Chapter 3

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

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Abstract

With increasing age, an individual is subjected to numerous changes at the molecular, cellular and tissue level. As blood is distributed throughout the body, disturbances in an organism should, at least in part, be reflected in the protein composition of the blood. To understand the mechanisms of aging, several studies have focused on identifying these changes, mainly by hypothesis-driven research. Although some aging-related changes in protein expression have been identified, these studies have failed to acknowledge the presence of numerous isoforms and protein fragments of a single gene product in this complex proteome. Here, we describe the proteomics analysis of pooled human plasma samples of young, middle aged and old individuals depleted of human serum albumin and IgG with 2D-DIGE and mass spectrometry, which enables accurate differential protein expression analysis. This resulted in the identification of a set of differentially expressed protein spots. Several were in line with data from others and confirmed a slight pro-inflammatory and pro-coagulant status upon aging. Intriguingly, in several occasions multiple differentially expressed protein spots were identified to originate from a single gene product. For some of these proteins the different isoforms exhibited divergent expression patterns. The most prominent of these proteins was complement C3 (CO3), which showed a clear increase in the presence of the CO3 fragment C3dg with increasing age. This study clearly indicates the importance of protein isoforms analysis in addition to total protein levels, and results in a set of proteins that might be used to define biological age.

Introduction

The aging process is one of the most common events in modern life and is accompanied by a decline in functionality. This imposes a huge impact on society, both socially and economically. Despite increasing efforts 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. 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 (Adams et al., 1982; Chance et al., 1979; Mackaness, 1970), or have an environmental origin, like radiation and chemicals (Cerutti, 1985; Cosgrove et al., 1985; Pollycove and Feinendegen, 2003; Stone and Pryor, 1994). Although an organism has several mechanisms to prevent and to repair damage caused by these agents, over time damage will accumulate. This ultimately leads to a decline in the ability of cells to respond to disturbances in cellular homeostasis, which has major consequences for the vitality of the organism.

Due to these events, changes in RNA as well as protein levels occur. Insight in these changes could aid in understanding the mechanisms underlying the aging process. Furthermore, it could provide a possibility to determine the biological age of an organism, rather than its chronological age, and it could facilitate in monitoring the effects of intervention studies to slow down the aging process.

The goal of the present study is to use advanced proteomics techniques to identify multiple independent biomarkers in human blood related to aging.

One of the most important and most accessible human clinical biological materials is blood plasma. For several plasma proteins, differences in expression during aging that have been described in literature involve mostly cytokines and other proteins involved in the immune response (Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Pedersen et al., 2000; Ritchie et al., 1998). Most of these results were obtained with immunological assays, which are usually very specific, sensitive and accurate in providing quantitative information. However, this approach neglects the presence of differences in posttranslational modification or the occurrence of protein fragments of a single gene product, which are very common for plasma proteins (Anderson, 2005; Misek et al., 2005).

Two-dimensional gel electrophoresis (2D-GE) is a well-established technology, which allows the separation of hundreds of proteins simultaneously. Moreover, 2D-GE is a highly suitable technique to detect protein isoforms. Accordingly, it is the most frequently used method despite the limitations towards sensitivity and reproducibility compared to immunological assays. Recent developments in 2D-GE analysis have paved the road to detect small expression differences with high accuracy. First of all, removal of highly abundant bulk proteins has improved the resolution of 2D gels and has increased the detection of low abundant proteins (Klooster et al., (Submitted)-a; Pieper et al., 2003a; Steel et al., 2003). Furthermore, advanced protein labeling techniques have become available, which enable the detection of small expression differences with a high confidence level. In Two-dimensional Difference Gel Electrophoresis (2D-DIGE) experimental variation is reduced due to the combination of multiple sample analysis in a single gel, and internal standard correction (Alban et al., 2003; Chen et al., 2005; Lilley and Friedman, 2004).

