent fibroblasts [16,17] and in replicatively aged yeast [18], which might attribute to the senescence-associated increase of oxidative stress [19-22]. The objective of the present study was to further validate the increase of cellular transferrin level upon senescence and to gain insight in the mechanism(s) responsible for transferrin cycling during the growth towards senescence in an endothelial cell system. The obtained data has provided biological information about the Tf pathway and enable the design of further studies to gain more knowledge about this mechanism in relation to endothelial aging.

Material and methods

Cell culture conditions

Initially, Human Umbilical Vein Endothelial Cells (HUVECs) were isolated from umbilical veins and cultured until senescence as described previously [3]. Additionally, each passage was suspended in EGM-2 (Cambrex, NJ, USA) containing 10% DMSO and frozen in -80 ^{0}C. The number of cell divisions towards senescence did not seem to be affected significantly by freezing and storage of the cells. Prior to analysis, cells were thawed and cultured in EGM-2 at 37 ^{0}C in 95% relative humidity and 5% CO_2 until a low Population Doubling (PD 4-7) or high Population Doubling (PD 15-17) was reached (Population Doubling (PD= (ln[number of cells seeded]-ln[number of cells harvested])/ln2)). If transferrin depletion was necessary, cells were subjected to serum-free growth medium (EGM-2) for 0, 0.5, 1 hr and overnight (o/n) at 37 ^{0}C in 95% relative humidity and 5% CO_2 .

Western blot analysis

Cells were scraped in HUVEC lysis buffer containing 25 mM Tris-HCL pH 8, 100 mM NaCl, 10 mM EDTA, 1 mM Na_3VO_4 and Complete Protease Inhibitors Cocktail tablet (Roche, Mannheim, Germany) at 4^{0}C and subjected to two freeze/thaw cycles in liquid nitrogen to ensure full cell lysis. Thereafter, extracts were centrifuged for 10 minutes at 14,000 g at 4 ^{0}C to remove cell debris. Supernatants were stored at -80 ^{0}C until further use. Prior to SDS-PAGE electrophoresis, samples were boiled in SDS sample buffer (150mM Tris-HCl pH 6.8, 2.5 % SDS, 100 mM DTT) for 5 minutes at 95 ^{0}C. Cell lysates were loaded on 10% SDS-PAGE gels of 8 cm by using a mini-Protean gel system (BioRad, Hercules, CA, USA). Separation was achieved at a constant voltage of 200 mV until bromo-phenol blue dye reached the end of the gel. Proteins were transferred onto a PVDF membrane in a Trans-Blot transfer cell (BioRad) within 80 minutes at 100V. Membranes were incubated in block buffer (TBS, 0.1% tween-20, 3% Marvel), followed by incubation with primary anti-body in block buffer for 1 hr at RT. Thereafter membranes were washed 3 times for 5 min in wash buffer (TBS 0.1 % tween-20, 0.3% Marvel) and incubated with the secondary anti-body for 1 hr in wash buffer. After the last anti-body incubation membranes were washed in wash buffer and subjected to Enhanced Chemiluminescence reagent (PerkinElmer) for 1 minute. Thereafter bands of interest were detected by exposure of the membranes to a light sensitive film (KODAK). Primary antibodies were diluted up to 1:2000 for the human anti-transferrin receptor (Stressgen Bioreagents, USA) and 1:1000 for

the sheep-anti-bovine transferrin (Bethel laboratory). Actin was used as loading control and detected with primary anti-actin (ICN Biochemicals) diluted upto 1:2500. Secondary antibody GAR-PO (Jackson Immunoresearch) was diluted 1:5000.

Transferrin internalization rate

HUVECs were cultured in 6 wells plates and transferrin depleted as described previously. EGM-2 medium was changed into EGM-Hepes (25 mM HEPES pH7.4) to perform further experiments under atmospheric conditions. Subsequently, cells were incubated with 2 µg/ml human ^{125}I-Tf (Amersham Biosciences, Upsalla, Sweden) in EGM-hepes for 0, 3, 6 and 9 minutes at 37 ^{0}C, followed by 5 minutes on ice. After washing with ice-cold PBS cells were subjected twice to an acid-wash (25 mM NaOH/ HAC pH 3.8, 150 mM NaCl) at 4^{0}C to remove and collect surface bound transferrin from the plasma membrane. Intracellular transferrin was obtained by solubilization of the cells with warm 1M NaOH. Acid wash (representing surface bound transferrin) and cell lysate were counted in a γ-counter (Perkin Elmer). The slope of the dependence of this ratio in time represents the specific rate constant for internalization [23].

Transferrin release into the medium

HUVECs were cultured in 6 well-plates and transferrin depleted as described in paragraph 2.1. Thereafter cells were incubated with 8 µg/ ml ^{125}I-Tf (Amersham Biosciences, Upsalla, Sweden) in EGM-2-hepes for 30 minutes, followed by cooling down on ice to stop the internalization. After washing with ice-cold PBS cells were subjected twice to an acid-wash (25 mM NaOH/ HAC pH 3.8, 150 mM NaCl) at 4 ^{0}C. Thereafter cells were washed once with ice-cold EGM-2-Hepes, followed by incubation with 2% serum containing EGM-2 at 37^{0}C. Medium was collected after 1, 3, 6, 9 minutes. Subsequently cells were subjected to ice-cold acid wash (representing surface bound transferrin), followed by cell lysis with 1 M NaOH at 37 ^{0}C. Collected medium, acid wash and cell lysate were counted in a γ-counter (Perkin Elmer, Boston, USA).

Fluorescence microscopy

Cells were grown on fibronectin-coated coverslips until subconfluency in EGM-2 at 37^0C in 95% relative humidity and 5% CO_2. Thereafter cells were subjected for 60 min to serum-free EGM-2 medium, to deplete cells from intracellular transferrin. Subsequently, cells were incubated with Alexa Fluor 488-conjugated human transferrin for 30 min. followed by fixation in 4% paraformaldehyde for 20 minutes and a wash in PBSG (1 x PBS, 50 mM Glycine). Subsequently, cells were washed in blocking buffer and incubated with 0.2 µg 4', 6-diamino-2-phenylindole (DAPI) in PBS at 37^0 C for 5 minutes Thereafter coverslips were washed once in distilled water and mounted with Mowiol-PPD. Examination of cells was performed with a Carl Zeiss Confocal microscope.

Results

Elevated levels of transferrin towards senescence

To confirm the increase in bovine transferrin (B-Tf) in HUVECs during the growth towards replicative senescence as shown in our previous proteomics study [3], HUVECs were grown towards senescence and analyzed on the presence of B-Tf with an anti-bovine transferrin antibody. The western blot (WB) results, shown in figure 1A and B, clearly show that towards senescence the intracellular bovine transferrin is increased and confirms the elevated abundance in our previous proteomic study. Two likely explanations for the increase in B-Tf in late passage HUVECs might be an increased expression of the Tf-receptor in senescent cells or differences in B-Tf internalization rates between young and old cells. As shown in figure 1, there is a decrease in the abundance of the transferrin receptor in late passage cells, which makes the first explanation unlikely. Tf recycling will be studied in the next paragraphs.

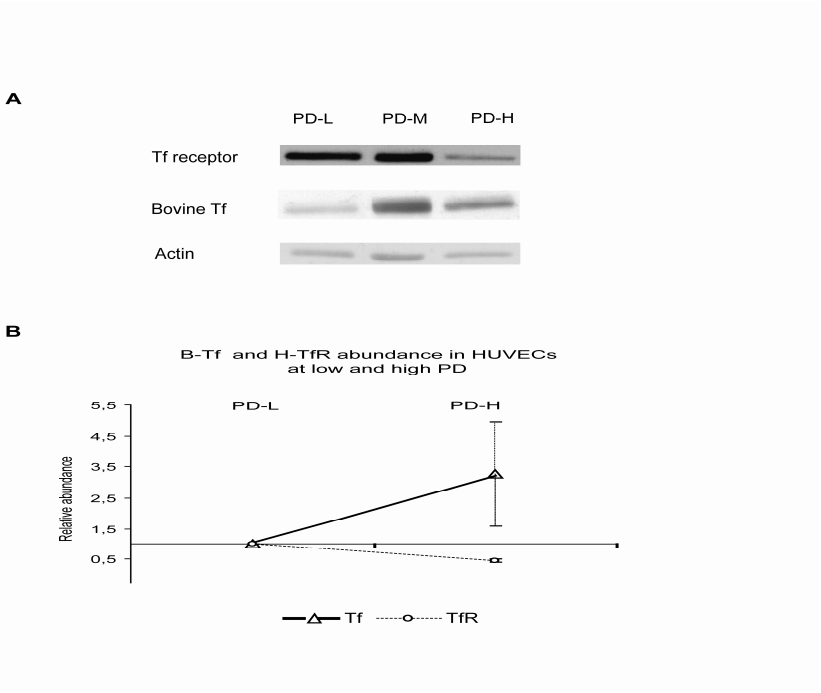

Figure 1 Increased intracellular abundance of bovine transferrin in late passage HUVECs.
(A) Bovine transferrin (B-Tf) abundance in early (PD-L), middle (PD-M) and late passage cells (PD-H) detected with sheep anti-bovine transferrin. Actin was used as loading control. Transferrin becomes upregulated towards replicative senescence. (B) Western blots were scanned and abundance was evaluated with densitometry. B-Tf and H-TfR abundances were compared to the abundance of the actin loaded control. Graph represents the result of two different HUVEC isolations.

Interaction of bovine Tf with human TfR

It is known that human transferrin is primarily endocytosed via the Tf- receptor (TfR) [24] and that the interaction between these two strongly depends on the c-lob domain of transferrin [25-29]. In order to find out whether bovine Tf (B-Tf) becomes internalized also via the human TfR system, we have performed a sequence alignment with Clustal W (EBI-Heidelberg) between the receptor binding domain of human transferrin [25,27] and the binding domain of bovine transferrin. In figure 2A is shown that there is extensive homology between the receptor binding C- lob domain of human Tf (H-Tf) and bovine Tf, which suggests that bovine Tf is able to interact with the human Tf receptor, which is in line with earlier findings [30]. Next to this, we have exposed early and late passage HUVEC to human serum medium conditions after the cells were grown with growth medium containing bovine serum. Western blotting in combination with anti- bovine Tf detection revealed that upon prolonged exposure of cells to human serum the intracellular amount of bovine -Tf becomes diminished (see figure 2B).

Internalization and externalization of human ^{125}I- Tf in young and old HUVECs

In order to unravel possible age-related differences in the transferrin cycling pathway we have conducted experiments with ^{125}I- labeled human transferrin (H-Tf). Cells were serum starved for 60 minutes and subjected to medium containing ^{125}I-labeled transferrin without serum. The plasma membrane bound transferrin fraction and intracellular transferrin fraction were determined at each sampling interval and used to calculate the internalization rate constant [23]. Figure 3A shows clearly that early passage cells seem to internalize transferrin at a slightly higher rate than late passage cells. In addition to this, we have determined also the amount of transferrin released in the medium by young and old HUVECs. For this purpose we have serum starved early and late passage cells for 1 hr. Thereafter cells were loaded with ^{125}I labeled human transferrin for 30 minutes followed by incubation with serum-enriched growth medium to prevent recycling of the labeled apo-transferrin. No radioactivity was recovered in the acid wash, indicating that no detectable amounts of Tf bound to the plasma membrane could be found. Externalization rates were based on the ratio between the amount of apo-transferrin in the medium and total Tf (= the amount of intracellular transferring plus amount released). Figure 3B shows clearly that the amount of released Tf by young and old HUVECs are similar.

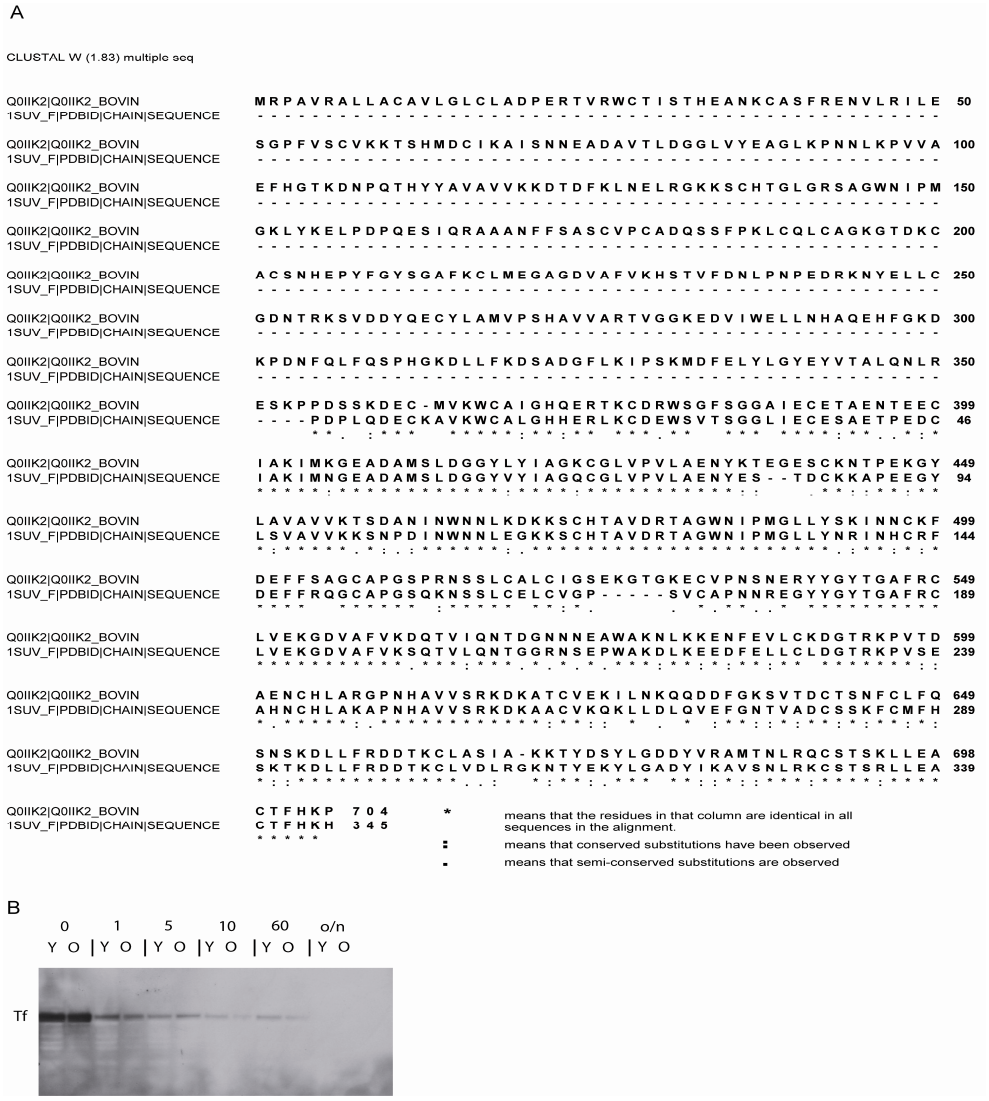

Figure 2 A) Alignment of human C-lob domain (1SUV-F) with bovine transferrin (Q0IIK2) with Clustal W (EBI, Heidelberg, Germany). There is sufficient homology of the C-lob domain in bovine Tf to conclude that bovine Tf will interact with the human TfR. B) Early (Y) and late (O) passage cells were cultured under normal bovine serum medium conditions. Subsequently cells were exposed to human serum for 0, 1, 5, 10, 60 min and overnight (o/n). Cell lysates were monitored with an anti-bovine transferrin antibody. Upon prolonged exposure to human serum the intracellular bovine-Tf abundance becomes less in time.

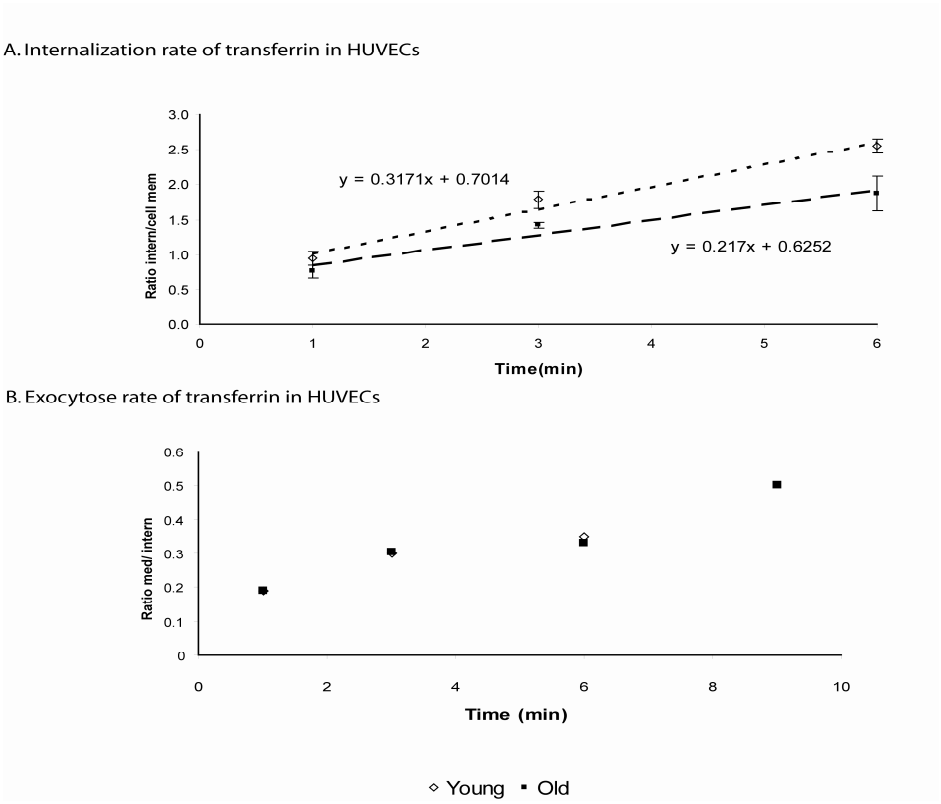

Figure 3 Transferrin recycling kinetics in early and late passage HUVECs (A) *Internalization rate of transferrin in young and old huvecs.* Tf depleted cells were incubated in a 6 well plate for 1, 3 and 6 minutes with culture medium containing ^{125}I-Tf. The ratio between intracellular Tf and surface bound Tf was calculated and plotted against time. The slope of the regression curve represents the internalization rate constant. Error bars represent the SD of a triplicate. Clearly, old huvecs showed a smaller internalization rate constant than young cells. (B) *Fraction of transferrin released into the medium by early (young) and late (old) passage huvecs*. Tf depleted cell were loaded with ^{125}I-Tf for 30 min. and subsequently subjected to serum-free growth medium for 1, 3, 6 and 9 minutes. The ratio between Tf released into the medium and intracellular Tf was calculated and plotted against time. There are no differences observed in amount of Tf released into the medium between young and old HUVECs.

Uptake of Alexa 488 human Tf conjugate visualized with confocal microscopy

Furthermore, to gain insight at individual cell level, we have performed immunofluorescent experiments in which we subjected transferrin-depleted HUVECs to a human transferrin-Alexa 488 conjugate (see figure 4) for 1, 5, 10 and 30 min. The results obtained were similar to the radioactivity assay and evaluation with confocal microscopy revealed that late passage cells take-up H-Tf at a lower rate as the early passage cells.

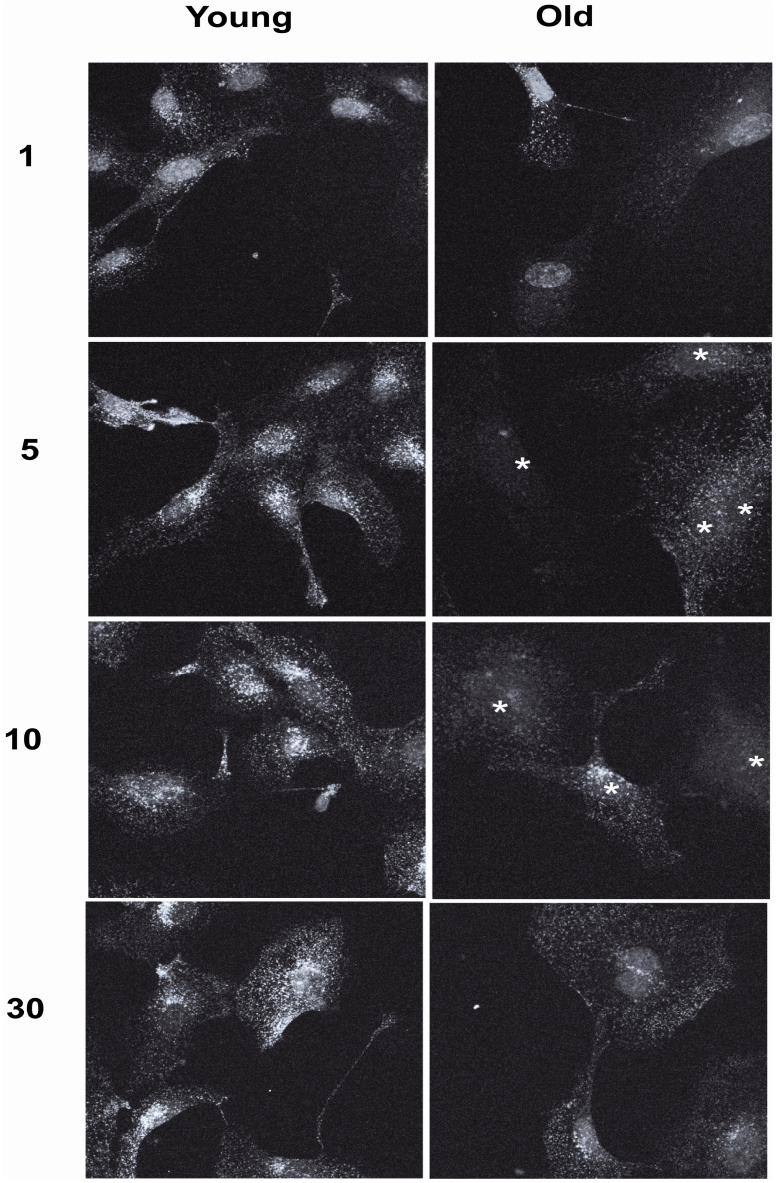

Figure 4 Internalization of transferrin in young and old huvecs followed with confocal microscopy Tf-depleted cells were subjected for indicated times (1-30 min.) to Alexa 488 conjugated human transferrin, whereas DAPI staining was applied for localization of nuclei (indicated with open star when not clearly visible). Confocal microscopy was used to monitor the internalization of transferrin in time. Obviously, young cells seem to endocytose transferrin much faster than old cells.

Discussion and conclusions

In the present study we have detected elevated levels of bovine serum transferrin in senescent HUVECs, which is in agreement with the proteomics results from our previous study [3]. Besides, others have detected increased concentrations of Tf also in retinas from older individuals exhibiting age-related macular degeneration [31]. Since transferrin is the major route for entrance of iron into the cell, an increase in cellular transferrin is also in line with findings that elevated iron levels were detected in senescent fibroblasts, HUVECs [16,17] and replicatively aged yeast [18]. A simple explanation for the increase in cellular Tf might be an increased expression of the Tf-receptor. However, the observations as shown in figure 1 indicate that this is not the case. In contrast, a down-regulation of the transferrin receptor is observed in senescent cells. So, to gain further insight in the mechanisms for the increased cellular Tf level, we evaluated the Tf recycling mechanism in relation to replicative senescence. Internalization experiments with human ^{125}I- transferrin revealed that early passage cells were able to endocytose Tf faster than the late passage cells (figure 4). Similar results were obtained when transferrin depleted cells were incubated with human Alexa 488-conjugated (holo)-transferrin. Furthermore, the exocytosis experiment (figure 4) with human ^{125}I-Tf did not reveal obvious differences between early and late passage cells. Taking together, the obtained results regarding receptor abundance and rate of internalization and exocytosis are not giving an explanation for the observed increase in cellular Tf content in old cells.

This suggests that either changes in the Tf-TfR stochiometry occurs with cellular senescence or an alternative pathway arises upon cellular senescence. Under normal conditions the Tf-TfR stochiometry is 2:1 [25], but unfortunately limited or no experimental data is available about this under senescence conditions. Another explanation might be the difference in transferrin species. The increased intracellular abundance was observed for bovine transferrin, whereas the recycling kinetics as well as the confocal experiments were performed with human transferrin. Although, clear indications exist that both forms of Tf can use the H-TfR pathway to enter the cell (see results section) it is still possible that the recycling capacity is less for the B-Tf/H-TfR compared to the H-Tf/H-TfR complex. Recycling experiments with ^{125}I-labelled bovine transferrin should be performed in the future to unravel this.

Non-receptor mediated Tf transport

As mentioned earlier, a possible explanation is that a part of the transferrin becomes internalized via a receptor-independent pathway, which would explain the observed decrease in TfR abundance in combination with elevated levels of B-Tf as depicted in figure 1. In view of this, we suggest that the clathrin-mediated transport of Tf is still active towards senescence, but that in time the endothelial cell becomes more susceptible for Tf internalisation via an alternative pathway, as depicted in figure 5.

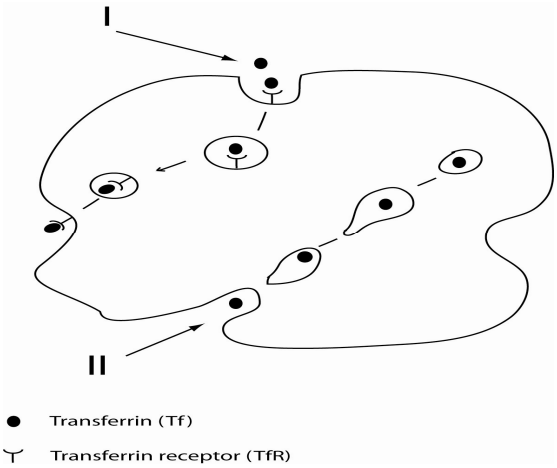

Figure 5 Senescence associated endocytosis of transferrin During the growth towards senescence cells might endocytose Tf via the clathrin mediated pathway (I) as well as an alternative receptor independent pathway (II).

One option might be fluid-phase endocytosis, in which non-specifically plasma membrane bound molecules are invaginated by the cell [32]. Interestingly, there are suggestions that severe oxidative stress can induce this process in endothelial cell systems [33]. Since senescence is accompanied with signs of increased oxidative stress [19,34], it might be possible that fluid phase endocytosis (FPE) becomes increased in course of time. In line with this we have performed some initial experiments with the well-known pinocytosis tracer dextran-FITC, which indicated that the uptake is somewhat faster in senescent endothelial cells. However, more experimental data is required to unravel this more thoroughly. Another alternative is proposed by Raje and colleagues, who found that in macrophages iron-loaded transferrin can be internalized also by the glycolytic enzyme GAPDH [35]. Although endothelial cells differ from macrophages it might be useful to evaluate this possibility also in future experiments.

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Chapter 4

Differential protein profiles during healthy aging in human plasma: Changes in protein expression and processing

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Manuscript in preparation

Abstract

With increasing age, an individual is subjected to numerous changes at the molecular, cellular and tissue level. Disturbances in an organism are expected to be reflected in part in the protein composition of the blood plasma (the plasma proteome). To understand the mechanisms of healthy aging, several studies have focused on identifying these changes, mainly by hypothesis driven research. Although some changes in protein expression have been identified, these studies have failed to acknowledge the presence of post-translational modifications (PTMs), protein isoforms and/or fragments of a single gene product in this complex proteome. Here, we describe the proteomics analysis of pooled human plasma samples, depleted of human serum albumin and IgG, of young, middle aged and old individuals by two-dimensional difference in gel electrophoresis (2D-DIGE), which enables accurate differential protein expression analysis including differences in (splice) isoform abundance. This resulted in the identification of an interesting set of differentially expressed protein spots, of which a number were primarily a result of post-translational modifications rather than protein expression. The most prominent differences were found for complement C3 (CO3), which turned out to be increasingly processed upon aging. Functional annotation in relation to the aging process showed that a pro-inflammatory as well as a pro-coagulant state might be associated with aging. Next to this, the results of this study suggest that protein isoform regulation might provide new knowledge about the aging process, which will contribute to the development of interventions to preserve healthy aging.

Introduction

The aging process is one of the inevitable events of life, although little is known about the precise mechanisms responsible for this phenomenon. Currently, it is generally accepted that aging is associated with a progressive malfunctioning of several biological mechanisms [1]. Over time, an organism is subjected to numerous damaging agents that either have an endogenous origin, like the generation of reactive oxygen species during oxidative phosphorylation in the mitochondria or during an immune response [2-4], or have an environmental origin, like radiation and chemicals [5-8]. Despite the presence of damage prevention and damage repair mechanisms, in time damage will accumulate, ultimately resulting in an overall decrease in the functionality of the organism. Consequently, aging is clearly accompanied with a decline in vitality. The decline in functionality is associated also with an increase in vulnerability towards age-related disease development, which has a huge impact on the society, both socially and economically.

Therefore, a lot of research has been focused on identifying the changes at the molecular level that occur during the aging process. Insight in these changes could aid in understanding the mechanisms underlying the aging process and facilitate the design of new intervention studies to preserve healthy aging. Still, the complexity of this biological process, amongst others caused by variations in life style and genetic make-up, demand multiple independent aging markers which cover the several aspects of the aging process [9]. In view of this, we aimed in this study for identifying molecular markers in relation to healthy aging in human blood plasma by using advanced proteomics techniques. Blood plasma is one of the most important and most accessible human clinical biological samples available and has already been used in a number of aging studies. In these studies age-related expression differences have been found, especially for several cytokines and other proteins involved in the immune response [10-14]. Most of these results were obtained by using immunological assays, which are usually very specific, sensitive and accurate in providing quantitative information. However, this approach is clearly focusing on predefined targets and is furthermore less well able to deal with the presence of differences in post-translational modifications and/or isoforms, which is very common for plasma proteins [9,15]. More information about these protein isoforms in relation to aging might be obtained when plasma samples are subjected to comprehensive proteome analysis. A well-established protein separation technology is two-dimensional gel electrophoresis (2DGE), which allows

the separation of hundreds of proteins simultaneously [16] and more importantly, is highly suitable to detect protein isoforms. Moreover, removal of highly abundant proteins such as serum albumin and IgG has significantly improved 2D gel resolution and has increased the sensitivity of this technique [17-19]. Furthermore, advanced protein labeling techniques have become available, which enable the detection of small expression differences with a high confidence level. In 2-D Difference In Gel electrophoresis (2D-DIGE) experimental variation is reduced due to the combination of multiple sample analysis in a single gel and internal standard correction [20-22]. Here, we describe the differential expression analysis with 2D-DIGE of plasma samples of young (17-28 years old), middle aged (45-55 years old) and old (85 years old) individuals all in good health. Proteins differentially expressed during aging were positively identified with mass spectrometry.

Materials and Methods

Plasma samples

Citrate plasma samples, which were part of the Leiden 85+ study, were obtained from 45 individuals that were divided in three different age groups, 17-28 years, 45-55 years and an 85 years old group, of 15 individuals each. Samples of each group were pooled and depleted of human serum albumin (HSA) and immunoglobulin G (IgG) before 2D-DIGE analysis.

HSA and IgG depletion

To obtain better resolution on a 2D-gel, human serum albumin (HSA) and IgG were removed from the plasma samples with affinity resin based on Llama heavy chain antibody (VHH) technology as described before [19]. The VHH anti-IgG resin (dynamic antigen capacity 10mg/ml) and anti-HSA resin (dynamic antigen capacity 12mg/ml) were mixed in a 1:2 ratio. The mixed affinity resin was washed extensively prior to use with ten times column volumes of Tris buffer (0.1M Tris/HCl pH 8.5; 0.5M NaCl) for three times followed by three washes with acetate buffer (0.1M sodiumacetate pH3.0; 0.5M NaCl). Thereafter, the affinity resin was washed three times in citrate buffer to neutralize the pH. Plasma samples were diluted 100 times in ice-cold citrate buffer and incubated at 4°C for 30 minutes with 50µl bedvolume per 100µl diluted sample. After incubation, the non-bound fraction was taken for differential protein analysis. The protein content of these samples was determined with a BCA protein assay (Pierce, Rockford, U.S.A.) according to the instructions of the manufacturer.

Protein labeling

In order to obtain the optimal reaction conditions, 50µg of each protein sample was treated with the Clean-up kit (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Protein pellets were dissolved in 15 µl buffer (30mM Tris/HCl pH8.5, 7M urea, 4% CHAPS). Cy5, Cy3 and Cy2 labelling was performed according to the instructions of the manufacturer (Amersham Biosciences, Roosendaal, The Netherlands). The labeled samples were diluted in rehydration solution (7M urea; 2M thiourea; 4% CHAPS; trace bromophenol blue; 0.5% (w/v) DTT; 0.5% (v/v) ampholytes pH 4-7; 1.2% (v/v) Destreak (Amersham Biosciences, Roosendaal, The Netherlands)) to a final volume of 450µl.

Two dimensional gel electrophoresis

Immobiline Dry strips pH 4-7 of 24 cm (Amersham Biosciences, Roosendaal, The Netherlands) were rehydrated overnight with the labeled protein samples and overlaid with 2ml Coverfluid in an Immobiline Drystrip reswelling tray. The first dimension of isoelectric focusing was run on an IPGphor system. Optimal protein focusing was achieved by starting at 500V for 1 hr, followed by 1500V for another hour. Subsequently, a constant voltage of 8000V was applied until a total of 48kV. Thereafter, the strips were equilibrated in equilibration buffer (50mM Tris/HCl pH8.8; 6M urea; 30% glycerol (v/v); 2% SDS (w/v)) containing 65mM DTT for 15 minutes followed by an incubation with 135mM iodoacetamide for 15 minutes. The second dimension was performed with lab-cast 24cm 12.5% polyacrylamide gels. Strips were loaded onto the gels and sealed with a solution of 1% agarose (w/v) containing a trace of Bromophenol Blue. The gels were run overnight on the Ettan DALT Twelve system (Amersham Biosciences, Roosendaal, The Netherlands) at 1W/gel till the Bromophenol Blue dye front reached the bottom of the gel.

Gel imaging and differential analysis

All gels were scanned in between low fluorescent glass plates at 100µm pixel resolution with the Typhoon image scanner 9400 (Amersham Biosciences). The Cy5 images were scanned using a 633nm laser and a 670nm BP30 emission filter. Cy3 images were scanned with a 532 laser in combination with a 580nm BP30 emission filter. Cy2 images were scanned using a 488nm laser and an emission filter of 520nm BP40. The photomultiplier tube was set to achieve maximum sensitivity without pixel saturation. Silver stained images were analyzed with a GS-710 Calibrated Imaging Densitometer (Biorad, Hercules CA, USA). Prior to differential analysis, images were cropped with ImageQuant (Amersham Biosciences, Roosendaal, The Netherlands) to remove insignificant parts of the gel. Subsequently, images were subjected to median filtering in Image Quant Tools to remove dust-related pixels. Differential analysis was performed with the Decyder V 5.01 (Amersham Biosciences) software package. Paired analysis of variance (RM)ANOVA was applied to extract relevant expression profiles.

Spot picking and in-gel digestion

Spot picking was performed with post-stained silver images of CyDye labeled gels to minimize the risk of mismatched spot picking. Silver staining was performed according to Shevchenko [23]. Spots of interest were subjected to in-gel tryptic digestion as described previously [24].

Nano LC-MS/MS and protein identification

Nano LC-MS/MS was performed with an Agilent 1100 series LC system (Agilent, Palo Alto, U.S.A.) coupled with a Thermo Finnigan LTQ (Thermo electron Company, Waltham, MA, U.S.A.) or LTQ-FTICR mass spectrometer as described previously in literature [24]. Briefly, peptide extracts were acidified with 0.1M acetic acid and injected on a trap column (Aqua^{TM} C18 RP (Phenomenex, Torrance, U.S.A.), 20 mm x 100 µm ID) at 5µl/min. Subsequently, the peptides were transferred with a split-reduced flow rate of 100nl/min to the analytical column Aqua^{TM} C18 RP (Phenomenex, Torrance, USA), 20 cm x 50 µm ID. Elution of the peptides was achieved with a linear gradient from 0-50% B (0.1M acetic acid in 80% (v/v) acetonitrile) in 60 minutes. The column effluent was directly introduced into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives, Woburn, MA, USA). The mass spectrometer was operated in the positive ion mode and parent ions were selected for fragmentation in data-dependent mode. The obtained mass spectra were subjected to a Mascot search engine [25] with 0.8 Da for LTQ or 10 ppm peptide tolerance in case of LTQ- FTICRMS, allowing two miss-cleavages, carbamidomethylation and methionine oxidation as variable modifications. Protein annotation was performed by using the SwissProt database.