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. Eighty-nine protein spots were considered as differentially expressed during aging of which 48 were positively identified with mass spectrometry. Several of the identified spots were in line with data described in literature, such as the differential expression of several acute phase proteins. In addition, remarkable differential expression of protein isoforms, originating from single gene products were detected upon aging, among which the differential expression of several complement C3 (CO3) fragments were the most striking. This illustrates the importance of developing methods to discriminate and analyze modified forms of a single protein. The differential expression of several of the identified proteins and their implications in the aging process will be discussed.

Materials and methods

Sample preparation

Citrate plasma samples 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 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 (Klooster et al., (Submitted)-a). 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 in 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). Next, 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 15µl 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 labelled 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 24cm (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 over night 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

All gels were scanned in between low fluorescent glass plates at 100µm pixel resolution with the Typhoon image scanner 9400 (Amersham biosciences, Roosendaal, The Netherlands). 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).

Differential analysis

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 (Amersham Biosciences, Roosendaal, The Netherlands) to remove dust-related pixels. Differential analysis was performed with the Decyder V 5.01 (Amersham Biosciences, Roosendaal, The Netherlands)

software package. Analysis of variance 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 (Shevchenko et al., 1996). Spots of interest were subjected to in-gel tryptic digestion as described previously (Kolkman et al., 2005).

Nano LC-MS/MS

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 or LTQ-FT (Thermo electron Company, Waltham, MA, U.S.A.) as described previously in literature (Kolkman et al., 2005). 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 on 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.

Protein identification

The obtained mass spectra were subjected to a Mascot search engine (Perkins et al., 1999). Probability-based protein identification was performed by searching sequence databases using mass spectrometry data with 0.8 Da peptide tolerance, 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 reducing or non-reducing 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer (2.5% protifar plus (w/v) in PBS), the membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or 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 polyclonal anti-CO3 antibody (Abcam, Cambridge, United Kingdom) diluted 2000 times 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 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 chemoluminescence (PerkinElmer, Boston, U.S.A.).

Results

Proteomic analysis

To analyze age related proteome changes, plasma samples of three different 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 subsequently, HSA and IgG were removed with VHH antibody technology (Klooster et al., (Submitted)-a) 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 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, 48 were positively identified *in duplo* and linked to their molecular weight, and expression dynamics (Figure 1 and Table 1). The majority of this subset showed an increased abundance towards aging, ranging from 1.2-10 (Figure 2A), and nineteen spots showed a decreased expression at old age (Figure 2B). Analyzing protein expression dynamics with 2D gel electrophoresis offers the possibility to visualize or evaluate the expression dynamics of protein isoforms. Plasma proteins are known for their variety in isoform abundance (Anderson, 2005; Misek et al., 2005). Protein identification of the spots of interest indicated that the majority of the abundance differences could be assigned to various protein isoforms or fragments of a restricted number of parent proteins (Figure 1 and Table 1). Twenty unique proteins were identified, among which proteins that are involved in coagulation, such as fibrinogen, the immune respons, such as CO3 and several acute phase proteins, as well as proteins that neutralize damaging compounds in the blood, such as gelsolin and vitamin D binding protein (DBP). The majority of the abundance differences were caused by haptoglobin (spots 13A-13I), fibrinogen (spots 10A-10G), transferrin (spots 17A-17D), alpha-1-antitrypsin (spots 1A-1C) and CO3 (spots 8A-8F). For these proteins, the trend of regulation of all isoforms originating from a single gene product was in general the same (Figure 3A-E). However, there were exceptions for some alpha-1-antitrypsin (Figure 3A), complement C3 (Figure 3B) and haptoglobin (Figure 3D) isoforms.