Western blot analysis

Equal amounts of plasma were size-separated on a 10 or 15% SDS poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer (2.5% protifar plus (w/v) in PBS), the membranes were soaked in MeOH and subsequently stained with Coomassie brilliant blue (CBB) [0.1% (w/v) Coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with a primary antibody for one hour and subsequently washed 5 times in PBST (0.05% Tween-20 in PBS). Thereafter, membranes were incubated with HRP-conjugated donkey anti-rabbit/donkey secondary antibody (Jackson Immunoresearch Laboratories, Westgrove, U.S.A.) for one hour and the wash steps were repeated. Two additional washes were performed with PBS and bound antibodies were visualized by enhanced chemo-luminescence (PerkinElmer, Boston, U.S.A.). Primary antibodies were diluted up to 1:2000 for the anti-CO3 (ab23891, Abcam, Cambridge, United Kingdom), 1:2000 for the anti-C3dg (kindly provided by M. Daha), 1:2000 for the anti-fibrinogen (F4639, Sigma), 1:4000 anti-Haptoglobin (H5015, Sigma) and 1:1000 for the anti-alpha-1- Antitrypsin (A-0409, Sigma). The secondary antibody GAR-PO (Jackson Immunoresearch) was diluted 1:5000.

Results

Proteomic analysis

To analyze age-related blood proteome changes, plasma samples of healthy individuals of three different age-groups were compared. These groups comprised a young (Y) (17-28 years old), a middle aged (MA) (45-55 years old) and an old (O) (85 years old) group, each consisting of 15 individuals. The plasma samples within each group were pooled and cleared from HSA and IgG by using VHH antibody technology [19] to increase 2D-gel resolution and sensitivity. Depleted protein extracts were subjected to the Ettan DIGE system and generated a plasma proteome map of about 750 protein spots. Analysis of variance (ANOVA) was used to compare protein expression differences between the three different age groups. Expression profiles with an ANOVA p-value of ≤ 0.05 were considered as significant and resulted in 89 protein spots which exhibited age related expression differences. Of these spots, 46 were positively identified *in duplo* and linked to their molecular weight and abundance dynamics (Figure 1 and Table 1). The majority of this subset showed an increased abundance towards aging, ranging from 1.2-10 fold and seventeen spots showed a decreased expression at old age Analyzing protein expression dynamics with 2D-DIGE offers the possibility to visualize or evaluate the expression dynamics of protein isoforms. Plasma proteins are known for their variety in isoform abundance [9,15] and protein identification of the spots of interest indicated that the majority of the abundance differences could be assigned to specific protein isoforms or protein fragments (Figure 1 and Table 1). Eighteen unique proteins were identified and the majority of the abundance differences were caused by haptoglobin, fibrinogen, transferrin, alpha-1-antitrypsin and complement C3. For most of these proteins, the trend of regulation of the different isoforms was in general the same (Figure 2A-E), although some revealed divergent patterns. Haptoglobin (HP), an acute phase protein that has many isoforms mainly caused by its glycosylation pattern, showed patterns that either gradually increased or decreased upon aging (Figure 2D).

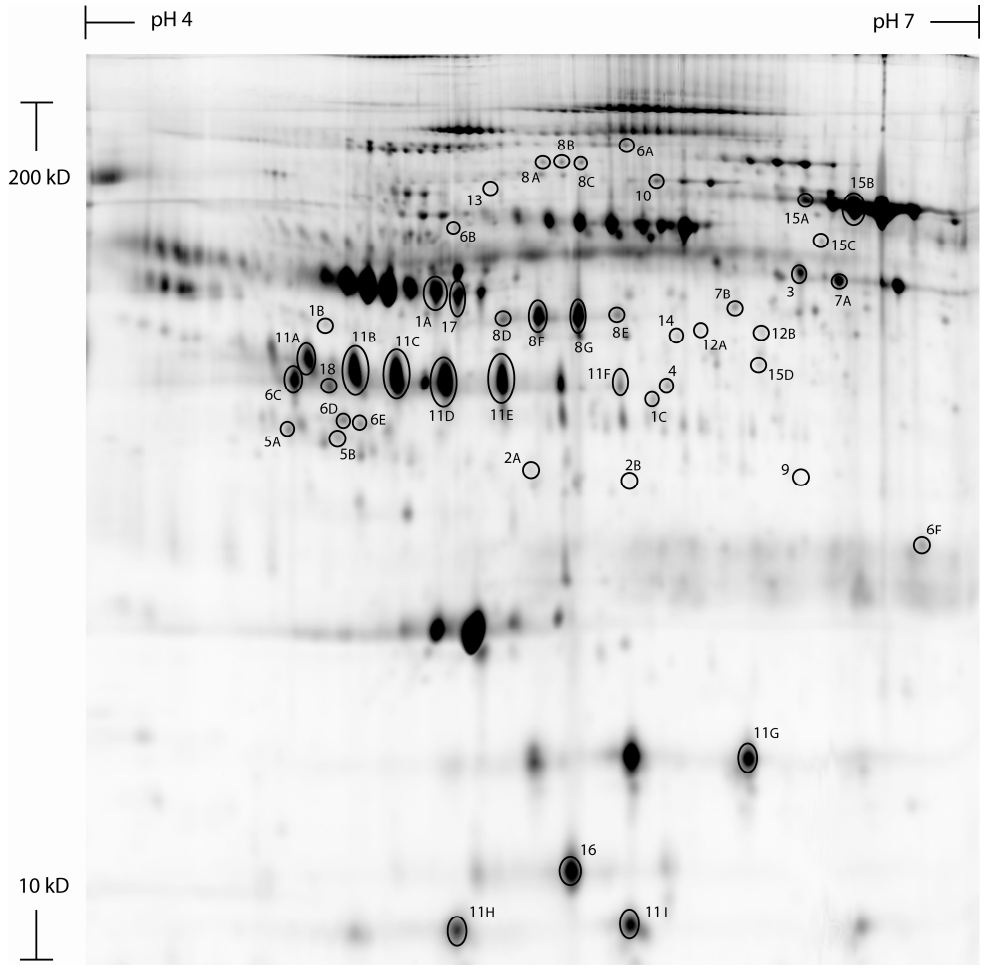

Figure 1: 2D-DIGE analysis reveals differential expression between the young and the old group of 89 different protein spots of which 46 were positively identified *in duplo*. Corresponding spot information is presented in Table 1.

Protein nr.		Protein name	Acc. nr.	Mw (th.)	Reg. O/Y	ANOVA	SC%	Pep
1	A	Alpha-1-anti-trypsin	P01009	46737	0.8	7.9E-04	62	23
	B				0.6	3.8E-05	23	11
	C				4.2	2.2E-03	23	12
2	A	Apo-E	P02649	36268	1.3	1.8E-03	54	22
	B				0.6	3.1E-04	58	23
3		Apolipoprotein H	P02749	39610	1.2	1.7E-02	52	19
4		Apolipoprotein-L1	O14791	43927	0.7	1.1E-03	24	23
5	A	Clusterin	P10909	53065	0.9	3.1E-02	32	14
	B				1.3	2.0E-02	28	14
6	A	Complement C3	P01024	188704	0.8	7.5E-04	27	48
	B				0.3	3.3E-03	17	39
	C				5.1	6.1E-04	16	32
	D				6.4	7.9E-06	7	14
	E				9.6	3.3E-07	7	14
	F				4.4	5.1E-07	3	8
7	A	Fibrinogen Beta chain	P02675	56613	1.4	5.5E-03	60	33
	B				1.8	1.1E-04	22	12
8	A	Fibrinogen gamma chain , alpha	P02679	52139	1.9	3.0E-04	39	20
	B				1.9	6.7E-05	35	18
	C				1.9	2.1E-03	38	19
	D				2.0	2.0E-04	50	26
	E				1.7	1.2E-04	37	20
	F				1.6	2.4E-05	52	32
	G				1.5	4.2E-05	62	35
9		Ficolin	O75636	33381	0.6	1.2E-03	17	6
10		Gelsolin	P06396	85698	1.3	2.5E-03	39	29
11	A	Haptoglobin	P00738	45205	1.2	1.4E-06	37	26
	B				1.3	7.6E-05	40	36
	C				1.3	9.8E-05	37	23
	D				1.3	7.4E-05	45	25
	E				1.3	3.2E-05	37	22
	F				0.7	1.2E-04	37	18
	G				0.8	8.3E-04	23	12
	H				1.7	2.5E-08	14	9
	I				2.5	7.0E-08	11	8
12	A	IgM	P01871	50242	1.5	3.6E-06	27	12
	B				0.7	2.0E-04	17	9
13		Inter-alpha-trypsin inhibitor H4	Q14624	103358	1.3	3.8E-04	16	14
14		Pigment epithelium-derived factor	P36955	46513	1.3	4.5E-04	36	15
15	A	Transferrin	P02787	79332	0.7	1.4E-06	46	48
	B				0.8	2.2E-02	70	82
	C				0.7	4.0E-02	45	41
	D				0.7	5.0E-02	20	15
16		Transthyretin	P02766	16001	0.8	2.5E-04	68	10
17		Vitamin D-binding	P02774	54561	0.7	9.9E-04	67	34
18		Zinc alpha 2 glycoprotein	P25311	33872	1.3	8.5E-04	54	22

Table 1: *In duplo* identified differentially expressed protein spots between the young and old group in human plasma. The differential expression of the old group is normalized to the expression in the young group (Regulation O/Y). The accession number, molecular weight of the unprocessed precursor (Mw unproc), the statistical significance (ANOVA), sequence coverage (SC%) and the amount of identified peptides (Pep) are indicated.

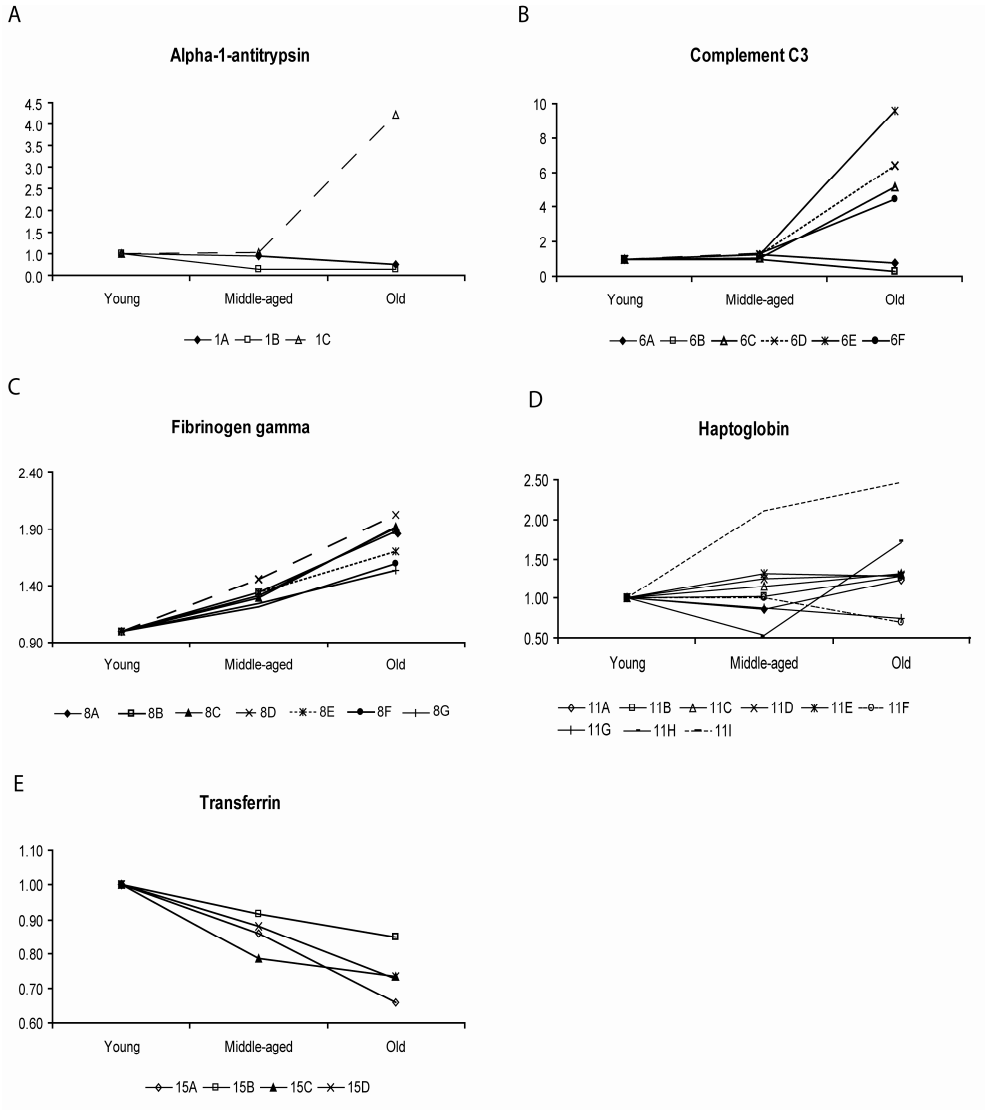

Figure 2: The expression patterns in the different age groups of several different isoforms of alpha-1-antitrypsin (A), CO3 (B), fibrinogen gamma (C), haptoglobin (D) and transferrin (E). Not all isoforms of a single gene product show a similar expression pattern. Some isoforms of alpha-1-antitrypsin (spot 1C), complement C3 (spots 6A and 6B) and haptoglobin (spots 11F, 11G and 11H), show an expression pattern that is not consistent with the majority of the isoforms.

The native form of HP has a molecular weight ± 90 kD and a tetramer consisting of two alpha and two beta subunits. Yet, *in vivo* HP is synthesized as a single polypeptide (known as the HP precursor) containing an alpha and beta chain, which become post-translationally processed extracellular to form the alpha and beta subunits of the native protein [26]. Sequence analysis revealed that spots 11A-F at ± 40-45 kD all consisted only of a beta chain, which is line with data from others [27-30]. Two isoforms of the alpha chain are presented by spots 11G and 11H/I respectively. To verify these results, we have subjected pooled as well as individual plasma samples also to western blotting and anti HP-antibody detection to determine whether the total HP blood concentrations are indeed increased towards healthy aging (Figure 3).

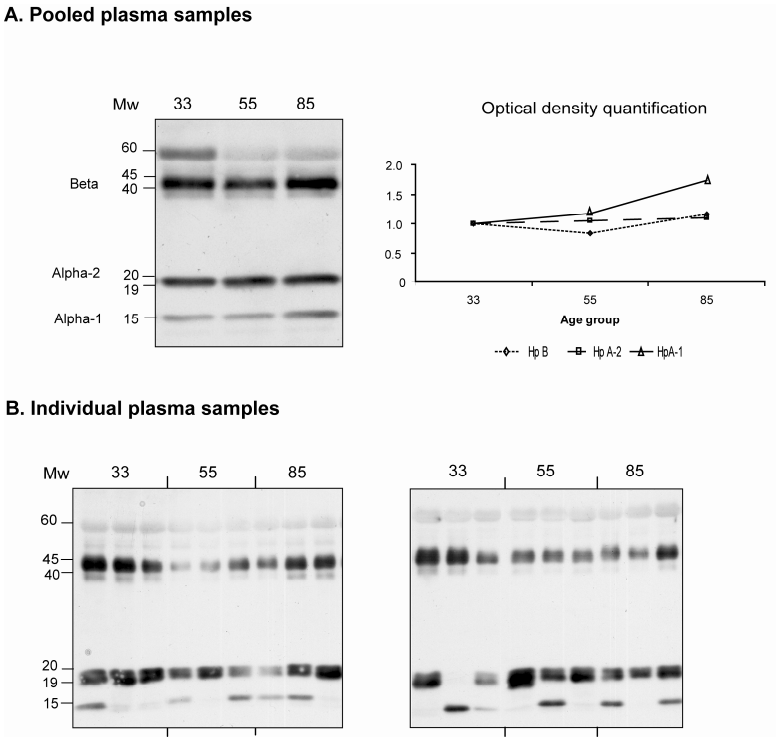

Figure 3. Validation of haptoglobin abundance levels in non-depleted pooled and individual plasma samples on Western blot. Multiple Hp bands at different molecular weights (Mw) could be distinguished. (A) Luminescence signal of pooled plasmas samples after ECL. In addition, the Fluor-S imager (Bio-Rad) was used to quantify the ECL signals. The intensity of all designated bands was increased in the old population. Intensities of 55 and 85 depicted are relative to the intensity at 33. (B) HP pattern in individual plasma samples after ECL.

The western blot analysis of the pooled samples, as depicted in figure 3A, showed that a band of 60 kD becomes strongly downregulated during aging. No HP spots could be found in the DIGE study with this mass, possibly because of masking by other spots. The 60 kD band most likely represents isoforms the HP precursor consisting of the alpha and beta chains. The band at 40-45kD corresponds with the mass of the spots 11A-F and represents the HP beta chain. Chemiluminescence signal quantification of this band showed that the abundance appeared to be altered towards aging, representing the summed effects observed for spots 11A-F and is in agreement with the results of the DIGE study. The bands at ± 20 and ± 15 kD might represent two isoforms of the alpha chain respectively. The spot 11G showed in the DIGE study a small decrease which cannot be confirmed with the band at 20kD. However, the trend of the optical density of the 15 kD band corresponds with the observed increased abundance of spots 11H and 11I in the DIGE. The observed trend from the pooled samples could not be distinguished in the individual plasma samples (Figure 3B) most likely due to the variability in HP abundance.

We also subjected plasma samples to western blot analysis using anti-fibrinogen and anti-alpha-1-antitrypsin antibodies. With the detection of fibrinogen only one band was observed, most likely representing spot 8D-G. In figure 4A analysis of the pooled samples clearly confirmed the upregulation of fibrinogen as observed in the DIGE study. This trend could also be observed in the individual samples of the different age-groups (see Figure 4B), although the abundance differences are less well-defined. Detection of alpha-1-anti-trypsin revealed one clear band, which is depicted in figure 4A for which a downward trend was observed. Although this is in agreement with the DIGE pattern observed for spot 1A, it has to be considered that the protein band will show the summed effect of all alpha-1-antitrypsin spots. So in spite of the heterogeneity it is obvious when analyzing individual samples a similar trend could be determined (see Figure 4B), which indicates that the total amount of alpha-1-antitrypsin might be down-regulated in the old population.

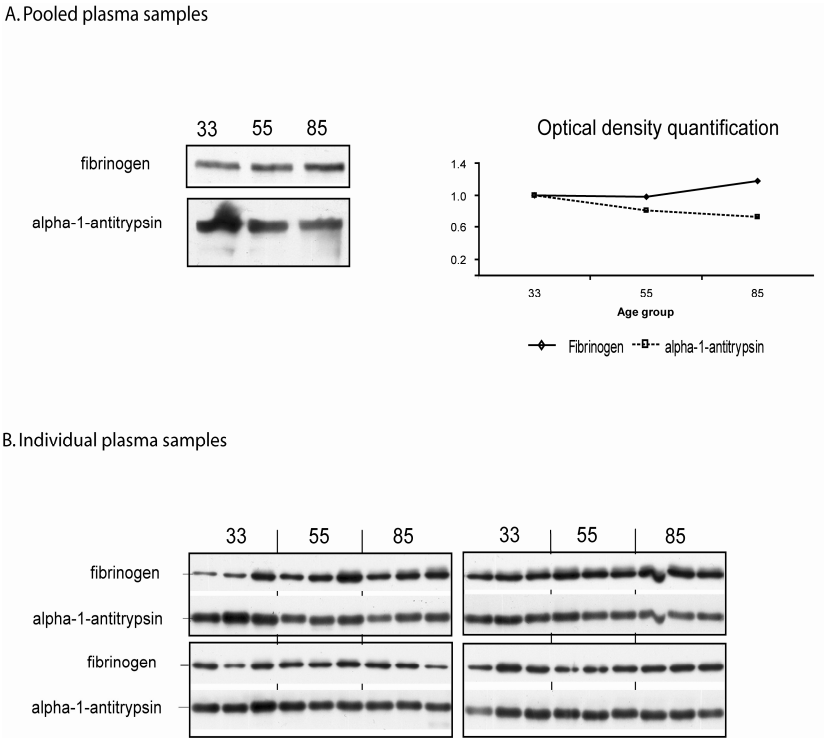

Figure 4. Validation of fibrinogen and alpha-1-antitrypsin abundance levels in non-depleted pooled and individual plasma samples on Western blot. (A) Luminescence signal of pooled plasmas samples after ECL. In addition, the Fluor-S imager (Bio-Rad) was used to quantify the ECL signals. Intensities of 55 and 85 depicted are relative to the intensity at 33. (B) Fibrinogen and alpha-1-antitrypsin abundance levels of individual plasma samples after ECL.

The most substantial differences between the young and old population were determined in a set of spots identified as complement C3 (CO3). In total, six differentially expressed spots were identified as belonging to CO3, of which four exhibited an above four times increase in expression between the young and old group (see Figure 2B). CO3 functionality is regulated by specific proteases that cleave the mature protein (Figure 5) and thereby induce conformational changes, which leads to several different active components, each with different specific functions [31,32]. During these cleavages, soluble polypeptide chains are released from the mature protein while others remain attached via disulfide bonds.

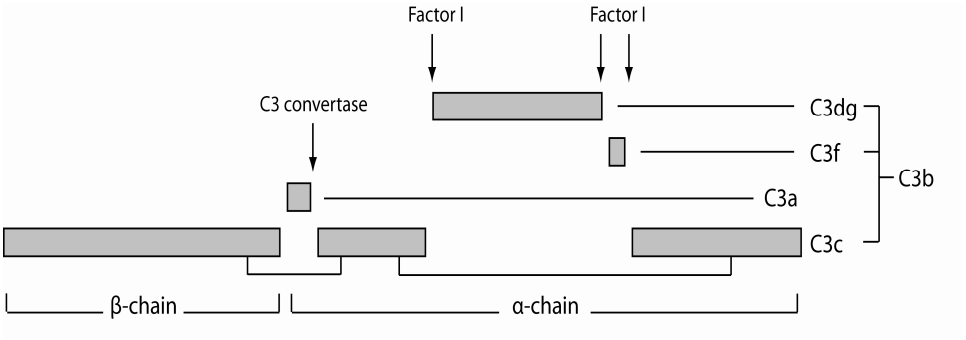

Figure 5: Complement CO3 undergoes several specific cleavages induced by specific proteases [33]. The mature protein is first cleaved by the enzyme complex C3 convertase into C3a and C3b. C3a is a mediator of local inflammatory processes, while C3b can bind covalently to target surfaces via a reactive thioester. Furthermore, C3b is involved in amplification of complement activity by formation of the C3 convertase C3bBb. Additional cleavages of C3b by factor I first releases C3f, which inactivates C3b into iC3b. Further cleavage by factor I releases C3dg, which is thought to be a key regulator in linking innate and adaptive immune responses.

Taking into consideration the Mw of CO3, the several differentially expressed CO3 spots, at different molecular weights on the 2D-gel, indicate that these spots were protein fragments of the mature protein. To confirm this, the sequences of the CO3 spots identified by FTICR-MS were examined in more detail, which enabled the annotation of the different CO3 fragments (Figure 6A and 6B). Spots that decreased upon aging were identified as the alpha chain of CO3 (spot 6A) and the alpha chain without the C3a fragment (spot 6B). The spots that increased upon aging were assigned to both CO3c fragments of the alpha chain (spots 6C and 6F) and C3dg (spots 6D and 6E). These results indicate that with increasing age, there is an increase in the cleavage of the CO3 alpha chain, which leads to an increased presence of C3dg and C3c in the plasma samples analyzed.

A Alpha-chain sequence

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 672             SVQLTEKRM DKVGKYPKEL RKCCEDGMRE NPMRFSCQRR TRFISLGEAC  C3a
 721 KKVFLDCCNY ITELRRQHAR ASHLGLARSN LDEDIIAEEN IVSRSEFPES WLWNVEDLKE
 781 PPKNGISTKL MNIFLKDSIT TWEILAVSMS DKKGICVADP FEVTVMQDFF IDLRLPYSVV  C3c
 841 RNEQVEIRAV LYNYRQNQEL KVRVELLHNP AFCSLATTKR RHQQTVTIPP KSSLSVPYVI
 901 VPLKTGLQEV EVKAAVYHHF ISDGVRKSLK VVPEGIRMNK TVAVRTLDPE RLGREGVQKE
 961 DIPPADLSDQ VPDTESETRI LLQGTPVAQM TEDAVDAERL KHLIVTPSGC GEQNMIGMTP
1021 TVIAVHYLDE TEQWEKFGLE KRQGALELIK KGYTQQLAFR QPSSAFAAFV KRAPSTWLTA
1081 YVVKVFSLAV NLIAIDSQVL CGAVKWLILE KQKPDGVFQE DAPVIHQEMI GGLRNNNEKD  C3dg
1141 MALTAFVLIS LQEAKDICEE QVNSLPGSIT KAGDFLEANY MNLQRSYTVA IAGYALAQMG
1201 RLKGPLLNKF LTTAKDKNRW EDPGKQLYNV EATSYALLAL LQLKDFDFVP PVVRWLNEQR
1261 YYGGGYGSTQ ATFMVFQALA QYQKDAPDHQ ELNLDVSLQL PSRSSKITHR IHWESASLLR  C3f
1321 SEETKENEGF TVTAEGKGQG TLSVVTMYHA KAKDQLTCNK FDLKVTIKPA PETEKRPQDA
1381 KNTMILEICT RYRGDQDATM SILDISMMTG FAPDTDDLKQ LANGVDRYIS KYELDKAFSD
1441 RNTLIIYLDK VSHSEDDCLA FKVHQYFNVE LIQPGAVKVY AYYNLEESCT RFYHPEKEDG  C3c
1501 KLNKLCRDEL CRCAEENCFI QKSDDKVTLE ERLDKACEPG VDYVYKTRLV KVQLSNDFDE
1561 YIMAIEQTIK SGSDEVQVGQ QRTFISPIKC REALKLEEKK HYLMWGLSSD FWGEKPNLSY
1621 IIGKDTWVEH WPEEDECQDE ENQKQCQDLG AFTESMVVFG CPN
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B CO3 fragment identification

Spot	Regulation O/Y	CO3 fragment	Seq. (Th)	SC (%)	Mw kD(th.)	pI(Th)
6A	0.8	C3 alpha chain	672-1663	58	113	5.55
6B	0.3	C3 alpha chain without C3c/C3a	955-1663	32	80	4.97
6C	4.4	C3c C-terminal fragment	1321-1661	80	39	4.79
6D	6.4	C3dg	955-1303	32	39	5.00
6E	9.6	C3dg	955-1303	32	39	5.00
6F	4.4	C3c N-terminal fragment	749-954	37	34	6.89

Figure 6: Identification of complement C3 cleavage products that are differentially expressed between the young and old group. For each CO3 spot, the amino acid sequence (A) was used to identify the precise splice product by matching the sequence data obtained by FTICRMS analysis (B). The sequence coverage (SC), theoretical molecular weight (Mw) and pI for each splice product are indicated.

To verify these results, the pooled plasma samples were further analyzed on Western blot with a polyclonal anti-CO3 antibody and a monoclonal anti-C3dg antibody (Figure 7). The protein bands running at ±118 and 72 kD correspond with the masses of the intact alpha and beta chain, respectively. At ± 42 kD a protein band is running which is in accordance with the C3c-C-terminal fragment as identified in spot 6C, whereas the C3c-N-terminal fragment (spot 6F) is possibly represented by the protein band at ± 35 kD. By using a specific anti C3dg antibody, the band at 39 kD could be identified as C3dg. Taking into consideration the intensity of these three protein bands it is obvious that prominent C3 processing occurs predominantly at higher age, which is in line with the results found in the 2D-DIGE study. More interesting is the question how these changes are reflected in the individual plasma samples.

Therefore, we have analyzed also individual plasma samples on the presence of C3 processing products. Rather interesting is that four individuals exhibited substantial C3 splicing in comparison to the other members of the pool. These individuals also revealed an inverse correlation between the alpha-chain at 118 kD and the 42, 39 and 35 kD proteins bands, which suggests that these splice products originate from the alpha-chain. Next to this, these results also imply that the abundance intensity of the observed splicing products in the pooled samples is strongly determined by these few individuals. Nevertheless, in spite of this inter-individual variability the distinct differences between the individuals of the old and the young populations is still observed and confirms the results obtained from the 2D-DIGE analysis.

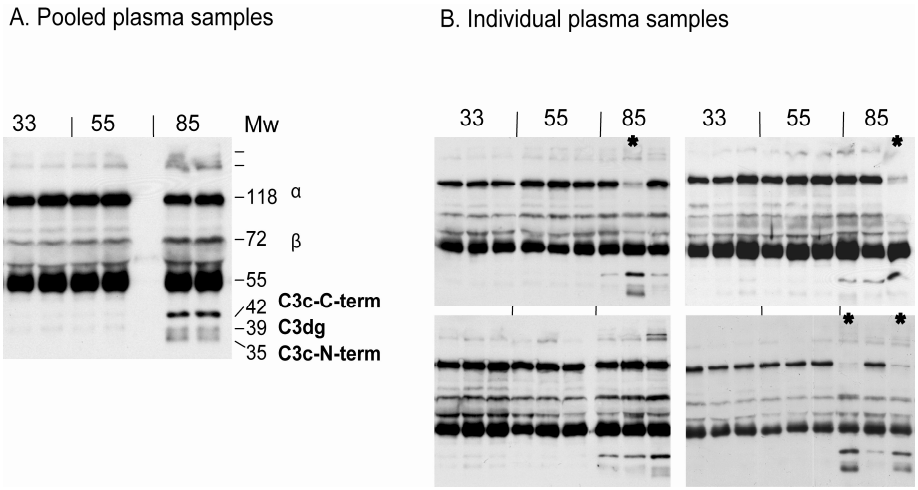

Figure 7. Validation of complement CO3 abundance levels with western blotting and antibody detection in non-depleted pooled and individual plasma samples. Clearly multiple C3 bands at different molecular weights (Mw) could be distinguished. (A) Luminescence signal of pooled plasmas samples after ECL. The molecular weight (Mw) at 118 kD and 72 kD correspond to the Mw of respectively the alpha and beta chain of CO3. Also distinct differences were found in bands with a molecular weight, which corresponds with the fragments found in the DIGE analysis. Bands possibly originating from C3c-C-terminal, C3c-N-terminal as well as C3dg become strongly increased in the old population (B) Luminescence signal of individual plasma samples after ECL. The asterisk indicates plasma samples which showed a remarkable increased abundance of CO3 splicing products.

Discussion

The aim of this study was to search for age-related protein changes in human plasma and is the first study in which age-related protein expressions in blood are analyzed with the 2D-DIGE approach. In search of general markers we decided to perform initial experiments with pooled plasma samples of individuals of three age classes. Although this discards variations within the groups, it will more likely lead to general applicable markers, rather than individual markers. Subsequent analysis of individual samples, for instance by western blotting will give further insight in individual variation. To increase the detection limits as well as gel resolution of 2DGE depletion of HSA and IgG was performed and in combination with 2D-DIGE this resulted in a relatively large set of differentially expressed proteins, which showed statistically relevant (ANOVA p-value ≤0.05) expression profiles between the three age-groups. The majority of the identified differentially expressed proteins were either protein fragments or post-translational modified proteins, which shows the added value of gel-based proteome analysis in detecting protein isoforms. As depicted in Table 1 we were able to find various differentially expressed proteins with different biological functions, which might be important in the healthy aging process.

A large part of the identified proteins showed small alterations towards aging and several of them agreed with results that have been described by others in studies focusing on one or a few blood components during aging. This included IgM [14], clusterin [3,34-36], transferrin and transthyretin [37]. Another set of proteins showing small changes during the aging process were gelsolin, vitamin D-binding protein, ficolin, pigment epithelium-derived factor, inter-alpha trypsin inhibitor and zinc alpha-2-glycoprotein and were not reported earlier in relation to aging. However in this study we focused our research efforts on a panel of proteins which were responsible for the most prominent alterations like, alpha-1-antitrypsin, haptoglobin, fibrinogen and complement C3. Alpha-1-antitrypsin is a circulating serine protease inhibitor and prevents damage from enzymes like elastase. The most distinct alpha-1-antitrypsin spot (spot 1A) shows a very slight decrease in expression, while the other two alpha-1-antitrypsin spots, which are less prominent show a decreased (spot 1B) and a marked increased expression (spot 1C) (Figure 2A). However, the effect of the strongly upregulated spot is not reflected in the pooled nor in the individual plasma samples. This might be caused by the high abundance of multiple alpha-1-antitrypsin isoforms in plasma [38]. Unfortunately, due to a lack of gel resolution, differential analysis of these spots could not be performed for

these abundant isoforms. However, they will be detected with an immunoassay and thus might contribute to the observed down regulation. Literature based data on immunoassays [39] showed no or at most a slight increase in alpha-1-antitrypsin expression after the age of 55. Haptoglobin is a glycoprotein and mainly responsible for clearance of free haemoglobin out of the blood plasma to prevent free radical formation. This protein becomes, amongst others strongly upregulated upon infection or other inflammatory processes. The Western blot analysis (Figure 3) of the pooled samples was consistent with the DIGE results (Fig 1 and Table 1). Although others have reported that towards healthy aging HP becomes upregulated [39], the Western blot analysis of the individual plasma samples revealed such strong differences even within the same age group that this protein cannot be used as a marker to specify the different age classes. More results were found for fibrinogen, which is involved in blood coagulation. The 2D-DIGE study as well as the western blot analysis (Figure 4) showed that in the pooled plasma samples fibrinogen appeared to be upregulated towards healthy aging. Although, this difference was less distinct in the individual samples the observed upward trend is in line with data from others, which have postulated also that increased plasma levels of fibrinogen will accelerate the development of cardiovascular diseases like thrombosis [40-42].

Furthermore, it has been suggested that a pro-coagulant state might be induced by an age-related low-grade inflammatory state [43,44]. This is interesting since there are indeed several indications that with increasing age circumstances arise that stimulate a more pro-inflammatory immune state [45], known as inflamm-Aging. This specific state is considered to be a result of an imbalance of the adaptive [46] and the innate immune system [47]. In this study we have detected protein (isoforms), which are related to the functionality of the innate immune response. The 2D-DIGE studies revealed clearly that complement C3 processing was increased in the pace to healthy aging. Complement C3 is an acute phase protein and plays a central role in the activation of the complement system, which is part of the innate immune response. Furthermore, it is widely appreciated that it plays a key role in several steps of the adaptive immune response and is considered to be the link between the separate immune response systems [31]. The most prominent regulations were caused by CO3 components (Figure 1 and Table 1). The Western blot results of the pooled as well as individual plasma samples (Figure 7) confirmed that an increase of C3c and C3dg is observed in the old population. As is depicted in Figure 5, the observed CO3 processing is mainly a result of proteolytic cleavage. Subsequently we can not completely exclude that, despite our precautions, intrinsic proteolytic activity triggered this during sample treatment, as

others have shown that blood plasma as well as serum samples are prone to intrinsic proteolytic activities and might introduce pre-analytical variability and plasma instability [48]. Nevertheless, the determined CO3 splicing products remain definitively specifically regulated upon aging. Moreover, others have found an increased expression level of cytokines such as IL-1 and IL-6, that induce the production of CO3 towards aging [10-13,49]. This increase would result in elevated CO3 concentrations in the blood circulation of the elderly. However, literature contains conflicting data about CO3 levels in relation to aging. The protein was reported as either upregulated [50] or constant [51-53]. Although total C3 levels are important, the present study has shown that CO3 processing products might be more suitable to be evaluated in the context of the healthy aging process. The increase in CO3 processing might also be the cause of the conflicting data presented in literature concerning the expression levels of this protein during aging. The most commonly used methods for CO3 quantitation are immunoassays (ELISAs), which are at the moment not suitable for detection of CO3 isoforms or spliceisoforms and antibodies that specifically recognize these forms would be of great help.