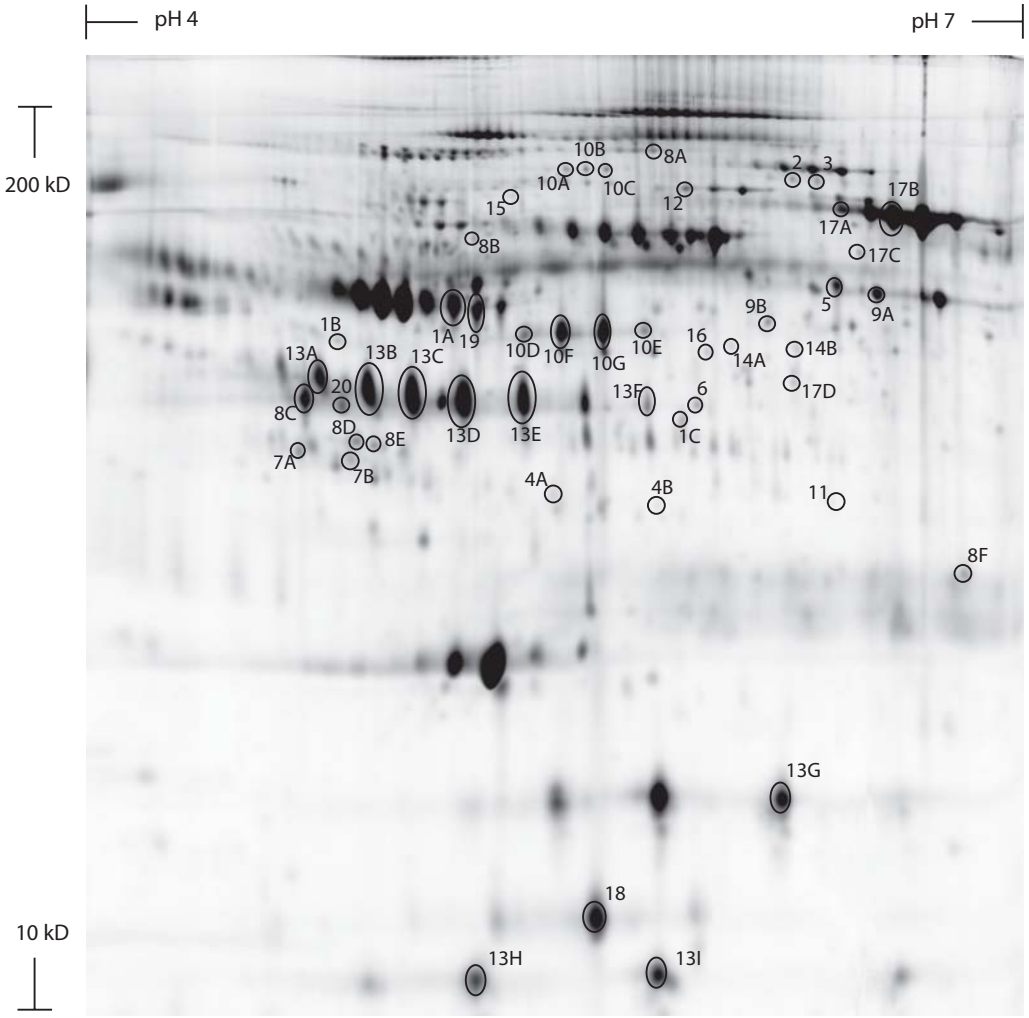


Figure 1: 2D-DIGE analysis of HSA and IgG depleted pooled plasma samples of young, middle aged and old individuals revealed differential expression between the young and the old group of 89 different protein spots of which 48 were positively identified *in duplo*. The numbers refer to the identified differentially expressed proteins and their isoforms, as shown in table 1.

Spot		Protein	Acc. nr.	Mw	Reg. O/Y	ANOVA	SC%	pept.
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		Alpha-2-macroglobulin	P01023	163278	0.7	1.7E-04	9	16
3		CFAB/alpha-2-macroglobulin	P00751	86902	0.7	1.6E-03	8	13
4	A	Apo-E	P02649	36268	1.3	1.8E-03	54	22
	B				0.6	3.1E-04	58	23
5		Apolipoprotein H	P02749	39610	1.2	1.7E-02	52	19
6		Apolipoprotein-L1	O14791	43927	0.7	1.1E-03	24	23
7	A	Clusterin	P10909	53065	0.9	3.1E-02	32	14
	B				1.3	2.0E-02	28	14
8	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
9	A	Fibrinogen Beta chain	P02675	56613	1.4	5.5E-03	60	33
	B				1.8	1.1E-04	22	12
10	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
11		Ficolin	O75636	33381	0.6	1.2E-03	17	6
12		Gelsolin	P06396	85698	1.3	2.5E-03	39	29
13	A	Haptoglobin	P00738	66058	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
14	A	IgM	P01871	50242	1.5	3.6E-06	27	12
	B				0.7	2.0E-04	17	9
15		Inter-alpha-trypsin inh. heavy ch. H4	Q14624	103358	1.3	3.8E-04	16	14
16		Pigment epithelium-derived factor	P36955	46513	1.3	4.5E-04	36	15
17	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
18		Transthyretin	P02766	16001	0.8	2.5E-04	68	10
19		Vitamin D-binding protein	P02774	54561	0.7	9.9E-04	67	34
20		Zinc alpha 2 glycoprotein	P25311	33872	1.3	8.5E-04	54	22