Concluding remarks

In the present study we have explored the use of 2D-DIGE in combination with HSA and IgG depletion to search for interesting age-related proteome regulations in blood plasma. This technology has enabled us to perform a dynamic proteomics study using blood plasma samples from three different age-groups. By using ANOVA statistics an interesting subset of proteins could be selected, which may play an important biological role during the healthy aging process. The application of HSA and IgG depletion prior to gel analysis allowed the detection of medium/low abundant proteins. The majority of the identified proteins were part of the acute phase response which indicated that towards aging a pro-coagulant as well as a pro-inflammatory state is present. This is in agreement with results from others.

However, in more detail the 2D gel approach has enabled also the evaluation of protein isoform regulation in relation to the aging process. Differential isoform regulations were found for proteins such as alpha-antitrypsin, haptoglobin, transferrin, fibrinogen and complement C3. The overall regulation of the isoforms per protein have been confirmed largely with western blot analysis of the pooled plasma samples and validate the results obtained with 2D-DIGE. However, it appeared to be more difficult to validate this in individual plasma samples.

Haptoglobin abundance in the individual plasma samples varies independently of age and is therefore not considered representative for healthy aging. This is not the case for complement C3, which is responsible for the most prominent age-related differences in this study. Although the intensity of the observed differences can be correlated to a few individuals of the old group it is obvious that all members exhibit more complement C3 fragments. This illustrates that more research emphasis should be put on unravelling the role of isoform regulation in relation to aging. Determination of complement C3 with antibodies, like in immunoassays, will result in detection of all C3 splicing products at once and might generate false positive results in case of severe C3 degradation. It is possible that the conflicting literature data concerning complement C3 in relation to aging could be caused by this problem. Only application of splice isoform specific antibodies would correct for this deviation. Unfortunately, this not always possible. Nevertheless, it is obvious that the present study has provided interesting knowledge about age-related blood plasma proteome regulations, which will contribute to the understanding of the aging process in relation to health and disease development.

Acknowledgements

We would like to thank the Bio Affinity Company, Naarden, The Netherlands (BAC) for kindly providing us with the VHH based anti-HSA and anti-IgG affinity ligands. We thank Martina O'Flaherty and Martijn Pinkse for their help and assistance with the LTQ/LTQ-FTICRMS analysis. We thank Prof. Daha from Leiden University Medical Centre (LUMC) for providing the specific complement C3dg antibody. This research was in part supported by Unilever Research Colworth (UK) and the Netherlands Proteomics Centre.

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Chapter 5

Human plasma proteome differences between healthy and unhealthy sub-populations at old age by using two-dimensional Difference In Gel Electrophoresis

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Abstract

Since the nineteenth century the life expectancy has increased as a result of improved life circumstances. However, this increase in life-span is accompanied with the increased occurrence of age-related diseases, like diabetes, Alzheimer and atherosclerosis. In order to design new treatments to support healthy aging more knowledge about the relationship between healthy and unhealthy aging is required. In view of this, we have explored the blood plasma proteome of healthy and unhealthy individuals at the age of 85 or higher by subjecting blood plasma samples from both groups to Difference In Gel Electrophoresis. Statistical evaluation of the data revealed a subset of proteins that showed a significant abundance difference ($P<0.05$) between the two populations. Seventeen unique proteins were identified with mass spectrometry of which a few can be putatively correlated with the later cause of death and might represent a disease marker. More interestingly, other proteins were found, which appeared to be independent of specific diseases and might represent possible general markers for (un)healthy aging. Although, the majority of the alterations were represented by proteins involved in the general acute-phase response, we obtained indications that unhealthy aging seems to be accompanied with a decline in oxidative stress response, anti-coagulant state, pro-inflammatory capacity and tissue damage response.

Introduction

Life circumstances and environmental conditions can be of great influence on the total life span of human individuals [1]. It is therefore not surprising, that the increase in economic prosperity in the industrialized countries since the late 1800s, which has initiated an improvement in life circumstances, can be correlated with an increase in life expectancy [2,3]. Although in general life expectancy is still increasing, the quality of those extra years may be in poor health, since the increase in life span is accompanied with an increase in age-associated diseases, like diabetes, Alzheimer and atherosclerosis [2]. Supporting healthy aging requires knowledge about the relationship between biological processes involved in advanced aging and development of particular age-related diseases. Therefore numerous studies have been performed in which populations of humans were assessed for the presence of specific metabolites or proteins correlating with aging in relation to disease development [4,5] [6-8]. This has resulted in a considerable amount of knowledge about the development of specific diseases in relation to aging. Although these early disease markers are very informative, more biomarkers related to the processes preceding the early disease status of an individual are needed. Those markers might be considered to be useful for the development of new intervention treatments to preserve healthy aging. Illustrative for this is the age-associated pro-inflammatory state. A pro-inflammatory state has been detected in populations at old age [9-11]. Yet, inflammation is involved also in the development of a wide variety of age-related disease pathologies like, atherosclerosis, diabetes, Alzheimer and Parkinson [12-14]. This implies that cytokines or interleukins are not disease-specific and that their abundance or expression regulation might be considered as representative for the pace to unhealthy aging [15]. In view of this, our aim is to find other general unhealthy aging markers independent of the presence of a specific disease. Proteomic studies can be highly suitable for this purpose, since they enable simultaneous detection and quantification of protein regulations, like expression or post-translational modifications (e.g. phosphorylation, glycosylation, glycation, carbonylation) in complex mixtures such as cells or tissues [16,17]. In aging research this type of analysis has been successfully applied already, but was mainly, as discussed above, focused on the development of specific age-related diseases, like Alzheimer or atherosclerosis [7,8]. Recently, we have performed a comprehensive proteomics study with blood plasma samples originating from various age-groups, which revealed interesting data concerning healthy aging [18]. We have now extended this type of research to search for proteome differences

between healthy and unhealthy populations older than 85 yr, in order to build a map of protein markers enabling to discriminate these sub-populations. All participants were 85 years of age at the time of blood withdrawal. The healthy population reflected those individuals still alive 5 years later, unhealthy indicated death within 2 years after blood withdrawal. Pooled plasma samples of each population were subjected to HSA and IgG depletion [19] to increase gel resolution and detection of medium abundant proteins. Thereafter samples were subjected to Difference In Gel Electrophoresis (DIGE) [20-22]. Proteins that statistically differ in intensity between the two sub-groups were identified with mass spectrometry.

Methods and materials

Plasma samples

Citrate plasma samples from 30 individuals, which were part of the Leiden 85+ study, were divided in two groups of 85 years old of 15 individuals each. Equal amounts of each sample of each group were pooled and depleted of human serum albumin (HSA) and immunoglobulin G (IgG) before 2D-DIGE analysis.

HSA and IgG depletion

To obtain better resolution on a 2D-gel human serum albumin (HSA) and IgG were removed from the plasma samples with affinity resin based on Llama heavy chain antibody (VHH) technology as described before [19]. The VHH anti-IgG resin (dynamic antigen capacity 10mg/ml) and anti-HSA resin (dynamic antigen capacity 12mg/ml) were mixed in a 1:2 ratio. The mixed affinity resin was washed extensively prior to use with ten times column volumes of Tris buffer (0.1M Tris/HCl pH 8.5; 0.5M NaCl) for three times followed by three washes with acetate buffer (0.1M sodiumacetate pH3.0; 0.5M NaCl). Thereafter, the affinity resin was washed three times in citrate buffer to neutralize the solution. Plasma samples were diluted 100 times in ice-cold citrate buffer and incubated at 4°C for 30 minutes with 50µl bed volume of affinity resin per 100µl diluted sample. After incubation, the non-bound fraction was taken for differential protein analysis. The protein content of these samples was determined with a BCA protein assay (Pierce, Rockford, U.S.A.) according to the instructions of the manufacturer.

Protein labeling

In order to obtain the optimal reaction conditions, 50µg of each protein sample was treated with the Clean-up kit (Amersham Biosciences, Upsalla, Sweden) according to the instructions of the manufacturer. Protein pellets were dissolved in 15µl buffer (30mM Tris/HCl pH8.5, 7M urea, 4% CHAPS). Cy5, Cy3 and Cy2 labeling was performed according to the instructions of the manufacturer (Amersham Biosciences). The labeled samples were diluted in rehydration solution (7M urea; 2M thiourea; 4% CHAPS; trace bromophenol blue; 0.5% (w/v) DTT; 0.5% (v/v) ampholytes pH 4-7; 1.2% (v/v) Destreak (Amersham Biosciences)) to a final volume of 450µl.

Two dimensional gel electrophoresis

Immobiline Dry strips pH 4-7 of 24cm (Amersham Biosciences) were rehydrated overnight with the labeled protein samples and overlaid with 2ml Coverfluid in an Immobiline Drystrip reswelling tray. The first dimension of iso-electric focusing was performed with an IPGphor system. Optimal protein focusing was achieved by starting at 500V for 1 hr, followed by 1500V for another hour. Subsequently, a constant voltage of 8000V was applied until a total of 48kV. Thereafter, the strips were equilibrated in equilibration buffer (50mM Tris/HCl pH8.8; 6M urea; 30% glycerol (v/v); 2% SDS (w/v)) containing 65mM DTT for 15 minutes followed by an incubation with 135mM iodoacetamide for 15 minutes. The second dimension was performed with lab-cast 24cm 12.5% polyacrylamide gels. Strips were loaded onto the gels and sealed with a solution of 1% agarose (w/v) containing a trace of Bromophenol Blue. The gels were run over night on the Ettan DALT Twelve system (Amersham Biosciences) at 1W/gel till the Bromophenol Blue dye front reached the bottom of the gel.

Gel imaging and differential analysis

All gels were scanned in between low fluorescent glass plates at 100µm pixel resolution with the Typhoon image scanner 9400 (Amersham biosciences). The Cy5 images were scanned using a 633nm laser and a 670nm BP30 emission filter. Cy3 images were scanned with a 532 laser in combination with a 580nm BP30 emission filter. Cy2 images were scanned using a 488nm laser and an emission filter of 520nm BP40. The photomultiplier tube was set to achieve maximum sensitivity without pixel saturation. Silver stained images were analyzed with a GS-710 Calibrated Imaging Densitometer (Biorad, Hercules CA, USA). Prior to differential analysis, images were cropped with ImageQuant (Amersham Biosciences) to remove insignificant parts of the gel. Subsequently, images were subjected to median filtering in Image Quant Tools (Amersham Biosciences,) to remove dust-related pixels. Differential analysis was performed with the Decyder V 5.01 (Amersham Biosciences) software package. A student T-test was applied to extract relevant abundance differences.

Spot picking and in-gel digestion

Spot picking was performed with post-stained silver images of CyDye labeled gels to minimize the risk of mismatched spot picking. Silver staining was performed according to Shevchenko [23]. Spots of interest were subjected to in-gel tryptic digestion as described previously [24].

Nano LC-MS/MS and protein identification

Nano LC-MS/MS was performed with an Agilent 1100 series LC system (Agilent, Palo Alto, U.S.A.) coupled with a Thermo Finnigan LTQ (Thermo electron Company, Waltham, MA, U.S.A.) or LTQ-FTICR mass spectrometer as described previously in literature [24]. Briefly, peptide extracts were acidified with 0.1M acetic acid and injected on a trap column (Aqua^{TM} C18 RP (Phenomenex, Torrance, U.S.A.), 20 mm x 100 µm ID) at 5µl/min. Subsequently, the peptides were transferred with a split-reduced flow rate of 100nl/min to the analytical column Aqua^{TM} C18 RP (Phenomenex, Torrance, U.S.A.), 20 cm x 50 µm ID. Elution of the peptides was achieved with a linear gradient from 0-50% B (0.1M acetic acid in 80% (v/v) acetonitrile) in 60 minutes. The column effluent was directly introduced into the ESI source of the mass spectrometer via a butt-connected nano-ESI emitter (New Objectives, Woburn, MA, U.S.A.). The mass spectrometer was operated in the positive ion mode and parent ions were selected for fragmentation in data-dependent mode. The obtained mass spectra were subjected to a Mascot search engine [25] with 0.8 Da for LTQ or 10 ppm peptide tolerance in case of LTQ-FTICRMS, two miss-cleavages, carbamidomethylation and methionine oxidation as variable modifications. Protein annotation was performed by using the SwissProt database.

Western blot analysis

Equal amounts of plasma were size-separated on a 10 or 15% SDS poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer (2.5% protifar plus (w/v) in PBS), the membranes were soaked in MeOH and subsequently stained with Coomassie brilliant blue (CBB) [0.1% (w/v) Coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with a primary antibody for one hour and subsequently washed 5 times in PBST (0.05% Tween-20 in PBS). Thereafter, membranes were incubated with HRP-conjugated donkey anti-rabbit/donkey secondary antibody (Jackson Immunoresearch Laboratories, Westgrove, U.S.A.) for one hour and the wash steps were repeated. Two additional washes were performed with PBS and bound antibodies were visualized by enhanced chemo-luminescence (PerkinElmer, Boston, U.S.A.). Primary antibodies were diluted up to 1:2000 for the anti-CO3 (ab23891, Abcam, Cambridge, United Kingdom), 1:2000 for the anti-C3dg (kindly provided by M. Daha), 1:2000 for the anti-fibrinogen (F4639, Sigma), 1:4000 anti-Haptoglobin (H5015, Sigma) and 1:500 for the anti-gelsolin (Stressgen). The secondary antibody GAR-PO (Jackson Immunoresearch) was diluted 1:5000.

Results and discussion

Blood samples

To determine plasma proteome differences between healthy and unhealthy aging we have used plasma samples from two populations of people at the age of 85. Each group consisted of fifteen individuals. People from one group (85 H) were considered healthy at 85 years and were still alive for at least five years, whereas individuals of the unhealthy group (85 UH) were initially selected on the fact that they died within 20 months after blood sampling. A detailed view in the health history revealed that the members of this group succumbed from various diseases like, cancer, cardiovascular disease (CVD), coronary artery disease (CAD) or intestinal disease (see Table 1). Therefore, this group was considered to represent unhealthy aging at 85 yr (85 UH). Since the aim of this study was to search for general unhealthy aging markers independently of a specific disease and in order to reduce the influence of disease specific protein regulations of each individual as well as analytical variation plasma samples were pooled per group prior to 2D-DIGE analysis.

Nr.	ICD-10 code	Description
1	K578	Disease of intestine with peritonitis
2	I219	Acute myocardial infarction (Heart attack)
3	I219	Acute myocardial infarction (Heart attack)
4	D469	Preleukemia
5	C900	Multipe Myeloma (Kahler's disease)
6	K579	Disease of intestine
7	C610	Malignant neoplasm of prostate
8	I219	Acute myocardial infarction
9	C349	Malignant neoplasm of bronchus or lung
10	K567	Disease of intestine
11	X599	External cause of morbidity
12	I639	Stroke (Cerebral Infarction)
13	I251	Atherosclerosis
14	G200	Parkinson
15	I640	Stroke (Brain attack or TIA)

Table 1 Health history of individuals of the 85UH group according to ICD10

Proteomics

In order to increase 2D-gel resolution, pooled plasma samples were subjected in triplicate to HSA and IgG depletion as described previously [19]. The Ettan-DIGE technology was applied and resulted in a plasma proteome map of approximately 750 protein spots (Figure 1). A Student t-test was used to determine significant ($P \leq 0.05$) protein abundance differences between the 85 H and the 85 UH group, which resulted in a set of 68 protein spots. Spot identification and characterization with mass spectrometry revealed a subset of 17 unique proteins, which were responsible for intensity variations of 41 protein spots (Figure 1).

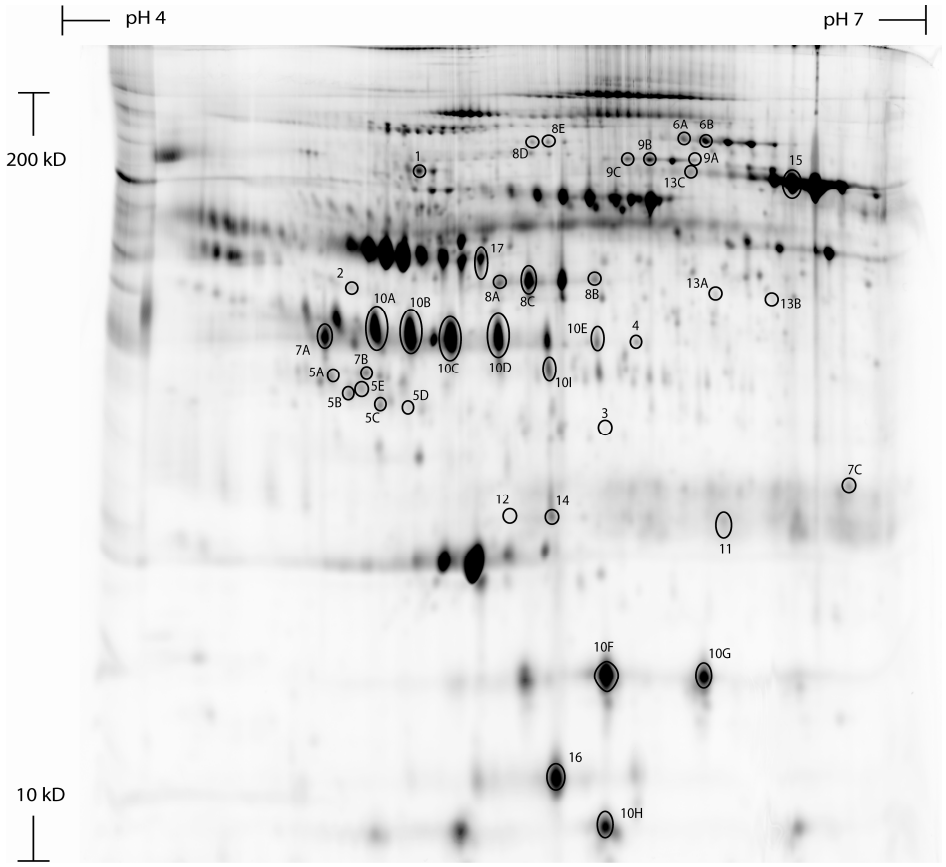


Figure 1 Representative Cy5 image of 50 µg pooled plasma after HSA and IgG depletion. Numbered spots exhibited significant expression changes between 85 and 85 UH population. Corresponding protein identification can be found in Table 2.