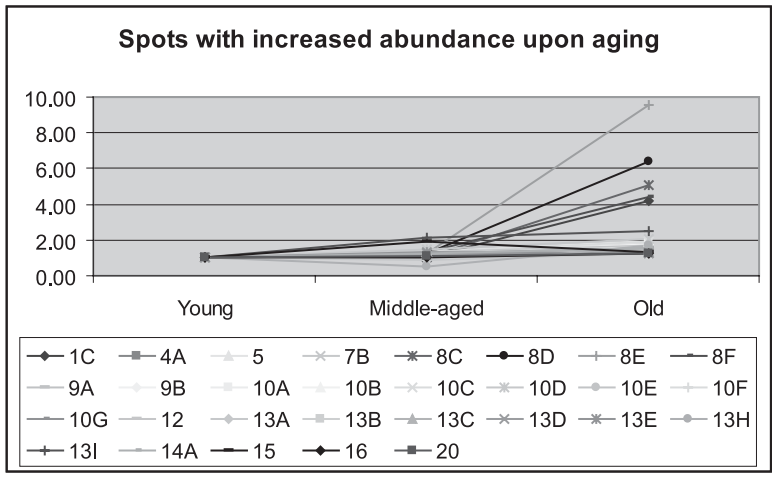
Table 1: *In duplo* identified differentially expressed protein spots between the young and old group in human plasma. The differential expression of the old group is normalized to the expression in the young group. The accession number (Acc. Nr.), molecular weight of the unprocessed precursor (Mw),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

For alpha-1-antitrypsin, three protein spots were identified. Two showed a small decrease in expression upon aging (spots 1A and 1B) and one showed a marked increase (spot 1C)(Figure 3A). Of these spots, spot 1A was the most abundant, while the other two spots were hardly visible.

Haptoglobin, an acute phase protein that has many isoforms mainly caused by its glycosylation pattern, showed mostly patterns that gradually increased

upon aging (Figure 3D). In contrast, a minor part of the differential expressed haptoglobin isoforms showed a divergent pattern (spots 13F-13I). All these isoforms were less abundant compared to the isoforms that showed a minor increase in expression upon aging (spots 13A-13D). Of the haptoglobin spots that showed a divergent expression pattern, two decreased slightly upon aging (spots 13F and 13G), one showed a marked decrease in the middle aged population and a marked increase in the old population (spot 13H), while the last spot showed an increased abundance in both the middle aged and the old population (spot 13I).

A



B

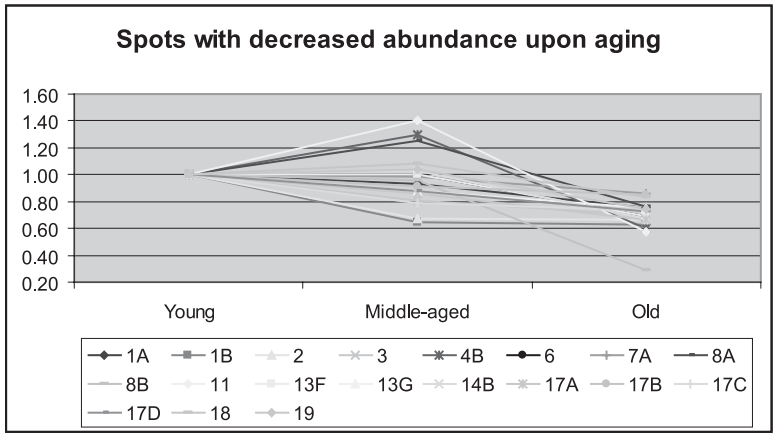


Figure 2: Differentially expressed protein spots with a significant increased (A) and decreased abundance (B) upon aging.