Spot nr.		Name	Swiss-Prot	Mw (kD)	85UH/85H	T-test	Ionscore	pep	SC(%)
1		Afamin	P43652	69069	0.7	1.6E-03	713	22	29
2		alpha-1-antitrypsin	P01009	46737	1.3	1.7E-03	652	11	23
3		Apo-E precursor	P02649	36268	1.4	1.2E-02	1505	23	58
4		ApoL1	Q14791	43927	1.3	3.4E-03	711	23	24
5	A	Clusterin	P10969	53065	0.8	1.1E-03	347	11	21
	B				0.8	3.9E-03	711	12	18
	C				0.9	3.3E-02	577	9	12
	D				0.9	4.2E-03	349	6	11
	E				0.7	7.5E-05	458	12	23
6	A	Complement B	P00751	86902	0.8	2.4E-02	1644	34	37
	B				0.8	1.7E-03	963	22	22
7	A	Complement C3	P01024	188704	0.6	6.4E-04	1900	16	32
	B				0.3	9.5E-04	887	7	14
	C				0.5	4.3E-05	386	8	3
8	A	Fibrinogen gamma chain , alpha	P02679	52139	0.7	1.1E-02	1640	26	50
	B				1.7	3.3E-04	1184	20	37
	C				0.9	2.2E-02	2203	32	52
	D				0.8	1.0E-02	1242	20	39
	E				0.8	8.4E-03	1242	18	35
9	A	Gelsolin	P06396	85698	0.9	8.7E-04	396	9	9
	B				0.7	4.8E-03	659	13	14
	C				0.7	2,1E-05	1959	29	39
10	A	Haptoglobin	P00738	45205	1.1	4.7E-02	1797	38	40
	B				1.1	1.3E-02	1322	23	37
	C				1.2	1.4E-03	1339	25	45
	D				1.2	1.9E-04	1327	22	37
	E				1.4	1.1E-04	1198	18	37
	F				1.6	2.6E-05	628	10	15
	G				1.8	3.9E-05	921	12	23
	H				0.4	2.6E-06	597	9	17
	I				1.3	7.6E-04	814	11	21
11		Ig kappa chain C	P01834	11609	1.3	2.3E-02	378	12	71
12		Ig lambda chain C	P01842	11236	16.6	5.2E-05	410	4	35
13	A	IgM chain C	P01871	50242	1.2	2.3E-02	597	8	11
	B				1.2	3.6E-02	156	3	7
	C				1.2	1.5E-02	1542	14	47
14		Serum amyloid p-component	P02743	25387	0.8	4.4E-03	516	5	22
15		Transferrin	P02787	77050	0.9	7.4E-03	5346	82	70
16		Transthyretin	P02766	15887	0.7	3.6E-05	739	10	68
17		VitD binding protein	P02774	52964	0.8	8.5E-03	1053	19	33

Table 2 Identified proteins with relevant (T-test P<0.05) abundance differences between healthy people (85H) and unhealthy people of 85 (85UH). Protein identity, ion score, unique peptides (pep) and sequence coverage (SC%) were obtained with the Mascot search engine in combination with the Swiss-prot database. Expression changes (Exp) were calculated using the average value of the 85 UH pool in comparison with the average values of the 85 pool.

The majority of the abundance differences could be assigned to multiple protein isoforms or splice products. These results clearly show the added value of two dimensional gel-electrophoresis, which enables the visualization of protein isoforms [26-28]. Protein identification revealed that some of the observed alterations might be related to a specific age-related disease (and possibly cause of death) whereas others might be representative of unhealthy aging and related to certain biological processes, as will be discussed further.

Possible disease-related protein regulations.

A part of the differentially expressed proteins could be associated with specific diseases, which were present in the 85 UH population (see Table 1). A remarkable observation in the 85 UH group was the upregulation of Ig Lambda light chain (IGL). Figure 2 clearly shows the on/off characteristic of this protein and as it might be an ideal marker for unhealthy aging. However, since we are dealing with pooled samples a strong upregulation of a specific protein(isoform) in one individual might account for the observed data. It has been reported that the ratio Ig Kappa light chain (IGK) and Ig Lambda light (IGL) chain may indicate whether patients are suffering from Multiple Myeloma (MM) or AL systemic amyloidosis [29,30]. Subjecting plasma samples from the 85 H and 85 UH population to a 10 % SDS-page gel with CBB staining revealed that one individual clearly showed a distinct different protein pattern than other group members, having similar IgG patterns. A detailed view in the cause of death history of the UH group (see Table 1) revealed that this individual member suffered from Multiple Myeloma, which explains the high abundance of the IGL spot in the 2D-DIGE study and thereby indicating that this protein is not a valid marker for unhealthy aging in general.

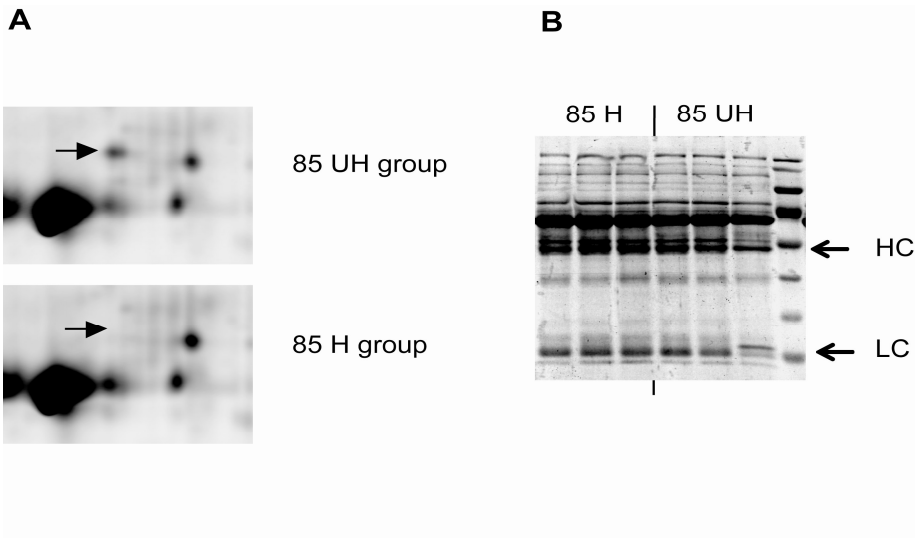


Figure 2 (A) Enlarged view of a part of the 2D image. A clear upregulation of IGK is determined in the unhealthy (85 UH) population as indicated with an arrow. (B) Plasma samples subjected to SDS-Page analysis stained with CBB. At the molecular height of Heavy (HC) and Light (LC) chain immuno-globulines a different protein band pattern can be distinguished for only one individual from the 85 UH group, whereas the rest of the groups showed similar patterns.

A second set of disease related proteins are those that are not necessarily markers of disease, but can be considered as risk factors. Some of these are described to be associated with aging, but these studies were focused on specific proteins rather than the proteome. In the present study, multiple protein isoforms of clusterin (CLU) appeared to be downregulated in the 85 UH group. This glycoprotein is expressed in a wide variety of tissues and subsequently determined in all human fluids. CLU is proposed to be involved in the suppression of amyloid fibril formation [31,32] and progression of carcinomas [33], diabetes and coronary heart disease [33,34]. Apo-lipoprotein E precursor (APOE) was upregulated in the 85 UH population and this protein plays a key-role in lipid transport in both plasma and the central nervous system. APOE is critical for lipid transport in the repair of myelin and neuronal membranes during development and after injury [35]. There are also indications that this protein correlates positively with the development of Parkinson [36] and different isoforms are supposed to be involved strongly in amyloid fibril formation in Alzheimer patients [37]. Next to this, others have found that APOE levels seem to be correlating also with cardiovascular mortality at old age [4]. So an increase in the APOE precursor might be indicative for the presence of the diseases listed in Table 1 in the unhealthy population.

Acute phase response

Upon disturbance of blood homeostasis resulting e.g. from particular injuries or infections an acute phase response is triggered. Positive acute phase proteins like haptoglobin, fibrinogen and alpha-1-antitrypsin are upregulated and negative acute phase proteins such as, transferrin and transthyretin are downregulated under those conditions [38]. The major difference between the 85 H and 85 UH group is the possible disease status at the moment of blood withdrawal, which would cause major disturbances in the acute phase. A detailed view in Table 1 shows that indeed a disturbance in the acute-phase response might be expected in the 85 UH population. Two of the proteins discriminating the 85 H and 85 UH groups are identified as transferrin and transthyretin and are known to be involved in the negative acute phase response [38]. Moreover, in this study multiple isoforms of the positive acute phase protein haptoglobin (HP) appeared to be elevated in the UH group. In the native form haptoglobin is a tetramer of 96 kD and consists of two alpha and two beta subunits. In vivo, HP is synthesized from a polypeptide of 40-45 kD (HP precursor), containing an alpha and beta chain, which are supposed to be post-translational cleaved to form the required subunits for the native HP [39,40]. Although spots 10A-F as shown in the DIGE gel correspond to the Mw of the HP precursor of ± 45 kD as extracted from the Swiss-prot database, it is more likely that these spots represent the beta chain since no sequence of the alpha chain

could be detected in the sequence analysis with mass spectrometry. Others have reported also that the HP beta chain has a molecular weight of ± 39-40 kD [41-44]. Two isoforms of the alpha chain are presented by spots 10G and 10H. Since the majority of the protein isoforms revealed similar regulation patterns we have subjected pooled as well as individual plasma samples also to western blotting and anti HP-antibody detection to find out whether the total HP blood concentrations are indeed increased in the 85 UH group (see Figure 3).

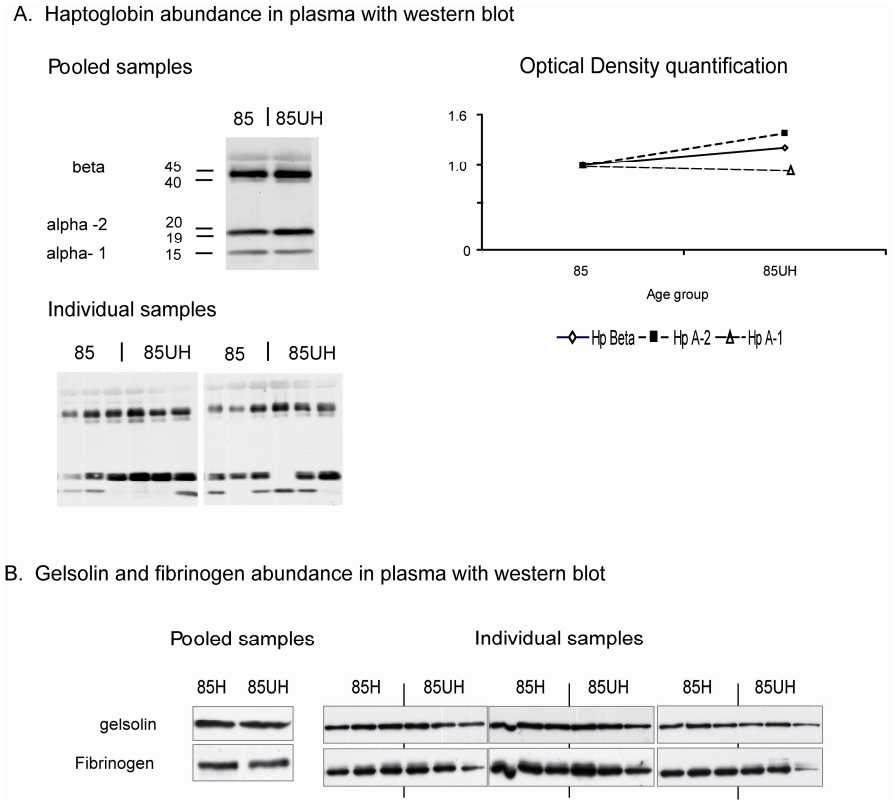


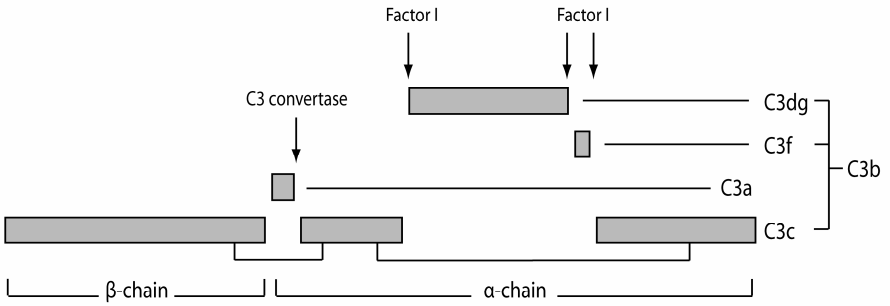
Figure 3 (A) In non-depleted pooled samples, application of anti haptoglobin (HP) revealed multiple bands which are supposed to be the HP beta chain (±40-45 kD) and two isoforms of the alpha chain (±20 kD and ± 15 kD). Densitometry of the proteins bands confirmed the increase in HP abundance in the 85 UH population. However, the variation in the individual samples was too high to determine properly abundance differences. (B) No distinct downregulation in gelsolin abundance could be observed in the pooled as well as the plasma samples. This is different for fibrinogen, which showed a decrease in abundance in the 85 UH population, which was confirmed by the densitometry analysis of the blot results. The same trend was observed in the individual samples.

In figure 3A the HP precursor as well as the two alpha chains can be distinguished properly, but the alteration in the (glycosylated) isoforms cannot be confirmed with this western blot analysis. Nevertheless, the observed alterations of the different isoforms in the DIGE study might still be valuable for the (un)healthy aging process. Haptoglobin (HP) is responsible for the removal of free hemoglobin from the circulation [45,46]. Free hemoglobin is able to increase the formation of ROS through iron mediated radical chemistry (Fenton-chemistry). Therefore, HP is regarded as a protector against hemoglobin mediated lipid peroxidation [47]. It is possible that in the unhealthy group elevated HP concentrations enable a fast response to liberated hemoglobin after hemolysis in order to prevent the formation of ROS. On the other hand it is also possible that the altered HP levels are already the result of increased hemoglobin concentrations. Another acute phase protein is fibrinogen, from which multiple isoforms were detected in this study (see Table 2 and Figure 1). The 85 UH group showed a general small decrease in abundance and suggests a small decline in the pro-coagulant state. Application of western blot analysis, as depicted in figure 3B, showed that in the pooled plasma samples the overall abundance of fibrinogen becomes less in the 85 UH, thus confirming the DIGE results. In the individual plasma samples this trend can also be observed, although there is a clear variation within the two groups (See Figure 3B).

Innate immune response

Remarkable abundance differences were detected in a number of proteins involved in the innate immune system, in particular isoforms of complement C3 (CO3) (see Table 2). This protein plays an important role in the activation of the immune system and specific proteolytic cleavages of the protein determine the functionality (see Figure 4A) [48,49]. Upon cleavage, different active proteins are released, which results in activation of different parts of the immune system. The differentially expressed CO3 protein spots were identified on the basis of sequence coverage, as listed in figure 4B. Particular fragments could be assigned to C and N-terminal fragments of C3c and C3dg. To confirm this, we have subjected non-HSA and IgG depleted plasma samples from both groups to a panel of antibodies (see Figure 4C). An interesting observation was the decrease in splicing activity of complement C3 in the 85 UH group, which influences the activity of the innate immune response. Immuno-detection with a polyclonal anti-CO3 antibody revealed various CO3 bands in MW ranges (figure 4C), corresponding to the CO3 mass values found with the 2D-DIGE approach. A band at 42 kD corresponds with the mass of spot 7A from the DIGE experiment, which appeared to be C3c-C-terminal. The C3c N-terminal fragment as sequenced in spot 7B is possibly represented by the band at 35 kD.

A. Schematic representation of complement C3 cleavage



B. Identification of complement C3 cleavage products

Spot	85UH/85H	Complement	Seq. (th)	SC%	MwkD (th)	pI
7A	0.56	C3c C-Term.	1321-1663	80	39	4.79
7B	0.32	C3dg	955-1303	32	36	5.00
7C	0.46	C3c N-Term.	749-954	37	24	6.89

C. Abundance evaluation with Western blot

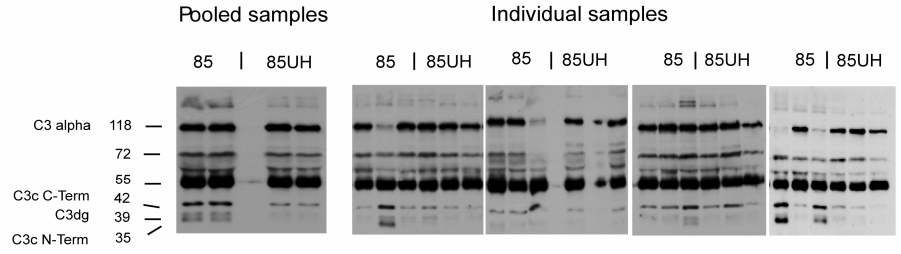


Figure 4 (A). Schematic representation of biologically induced cleavage of complement C3. Mature protein is first cleaved by C3 convertase into C3a and C3b. Subsequently, factor releases first C3f followed by C3dg. More information can be found in Janssen et al [50].(B) Complement C3 related spots found in this study. For each spot sequence coverage (SC), molecular weight and pI are indicated. (C) Western blot analysis of non-depleted pooled plasma samples with a polyclonal anti-CO3 antibody. C3dg was determined with a specific mono clonal antibody, which was kindly provided by M. Daha. Clearly several bands were differentially regulated, which showed the same trend as observed in the DIGE study.