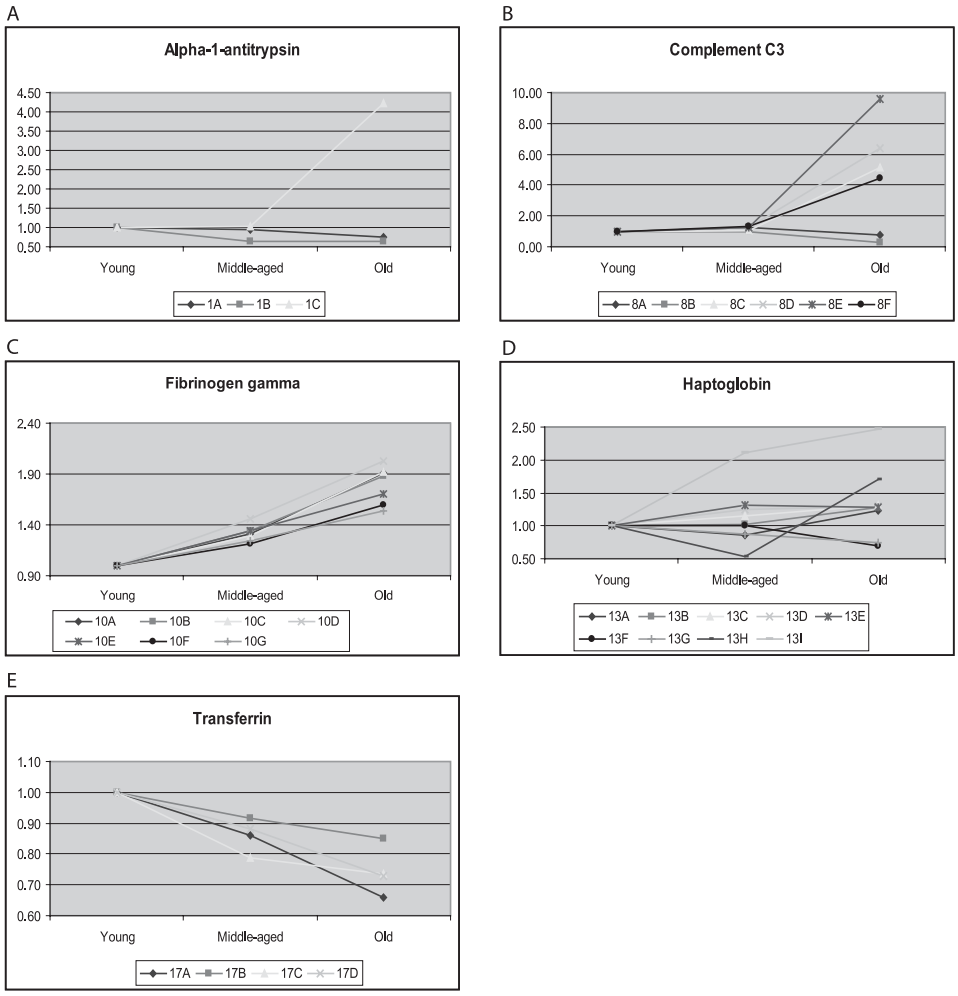


Figure 3: The expression patterns in the different age groups of several different isoforms of alpha-1-antitrypsin (A), CO3 (B), fibrinogen gamma (C), haptoglobin (D) and transferrin (E). Not all isoforms of a single gene product show a similar expression pattern. Some isoforms of alpha-1-antitrypsin (spot 1C), complement C3 (spots 8A and 8B) and haptoglobin (spots 13F, 13G and 13H), show an expression pattern that is not consistent with the majority of the isoforms.

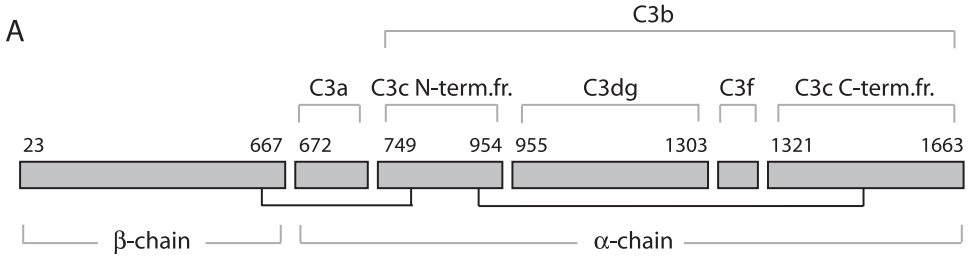
These data show that in addition to total protein levels, levels of protein isoforms could be important determinants with respect to aging.

Strikingly, the most substantial differences between the young and old population were found in six spots identified as CO3, of which four gave an increase in expression above a factor four between the young and old group (spots 8C-8F), while two showed a marked decrease in expression (spots 8A and 8B)(Figure 3B).

Differential expression of CO3 protein fragments

CO3 plays a key role in the activation of the immune system. Its functionality is regulated by specific proteases that cleave the mature protein (Figure 4A) and thereby induce conformational changes, which leads to several different active components, each with different specific functions. During these cleavages, soluble polypeptide chains are released from the mature protein while others remain attached via disulfide bonds.

A



B

Spot	Regulation O/Y	CO3 fragment	Seq. (Th)*	SC (%)*	Mw kD(th.)	pI(Th)*
8A	0.8	C3 alpha chain	672-1663	58	113	5.55
8B	0.3	C3 alpha chain (no C3c/C3a)	955-1663	32	80	4.97
8C	4.4	C3c C-terminal fragment	1321-1663	80	39	4.79
8D	6.4	C3dg	955-1303	32	39	5.00
8E	9.6	C3dg	955-1303	32	39	5.00
8F	4.4	C3c N-terminal fragment	749-954	37	34	6.89

Figure 4: Identification of complement C3 cleavage products that are differentially expressed between the young and old group. During activation, complement C3 undergoes several conformational stages induced by specific proteases that cleave the mature protein (A). 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. For each CO3 spot found in our study, the precise splice product was identified by matching the recovered peptides from the FTMS to the amino acid sequence of CO3 (B). The sequence coverage (SC), theoretical molecular weight (Mw) and pI for each splice product are indicated.

In this study we have identified several differentially expressed CO3 spots, at different molecular weights on the 2D-gel, indicating that these spots were protein fragments of the mature CO3 protein. To confirm this, the sequences of the CO3 spots identified by the LTQ-FT were examined further, which

enabled the annotation of the different CO3 fragments (Figure 4A and 4B). Spots that decreased upon aging were identified as the alpha chain of CO3 (spot 8A) and the alpha chain without the C3a fragment and the N-terminal C3c fragment (spot 8B). The spots that increased upon aging were assigned to both C3c fragments of the alpha chain (spots 8C and 8F) and C3dg (spots 8D and 8E). These results indicated that on average 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 circulation.