This is interesting since others have found that C3c becomes differentially regulated in patients suffering from Parkinson [51]. Application of a antibody specific to C3dg revealed that the band at 35 kD contained the C3dg fragment and confirmed the findings of the DIGE study. C3dg plays an important role in the activation of the adaptive immune response [52]. Although pooling of samples is very useful to minimize analytical variation, it is possible that the variations in protein isoform abundances are caused by a few individuals. To evaluate this we subjected also individual plasma samples to CO3 immunodetection. Clearly there are abundance differences between the individuals, but the overall trend of a decrease in splicing in the unhealthy individuals is definitively observed. The cleavage of CO3 is mainly performed by determined complement B or Factor I (see Fig 4A). The decline in abundance of complement B in the DIGE study might result in a decrease of CO3 cleavage products, as we have noticed in our study as well. The downregulation of multiple isoforms of CO3 and factor I suggests a reduction in the innate immune response in the 85 UH group. It is possible that due to the observed decline of pro-inflammatory capacity people are more sensitive for pathogen invasion. Although upon aging an increased pro-inflammatory status production has been described widely [11], there are indications that upon LPS stimulation at advanced age the mortality rate becomes increased due to a decreased response of inflammatory mediators like cytokines [53].

Tissue and cell damage response.

Tissue or cell damage causes a release of cellular actin, which is capable of obstructing the microvasculature. Gelsolin (GEL) is involved in scavenging free actin molecules in the blood circulation [54,55]. The downregulation of gelsolin in the 85 UH population implicates a decline in actin-scavenging properties. Others have suggested that reduced gelsolin levels in the circulation of critically ill patients reduce the survival rate after surgery or serious injury [56]. In figure 3B we have analyzed pooled plasma samples on gelsolin abundance, which shows that the small isoform differences observed by DIGE cannot be confirmed by western blot detection. This might be due to the presence of other gelsolin isoforms not detected by DIGE and the possibility that the antibody applied does not specifically detect one of the affected isoforms. To validate the observed gelsolin isoform differences demands further development of isoform specific antibodies. Another protein possibly related to tissue or cell damage is serum-amyloid-p- component (SAP), which was downregulated in the 85 UH population also. SAP is partially responsible for removal of late-apoptotic cells [57]. Abundance of late-apoptotic cells will negatively influence tissue functionality, which will accelerate the mortality rate of an individual.

Healthy aging vs unhealthy aging

In the previous chapter we have evaluated the influence of healthy aging on the blood plasma proteome. First the 2D-DIGE analysis showed that in the pace to healthy aging the majority of differences could be seen in haptoglobin, fibrinogen and complement C3. Interestingly, these proteins were also responsible for the majority of the changes observed in the 85 UH group. Although in both studies western blot analysis could not confirm the isoform regulation of haptoglobin, as seen with DIGE, it is clear that the observed upward trend during healthy aging is continued in the unhealthy subgroup. This is not the case for complement C3 and fibrinogen. Clearly, the C3 processing activity was elevated towards healthy aging, but apparently this activity becomes less in the unhealthy subgroup. Actually the same trend can be observed for regulation of the fibrinogen isoforms. Similar observations were seen with the regulation of gelsolin. One isoform of this tissue damage response protein appeared to be upregulated during healthy aging, whereas other isoforms were downregulated in the unhealthy pool. Taking all these changes into consideration it is obvious that during healthy aging people will develop a pro-coagulant as well as a pro-inflammatory state to be able to respond more adequately to sudden changes in environment or pathogen invasion, which is in line with results from others [11,58]. More interestingly, the present study suggests that upon a decrease of these pro-states, healthy aging cannot be preserved. As a result of this the old individual might become more susceptible to disease development, which will finally increase the mortality rate.

Concluding remarks

In this study, we have explored the applicability of 2D-DIGE analysis after HSA and IgG depletion to visualize differences between plasma samples from healthy and unhealthy elderly at the age of 85. This has resulted in a subset of proteins, which show statistically significant differences in abundance levels between the two sub-populations. The most prominent protein regulation could be correlated directly to multiple myeloma (MM), which appeared to be present amongst the unhealthy individuals. Furthermore, disturbances in the acute-phase response revealed interesting biological processes, which might be representative for unhealthy aging in general. Tissue damage response as well as pro-coagulant state seems to be reduced in the unhealthy population. The proteomics study revealed also that various complement C3 cleavage products were downregulated in the unhealthy population, which might indicate that the pro-inflammatory status becomes reduced. In comparison with healthy aging it seems that the overall adaptive capacity of healthy individuals as found in a previous study might be reduced, which might increase the susceptibility of an individual towards disease development or pathogen invasion and will accelerate the mortality rate. It is obvious that the application of 2D-DIGE in this study has provided knowledge, which will be of benefit for development of intervention studies to preserve successfully healthy aging. However, the majority of the regulations concerned changes in isoform abundance, which could not be confirmed directly with western blot analysis. This is probably caused by the lack of specificity of the applied antibodies towards these isoforms and clearly illustrates the added value of gel-based proteomics in plasma research. Since the biological impact of many isoforms regulations is largely unknown, future studies have to be focused also on unraveling the biological effects of blood plasma protein isoform regulation.

Acknowledgements

We would like to thank the Bio Affinity Company, Naarden, The Netherlands (BAC) for kindly providing us with the VHH based anti-HSA and anti-IgG affinity ligands. We thank Martina O'Flaherty and Martijn Pinkse for their help and assistance with the LTQ/LTQ FTICRMS analysis. We thank Prof. Daha from Leiden University Medical Centre (LUMC) for providing the specific complement C3dg antibody. This research was in part supported by Unilever Research Colworth (UK) and the Netherlands Proteomics Centre.

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Chapter 6

Summarizing discussion

In general human aging is considered one of the most complex and less-well understood processes in biology. The complexity of the aging process demands a multidisciplinary approach to understand the effects on all levels in the human body. This thesis was part of a multidisciplinary project in which the possibilities of proteomics technology in the field of aging of the human body were explored and evaluates the effects of aging on the proteome in an endothelial cell model system as well as blood samples. The complexity of the aging process was supposed not to be accompanied with huge proteome variations, which made the application of the proteomics technology at that time a real challenge. Initial experiments with two-dimensional electrophoresis in combination with silver staining confirmed this, since the analysis was hampered by a high analytical variation. However, the use of 2D-DIGE enabled a reduction of the analytical variation and even allowed the use of biological replicates, which supported the biological relevance of the observed proteome differences.

I Endothelial senescence

Cellular aging of endothelial cells (endothelial senescence) has been detected *in vivo* in atherosclerotic lesions [1,2] and is therefore considered to be important in the development of the age-related disease atherosclerosis. Therefore, a part of the aim of the work described in this thesis was to analyze the influence of replicative senescence (i.e. cellular aging) on protein expression in an endothelial cell model system, called HUVECs. The results in ***Chapter 2*** clearly pointed out the importance of using paired ANOVA statistics to reduce the biological variability of cell cultures originating from three independent isolations. This study showed that endothelial replicative senescence might be accompanied with increased cellular stress, protein biosynthesis and reduction in DNA repair and maintenance. In addition, remarkable differences were observed concerning cytoskeletal structure and nuclear integrity. The down-regulation of Lamin B in relation to endothelial senescence has, to our knowledge, never been reported before and it would be interesting to further evaluate with additional techniques the role and effects of this protein during replicative senescence in endothelial cells. Another observation was the upregulation of the proton pump V-ATPase, which is abundantly present in lysomes and as such correlates with lysosomal body increase. Interestingly, electron microscopy of early passage and senescent HUVECs as presented in the **addendum** of chapter 2, clearly showed that there is an increase in lysosomal bodies and confirmed the conclusions from the proteomics study. Next to this, the EM study also showed that senescent cells contain more fat droplets and branched mitochondria than young endothelial cells.

In ***Chapter 3*** we were able to confirm with Western blot analysis the observation of the 2D-DIGE study that towards aging endothelial cells contain elevated levels of bovine transferrin (B-Tf). Remarkably, a down regulation of the Tf receptor was observed in old HUVECs, which suggests a non-receptor mediated endocytosis of bovine Tf. Experiments with human ^{125}I labeled Tf did not reveal age-related differences in the Tf recycling pathway. Although there are indications that the human Tf receptor interacts with bovine as well as human Tf it might be possible that the recycling capacity for bovine Tf is less than for the human form. Incubation of early and late passage cells with labeled bovine Tf should be performed to elucidate this more properly. Another option might be that in old cells, transferrin is endocytosed also via a receptor-independent pathway, like fluid phase-mediated endocytosis.

Taking all together the application of 2D-DIGE in cell systems clearly has provided interesting knowledge about a variety of biological processes occurring during growth towards replicative senescence of endothelial cells, of which some were further studied with additional technologies like electron microscopy and western blot analysis.

II Aging markers in blood

Since 2D-DIGE has proven to be able to correct for analytical as well as biological variation we have applied this technology also to evaluate age-related proteome differences in blood plasma samples from three different healthy age-groups extracted from the Leiden longevity study. In ***chapter 4*** pooled plasma samples from three different healthy age groups were depleted from serum albumin and IgG to increase gel resolution as well as improved detection of medium abundant proteins. The majority of the differentially regulated protein spots could be accounted to a small subset of proteins, which indicated that the observed protein regulations are primarily a result of isoform or splice-isoform activity. Functional analysis revealed that during healthy aging a pro-inflammatory as well as an increased pro-coagulant state are involved in aging. The most remarkable observation was the strong regulation of complement C3 (CO3) splicing products, which suggests that the immune response becomes increased in the healthy elderly group. Splicing products CO3dg and CO3c were clearly more abundant in this population and are known for their role in activating other parts of the immune system. Some of the CO3 spots could be observed only because of the use of depletion of IgG and HSA.

Next to information about healthy aging, more knowledge would be valuable also about the unhealthy aging. ***Chapter 5*** describes an explorative study in which we have compared plasma samples from healthy and unhealthy individuals at the age of >85. People who died within two years after blood withdrawal and suffered from various diseases were considered unhealthy, whereas the healthy individuals were still alive after 5 years after blood withdrawal. Pooled samples of both populations were subjected to 2D-DIGE, which revealed that apparently the pro-coagulant as well as the pro-inflammatory state as was observed during healthy aging becomes reduced in the pace to unhealthy aging. As a result of this, old individuals might become more susceptible to disease development, which will finally increase the mortality rate.

Although interesting age-related biological process have been determined with our DIGE approach in both blood plasma studies, it is obvious that the majority of the regulations are concerned with alterations in the abundance of protein isoforms or splice isoforms. So, next to detection of extremely low abundant proteins, more research emphasis should be put in unraveling the effect of the huge variety of protein isoforms regulations in the blood plasma or serum during aging. In disease development the importance of glycosylation has become already more evident and people have found that the degree of glycosylation of alpha-1-glycoprotein can be correlated with inflammation and diabetes [3]. By using lectin-affinity chromatography others have found also interesting differences in the glycosylation patterns of serum proteins between healthy patients and patients suffering from breast [4] or pancreas cancer [5]. Taking into consideration also that aging might be accompanied with non-enzymatic glycation, it would be beneficial to put more effort in analyzing the influence of age on isoform regulation of blood plasma or serum proteins.

III Future prospects of proteome analysis in aging research.

To be able to extract all possible biological information embedded in the proteome, analytical proteome strategies have to deal with the diversity of the proteome, e.g. post-translational modifications, as well as the huge dynamic range of protein abundance, e.g. 10^6 for cells [6] and 10^9 in case of human serum or plasma [7]. In cell proteome research the reduction of proteome complexity might be obtained by the development of organelle extraction protocols. Eukaryotic cells consist out of multiple cell organelles with organelle specific proteins or in some cases DNA (mitochondria), which will be influenced differently in each organelle, upon perturbation. In order to search also for specific low abundant proteins, fractionation and purification of cell organelles in combination with proteome

analysis have been developed and resulted already in valuable information about specific cellular processes [8,9]. In view of this, future proteomics experiments should be focused on cell organelles involved in age- or senescence related processes like endothelium integrity (plasma cell membranes), ROS production (mitochondria) and protein accumulation (endoplasmic reticulum and lysosomes). However, this approach has also some major drawbacks, which might be the limiting factor in aging research. Fractionation and purification of cell organelles is always accompanied with contamination and unwanted protein losses, but more importantly is that cell organelle integrity might be compromised upon each perturbation. In case of replicative senescence it might be possible that due to a difference in membrane composition the plasma membrane of old cells reacts differently during the extraction than the membranes of young cells. Another issue is that isolation of specific proteins out of their biological environment might result in information, which might not be related to the proper biological function, but is a result of the applied extraction procedure. Therefore, we initially focused in this thesis on the analysis of whole cell extracts with 2D-DIGE despite some intrinsic limitations towards protein analysis, like limited linearity and under-representation of specific protein classes, like plasma membrane proteins [10,11].

In blood plasma or serum proteome research, fractionation of specific sets of proteins seems to be inevitable since the huge dynamic range of plasma proteins is at least 10 orders of magnitude and can simply not be covered by one analytical methodology. In this thesis we have depleted high abundant proteins like HSA and IgG, which still resulted in a plasma proteome representing 99% of the total blood plasma proteome. Although interesting biological information could be found, more relevant proteins might be determined, when protein classes are fractionated based on their pI, hydrophobicity or abundance [12-16]. Next to this, application of fractionation technology to large sample batches would be too laborious and will introduce more analytical variation.

Another approach to extract more information from the proteome is to increase the sensitivity of protein detection of the analytical methodology. In this way, less or no protein will be lost as a result of poor extraction efficiency. To overcomes this, people have initiated other approaches in which the sensitivity of detection plays an important role and where the mass spectrometer is responsible for the quantification as well as the identification of the proteins. In these approaches, protein extracts are subjected to proteolytic cleavage and subsequently LC-MS/MS analysis for peptide identification. However, differential peptide analysis with mass spectrometry is inherently not quantitative, since the diversity in physical properties

of (bio)molecules will result in different MS responses. Therefore, in these so-called LC-based approaches, peptides are labeled with stable isotope tags, to improve accurate quantifications. Stable isotopes are physically similar to each other with exception of the masses. Incorporation of stable isotope tags allows relative comparison of two different cellular states or tissues, because the labeled and unlabeled samples can be mixed and analyzed in parallel with LC-MS (see Figure 1). In the mass spectrometer discrimination between labeled and unlabeled peptides is possible which enables differential peptide/protein analysis of in our case two different age groups. In the last decade various stable isotope incorporation strategies have been developed, which can be divided in two classes, biological or chemical incorporation.

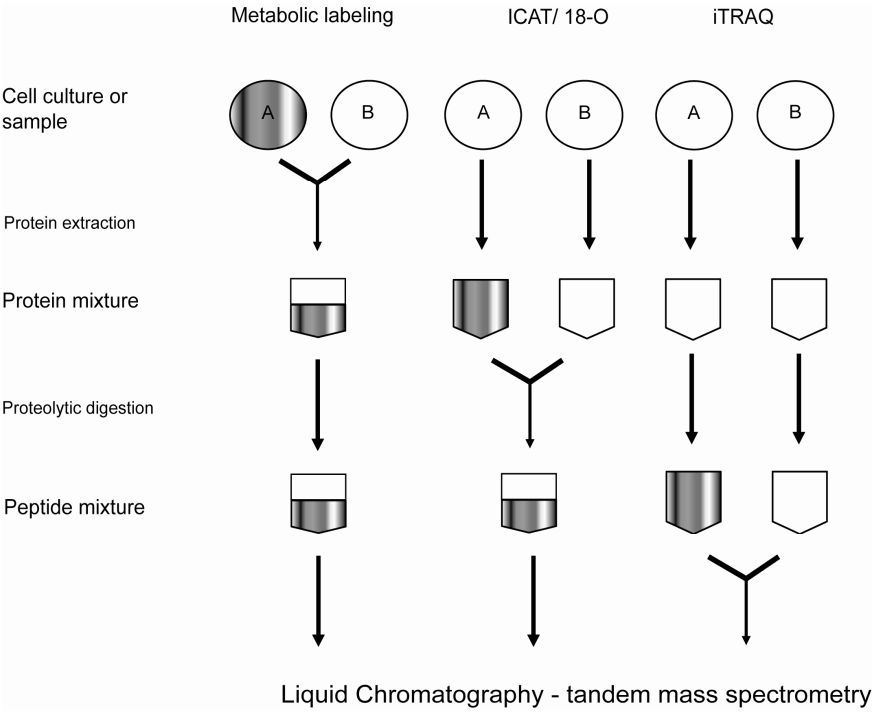

Figure 1. Various strategies for labeling proteins or peptides with stable isotopes for accurate quantitation.

In biological isotope incorporation, stable isotope labeled amino acids or metabolites are added to the cell culture, which allows full isotope incorporation in the whole cellular proteome. In this strategy the labels are introduced at the earliest moment in time and provide the most accurate biologically relevant protein quantitation. So with respect to cellular replicative senescence this would be the preferred method in the future to gain additional proteomic data. However, application of this labeling strategy requires adjustments to the cell growth medium, which is not always possible for all type of cells. Furthermore, labeling of whole organisms and subsequently tissues is limited to small mammals like mice or rats [17], whereas isotope incorporation in human tissues is certainly beyond ethical limits. This problem is more easily addressed when stable isotopes are chemically linked to the peptide via reactions with functional groups of the peptide or protein, such as lysine side chains or free N-termini. In these post-labeling methods (see Figure 1) the stable isotope is introduced after several steps of sample preparation, such as sample lysis or proteolytic digestion. Biotinylated Isotope Coded Affinity Tags (ICAT) react selectively with free cysteines thereby reducing the complexity of the peptide mixture since only labeled peptides or proteins can be enriched prior to MS analysis [18]. Other approaches are the incorporation of ^{18}O via tryptic digestion in $H_2{}^{18}O$, at the C-terminal side of the peptide [19] or iTraq [20]. One disadvantage with these strategies is that the stable isotope label will be introduced after several steps of sample preparation, like cell lysis or proteolytic digestion, which will cause partial losses of quantitative information.

In conclusion, it is obvious that each approach discussed in the previous paragraph will have its own advantages and that the research interest strongly determines the choice of methodology. However, it can be expected that the emerging proteomics research field will certainly provide us with more in-depth age-related proteome information in the coming decades, which will contribute to the development of new therapies to increase the quality of life at old age.