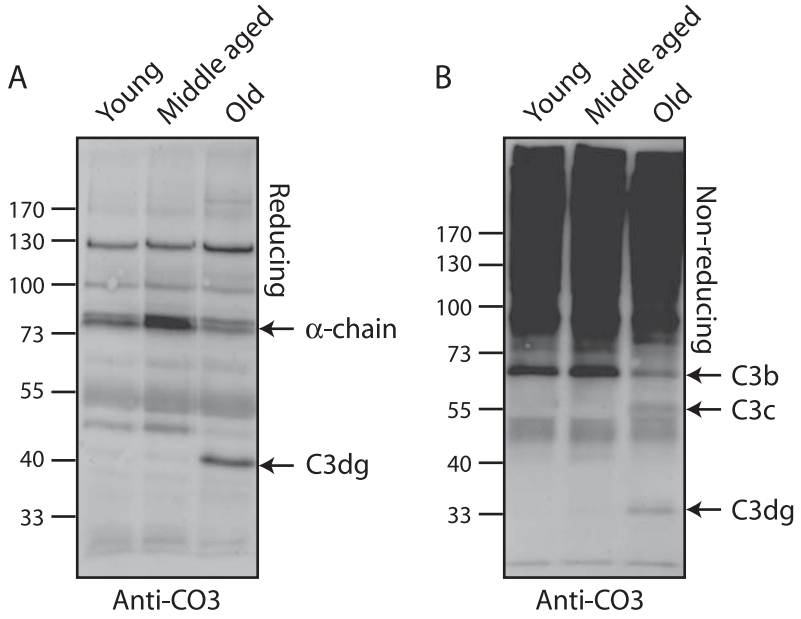


Figure 5: Verification of differential expression of CO3 splice products during aging. The pooled plasma samples of the different groups were analyzed on Western blot with a polyclonal anti-CO3 antibody. Samples were analyzed on blots that were obtained by running the samples on a reducing and a non-reducing gel. On both blots there was a clear difference in expression of several protein bands (arrows).

To verify these results, the pooled plasma samples were further analyzed on Western blot with a polyclonal anti-CO3 antibody. First, samples were run on a reducing gel which showed a protein band running at ~40kDa that showed an apparent increase in expression in the old group compared to the young and middle aged group (Figure 5A). Furthermore, there was an inverse correlation with protein bands running at ~45 and ~80kDa, suggesting that the 40kDa product was a protein fragment originating from the 45 or 80kDa proteins.

Experiments with non-reducing gels showed a similar pattern (Figure 5B). Two bands were observed at ~33kDa and ~55kDa that showed a clear increase in the old group compared to the young. Again an inverse correlation was seen with a protein band running at ~70kDa. These data were in line with the degradation of the alpha chain of CO3 into the components C3dg and C3c with increasing age.

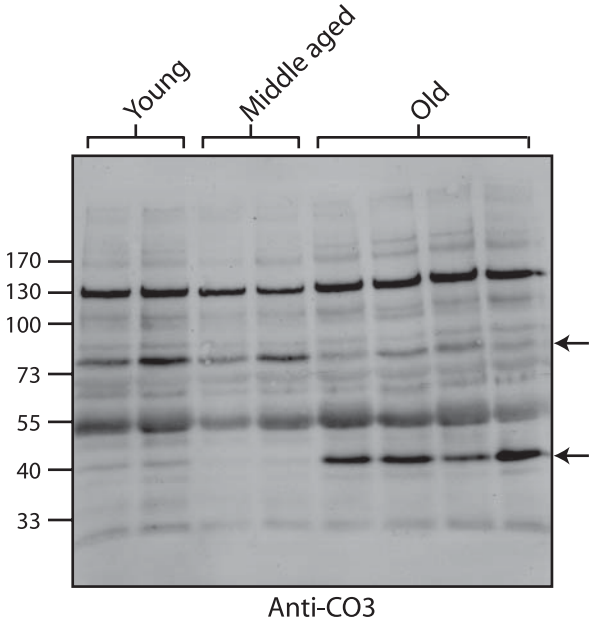


Figure 6: Confirmation of differential CO3 splice product expression with increasing age. All individual plasma samples were analyzed on Western blot with a polyclonal anti-CO3 antibody. Although there is individual variability within each group, there is a clear difference between the individuals in the old and the young and middle aged groups (arrows).

Because this observed difference could be caused by the presence of a few individuals with a pro-inflammatory immune state, the original citrate samples of all individuals (Figure 6), as well as heparin plasma samples of young and old individuals that were not included in the 2D-DIGE analysis (data not shown), were analyzed on Western blot. Although variability between individuals was observed, there was a clear difference between the individuals from the old and the young group that corresponded to the results obtained with the pooled samples. Especially the protein band running at ~40kDa was clearly up-regulated in all samples from the old individuals.