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Samenvatting

Samenvatting

In het algemeen wordt veroudering van het menselijk lichaam verondersteld als één van het meest complex en minst begrepen proces in de biologie. De hoge complexiteit van het verouderingsproces vereist een multidisciplinaire onderzoeksaanpak om het effect op alle niveaus in het menselijk lichaam te kunnen begrijpen. Dit proefschrift maakte deel uit van een multidisciplinair project, waarin o.a. de mogelijkheden van proteomics technologie in het onderzoeksveld van veroudering van het menselijk lichaam werden onderzocht en beschrijft hoofdzakelijk de effecten van veroudering op het proteoom in een modelsysteem van endotheelcellen alsmede in bloedmonsters. Door de complexiteit van het verouderingsproces werd verondersteld dat grote veranderingen in het proteoom niet zouden plaatsvinden, wat de toepassing van proteomics technologie tot een grote uitdaging maakte. Dit werd merendeels bevestigd door de eerste resultaten met 2D-gelelectroforese in combinatie met zilverkleuring, waarbij de resultaten duidelijk gepaard gingen met een hoge analytische variatie. Echter, de komst van 2D-DIGE heeft het mogelijk gemaakt om analytische variatie in gel-analyses sterk te reduceren. Hierdoor werd zelfs de mogelijkheid gecreëerd om gebruik te maken van biologische replica's, wat de biologische relevantie van de proteoom veranderingen ondersteunt.

I Veroudering van endotheelcellen
Endotheelcellen vormen de barrière tussen het bloed en het omliggende weefsel. Bij beschadiging van het endotheel worden de cellen vervangen door aangrenzende endotheelcellen of door specifieke cellen die circuleren in het bloed. Endotheelcellen kunnen niet ongelimiteerd delen en bereiken op een zeker moment een irreversibele groeistop, wat senescence wordt genoemd. Cellen in deze toestand zijn nog steeds metabolisch actief zijn en er treden veranderingen op in genexpressie, welke nadelige gevolgen kunnen hebben voor de functie van het endotheelweefsel. Deze verstoring kan leiden tot het ontstaan van aandoeningen zoals atherosclerose. Senescente endotheelcellen zijn reeds *in vivo* aangetoond in atherosclerotische plaques en spelen mogelijk een belangrijke rol in de progressie van deze ouderdomsziekte. Om deze reden was een gedeelte van het onderzoek gericht op het analyseren van eiwitveranderingen gedurende veroudering in een humaan endotheel celmodel (HUVEC) met behulp van 2D-DIGE. De resultaten uit **hoofdstuk 2** laten duidelijk zien dat een statistische bewerking met ANOVA noodzakelijk is om de biologische variatie van drie onafhankelijke celisolaties te kunnen reduceren, wat de betrouwbaarheid van de

gevonden eiwitregulaties aanzienlijk heeft verhoogd. Deze studie heeft ook aangetoond dat replicatieve senescence van endotheelcellen gepaard gaat met een verhoging van intracellulaire oxidatieve stress, een verlaging van de eiwitsynthese en veranderingen in de DNA reparatie- en onderhoudscapaciteit . Andere opmerkelijke veranderingen betreffen structurele veranderingen in het cytoskelet en eiwitten die verantwoordelijk zijn voor de integriteit van de kern, zoals lamine B. Het is nog nooit eerder vermeld dat dit eiwit in oude (senescente) cellen lager tot expressie komt dan in jonge cellen en het zou interessant zijn om met additionele technieken zoals microscopie of RNAi de rol van dit eiwit tijdens celveroudering verder te onderzoeken. Een andere interessante regulatie die gevonden is, is de verhoogde expressie van de V-ATPase B2 subunit. Dit eiwit is onderdeel van de V-ATPase protonpomp, welke veel in lysosomen voorkomt. Een verhoogde aanmaak van deze celorganellen is een van de kenmerken van senescente cellen. Ook in onze studie vertoonden veel sterk verouderde cellen een verhoogde SA-beta-galactosidase activiteit, hetgeen correleert met de hoeveelheid lysosomen in een cel. In het **addendum van hoofdstuk 2** is beschreven dat met behulp van elektronen- en lichtmicroscopie duidelijk te zien is dat oude (senescente) endotheelcellen meer lysosomen bevatten dan de jonge cellen. Ook was duidelijk waarneembaar dat senescente endotheelcellen meer vetdruppels en vertakte mitochondria hebben. Dit bevestigt dat er tijdens celveroudering een verstoring plaatsvindt van verscheidene metabole routes.

In **hoofdstuk 3** hebben we met Western blot kunnen bevestigen dat tijdens veroudering endotheelcellen verhoogde concentraties van runder-transferrine bevatten. Daarentegen blijkt de transferrine receptor lager tot expressie te komen in oude cellen, wat suggereert dat transferrine wordt opgenomen zonder receptor of dat de opnamesnelheid verschilt tussen jonge en oude HUVECs. Incubatie-experimenten met ^{125}I gelabeld humaan transferrine heeft geen verschillen aangetoond tussen jonge en oude cellen. Alhoewel is aangetoond dat runder-transferrine kan worden opgenomen via de humane transferrine-receptor, kan het nog steeds mogelijk zijn dat de recycling-capaciteit voor runder-transferrine toch afhankelijk is van de celleeftijd. Incubatie-experimenten met ^{125}I gelabeld runder-transferrine zal in de toekomst hiervoor uitsluitsel moeten geven. Een andere optie is dat transferrine in oude cellen niet via een receptor wordt opgenomen maar door middel van pinocytose.

II Verouderingsmarkers in bloed

Gezien de mogelijkheden van 2D-DIGE om analytische alsmede biologische variatie te kunnen reduceren, hebben we deze technologie ook toegepast om verouderingsmarkers te kunnen vinden in bloed. Hiervoor zijn bloedplasma monsters van drie gezonde leeftijdscategorieën uit de Leiden Longevity studie gebruikt. In **hoofdstuk 4** zijn, met behulp van affiniteitschromatografie, bloedplasma monsters gedepleteerd van HSA en IgG teneinde hogere gelresolutie te verkrijgen. Tevens was het hierdoor mogelijk om minder abundante eiwitten te evalueren. Dit heeft geresulteerd in een groep eiwitten die relevante regulaties vertoonden welke mogelijk correleren met gezond verouderen en waarvan het grootste deel van de eiwitexpressies veranderingen in eiwit- en/of splice-isovormen betrof. Functionele annotatie heeft laten zien dat gezond verouderen gepaard gaat met een licht verhoogde pro-coagulante en pro-inflammatiore staat. Belangrijke regulaties zijn gevonden in de verschillende complement C3 splicing-producten. Dit eiwit is betrokken bij de activatie van het immuunsysteem en de verhoogde aanwezigheid van verscheidene fragmenten suggereert dat de immuun-respons is verhoogd in de gezonde ouderenpopulatie. In andere verouderingsstudies is nooit melding gemaakt van dit fenomeen, wat mogelijk wordt veroorzaakt door het feit dat de gebruikte technologieën niet geschikt waren voor de detectie van complement C3 fragmenten. Dit toont de toegevoegde waarde van gel-electroforese in het verouderingsonderzoek aan.

Naast informatie over gezond verouderen is het ook noodzakelijk om kennis te verkrijgen over ongezond verouderen. In **hoofdstuk 5** hebben we een verkennende studie uitgevoerd met bloedplasma's van ongezonde en gezonde ouderen. De ongezonde pool bestond uit mensen die binnen 20 maanden overleden na bloedafname en leden aan verscheidene aandoeningen. De gezonde pool waren mensen van 85 jaar oud en waren 5 jaar na bloedafname nog steeds in leven. De gepoolde monsters zijn geanalyseerd met 2D-DIGE, wat heeft geresulteerd in interessante eiwitregulaties. Één eiwit kon worden gecorreleerd aan een ziekte waar een van de mensen uit de ongezonde pool aan leed. De meest prominente regulatie was de zeer hoge expressie van Ig Kappa light chain, welke kon worden gecorreleerd met de ziekte van één persoon uit de ongezonde pool. Minder ziekte-specifieke eiwitregulaties lieten zien dat in de ongezonde pool er een enigszins verlaagde pro-inflammatoire alsmede een verlaagde pro-coagulante staat aanwezig was. Het is bekend dat ouderen met een verlaagde weerstand vatbaar zijn voor de ontwikkeling van verscheidene ziekten. Het is dus mogelijk dat deze verlaging de mortaliteit in de ongezonde pool heeft verhoogd.

Curriculum vitae

Michael Eman werd geboren op 9 maart 1974 te Zeist. Na het behalen van zijn MAVO diploma aan de Gemeentelijke MAVO te Tiel in 1990, werd begonnen met een MLO studie aan het Versfelt-Ruygenhoek college in Utrecht. Na het behalen van het diploma startte Michael in datzelfde jaar met de HLO aan de Hogeschool van Utrecht. Deze studie werd, na een stage van 9 maanden bij Gist-brocades in Delft, afgerond in 1998 met Biochemie als afstudeerrichting. Na het afstuderen heeft Michael bijna 4 jaar gewerkt bij het Kluyver laboratorium voor de Biotechnologie van de Technische Universiteit Delft als onderzoeksanalist. Hier heeft hij vooral ervaring opgedaan met het ontwikkelen van LC-MS/MS methodes voor het kwantificeren van intracellulaire metabolieten in gist. Na deze leerzame periode begon Michael in 2002 een promotieonderzoek bij de sectie Cellular Architecture & Dynamics en Biomoleculaire Massaspectrometrie onder begeleiding van dr. Jan Andries Post, prof. Arie Verkleij en prof. Johan Haverkamp, wat heeft geleid tot de in dit proefschrift beschreven resultaten. Sinds 15 november 2007 is Michael werkzaam als studieleider Bioanalyse bij NOTOX BV in ’s Hertogenbosch.

List of publications

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Dankwoord

Een promotie doe je nooit alleen en er zijn veel mensen die mij geholpen hebben tijdens de afgelopen vier jaar. Een AiO-schap bij twee totaal verschillende onderzoeksgroepen heeft als leuke bijkomstigheid dat je echt veel collega's hebt. Om nu niemand te vergeten wil ik bij deze iedereen van Biomassa en MCB (of CAD) bedanken voor de gezelligheid en de leuke "wetenschappelijke" discussies.

Uiteraard zijn er natuurlijk ook een aantal mensen die ik speciaal wil bedanken. Allereerst mijn promotor Arie Verkleij. Arie jouw onuitputtelijke stroom van ideeën heeft mij menigmaal verder geholpen tijdens het promotietraject. Jouw manier van communiceren heeft mij altijd aangesproken en ik wil je ook hartelijk bedanken voor de steun tijdens mijn promotietijd. Jap, Jappie of gewoon Jan Andries het maakt jou niet uit hoe je wordt genoemd. Bedankt voor de persoonlijke manier waarop je mij de afgelopen vier jaar hebt begeleid. Altijd een geduldig en welwillend oor voor de vele "biologische" vragen die ik stelde. Mede door jouw geduld en kundige uitleg is mijn kennisgat betreffende de celbiologie (gedeeltelijk) opgevuld. Dat mijn interesse voor biologische processen is aangewakkerd, kun je wel op jouw conto schrijven. Johan, als mijn andere promotor wil ik je graag bedanken voor de vrijheid en steun die je me hebt gegeven tijdens mijn promotietraject. Je hebt me altijd veelvuldig gewezen op het voordeel van multidisciplinair werken en denken, een advies wat mij nog goed van pas gaat komen in mijn huidige baan. Albert, ik wil jou bedanken voor het feit dat ik gebruik mocht maken van alle onderzoeksfaciliteiten en ideeën/adviezen die jouw groep te bieden had. Tevens ben jij een belangrijke katalysator geweest bij het tot stand komen van mijn eerste publicatie als eerste auteur. Bedankt daarvoor!

Uiteraard wil ik ook Rinse bedanken. In tegenstelling tot wat ik eerst dacht is het bloedplasma project toch een belangrijk onderdeel geworden van mijn proefschrift. Ik wens je veel succes met je post-doc baan en je gezinsleven. ("Het leven begint pas echt als je vader bent geworden", …. toch?). Ook wil ik graag Jord bedanken voor het kritisch doorlezen van mijn plasma manuscripten en het uitvoeren van de vele blotjes. Also I would like to thank Elsa for her contribution to my first publication in Electrophoresis. Elly hartelijk bedankt voor die verschrikkelijk mooie EM plaatjes!! Echt vakvrouwschap zullen we maar zeggen. Martijn wil ik ook bedanken voor het analyseren van wéér die verdraaide spotjes!! Jouw vakmanschap heeft er zeker voor gezorgd dat menig spotidentificatie in één keer

goed ging. Mijn studenten, Judith, Elaine en Andree wil ik ook graag bedanken voor hun inzet.

Het grootste gedeelte van de tijd heb ik fysiek bij Biomassa doorgebracht op de mooiste en gezelligste kamer van het Wentgebouw; Z301. Graag wil ik natuurlijk de andere Z301 kamerbewoners van het eerste uur bedanken. Esther, hartelijk bedankt voor de gezelligheid, het “groen/paars” houden van de kamer, de kerstboomverlichting en ….voor het veelvuldig aflezen van de buitentemperatuur op 15 km afstand. Eef, apa kabar? Hoe gaat het met je Indonesisch, weet je al wat je NIET hardop in de bus moet roepen? Ook jij bedankt voor de leuke gesprekken die we altijd hadden en ik weet het .... ook ik mis de tijd dat we in één bed sliepen (…☺). Judith, ook jij hartelijk bedankt voor de gezelligheid en …. het verzorgen van de planten van Esther als ze op vakantie was, dan bleef de kamer nog enigszins groen in plaats van lichtbruin!

Het laatste jaar van mijn promotie heb ik doorgebracht bij MCB, op de vijfde verdieping. Ook hier zijn er natuurlijk een aantal collega’s die hebben bijgedragen aan dit boekje. Miriam en Bart bedankt voor het isoleren en kweken van “mijn” cellen!! Zonder jullie vakkundigheid waren die cellen nooit oud geworden!! Ook Maarten wil ik bedanken, voor de enthousiaste begeleiding op de confocale microscoop. Ondanks dat ik lang niet alles heb kunnen gebruiken, vond ik het bijzonder leerzaam!

Graag wil ik ook wat oud-collega’s van het Kluyverlab bedanken. Rosanne, Merle en Martijn, volgens mij ben ik de laatste van “ons” groepje die moest promoveren. Nu zijn we eindelijk allemaal klaar!! Bedankt voor de leuke tijd op het Kluyverlab. Uiteraard kan ik ook Jan niet vergeten. Jan, hartelijk bedankt voor de vele adviezen, steun en onderzoeksvrijheid die je me hebt gegeven tijdens mijn analistentijd. Mede daardoor ben ik toch een promotiestudie gaan doen.

Naast het werk zijn er ook een heleboel mensen die voor mij belangrijk zijn geweest tijdens mijn AiO-schap. Klussen is voor mij een beetje een hobby, maar er zijn van die klussen waar je toch echt hulp bij nodig hebt. John, Erik en Frank hartelijk bedankt voor de hulp tijdens mijn keukenverbouwing en het slap geklets naderhand. Die ontspanning had ik echt nodig!! Als we dan toch over slap kletsen praten dan moet ik zeker Haas niet vergeten. Sandor, ook jij bedankt voor de ontspanning en lol. Onze conversaties gingen echt werkelijk *nergens* over en nu de promotieperiode voorbij is, wil ik je dan ook uitdagen voor een serieuze kart-sessie met als inzet een broodje Mario!! Thamara wil ik ook bedanken voor het ontwerpen

van de omslag. Geweldig om te zien dat jij aan een half uurtje praten genoeg hebt om de mooiste ontwerpen te maken, echt super!! Uiteraard zijn er nog een heleboel vrienden, ooms, tantes, neven en nichten die ik ook wil bedanken voor hun getoonde interesse in de voortgang van mijn promotie. Ik hoop dat dit boekje jullie meer helderheid verschaft over wat die “Eman” nou eigenlijk de afgelopen jaren heeft gedaan.

Dan is er natuurlijk ook nog de naaste familie. Beide families, de Emannetjes en de Moretjes, betekenen heel veel voor mij en verdienen zeer zeker een vermelding in dit laatste hoofdstuk. Lieve ouders en schoonouders, graag wil ik jullie bedanken voor de steun en gezelligheid. De “studie” is eindelijk af!!! Koos bedankt voor de tip om de “pakketjes” maar gewoon aan te nemen. Het heeft het schrijven van mijn proefschrift een stuk leuker en makkelijker gemaakt!! Ook mijn broertje, schoonzussen en zwagers hebben kunnen meegenieten van mijn schrijfperikelen, als Cathérine en de kids wéér alleen naar een feestje kwamen. Ook jullie bedankt voor de steun en gezelligheid in de afgelopen jaren. Fréderique, ik vind het echt super dat je mijn paranimf wilt zijn! Lieve familie, gezien de lol en vreugde die we altijd hebben, verheug ik me nu al op de eerste verjaardag of het eerste familieweekend NA mijn promotie!!

Tsja, en nu komen de mensen die mij tijdens de afgelopen vier jaar wel van héél dichtbij hebben meegemaakt. Marise en Louisa, papa is EINDELIJK KLAAR met zijn werk in Utrecht!! Ladies, we gaan snel zwemmen, balletten, paardrijden voetballen, knutselen, fietsen, etc …. papa heeft nu zeeën van tijd!!! Dames, jullie zorgeloze en vrolijke kijk op het leven (papa, kun je een paard voor me kopen? Je werkt toch?) heeft mij altijd de energie gegeven om met vreugde wéér een gelletje te draaien. Lieve kleine Sarah, óók jij bent mijn kleine oogappel. De zes maanden die jij bij ons was, heeft een grote indruk bij papa achtergelaten. Jouw wilskracht en liefde zal ik nooit vergeten……Tot ziens in de Hemel! .

Lieve Cathérine, we kennen elkaar al heel lang en ik weet nu al dat er op z’n minst nog een extra katern in het proefschrift bij moet komen om al mijn dank te uiten. Zonder jouw steun en toeverlaat had ik dit traject überhaupt nooit kunnen opstarten. Je bent natuurlijk niet voor niets één van mijn paranimfen en één ding weet ik héél zeker ….*met jou zie ik de route van veroudering met vertrouwen tegemoet!!* Lieve schat, ik hou van je…

Michael