Discussion

The aim of this study was to search for age-related protein changes in human plasma. Proper depletion of HSA and IgG, and subsequent analysis with 2D-DIGE resulted in a set of proteins, which showed expression profiles with a significant ANOVA p-value ≤ 0.05. Some of the identified differentially expressed proteins were either protein fragments or post-translational modified forms of single gene products.

To our knowledge, the present study is the first to study age-related protein expression in blood with a 2D-DIGE approach, resulting in a relatively large panel of differentially expressed proteins. Several of the identified proteins agreed with results that have been described by others in studies focusing on one or a few blood components during aging. This included IgM (Ritchie et al., 1998), clusterin (Ishikawa et al., 1998; Mackness et al., 1997; Trougakos and Gonos, 2002; Witte et al., 1993), alpha-2-macroglobulin (Ritchie et al., 2004a) and several acute phase proteins, which are involved in the maintenance of blood homeostasis, such as haptoglobin (Ritchie et al., 2000), fibrinogen (Aillaud et al., 1986; Ernst and Resch, 1993; Tofler et al., 2005), transferrin and transthyretin (Ritchie et al., 1999). Next to this, our approach resulted in several other proteins, which have not been described earlier as being differentially expressed during aging. These included the proteins gelsolin, DBP, ficolin, pigment epithelium-derived factor, inter-alpha trypsin inhibitor and zinc alpha-2-glycoprotein. Some of these expression differences are in line with the accepted view of increased cellular damage and an increased pro-coagulant (Tofler et al., 2005) and pro-inflammatory (Gomez et al., 2005) state with increasing age.

The identification of plasma gelsolin and DBP are intriguing with respect to tissue damage, as they are thought to act in concert with each other to neutralize the potential damaging effects of cellular actin released in the blood. Plasma gelsolin is an actin severing molecule that caps actin released in the blood by tissue or cell damage. Thereby, it prevents the formation of actin filaments that can obstruct the microvasculature. The up-regulation found in the present study could reflect an attempt of the system to cope with increased cell damage with increasing age and thereby prevent further tissue damage induced by microvascular obstruction. Monomeric G-actin molecules that dissociate from the pointed filament ends of the actin polymer bound by gelsolin are sequestered by DBP (Vasconcellos and Lind, 1993). This could

induce an increased uptake and degradation of DBP when bound to actin, as has been shown in rats (Dueland et al., 1990) and would be in line with reports that describe decreased DBP blood levels upon acute tissue damage (Dahl et al., 1998; Schiodt et al., 1997).

The many different up-regulated fibrinogen spots are indicative of a pro-coagulant state with increasing age. This is in line with data from others (Aillaud et al., 1986; Ernst and Resch, 1993; Tofler et al., 2005) who have described up-regulation of fibrinogen in aging subjects, and which has also been shown to be a risk factor for developing cardiovascular diseases like thrombosis. This pro-coagulant state has also been shown to correlate to increased levels of other pro-coagulant factors, such as von Willebrand factor, PAI-1 and t-PA antigen (Haverkate et al., 1995; Thompson et al., 1995), which were not observed in the present study, and which is thought to be caused by a low-grade inflammatory state associated with aging.

Indeed, many of the identified proteins in our study are related to the immune response and are also indicative of a pro-inflammatory immune state. Some differentially expressed proteins belong to the acute phase proteins, such as fibrinogen, haptoglobin, alpha-1-antitrypsin, transthyretin and transferrin, and are critical for maintaining or re-establishing homeostasis. The hepatic synthesis of these proteins is regulated by the primary cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor alpha (TNFα) (Ruminy et al., 2001). Upon infection, injury or other physiological conditions that distort homeostasis, an acute phase response is triggered in which the positive acute phase proteins, including haptoglobin, fibrinogen and alpha-1-antitrypsin are up-regulated and the negative acute phase proteins, including transferrin and transthyretin, are down-regulated (Ruminy et al., 2001). Although there is some variation in differential expression between different protein isoforms, in general the overall average expression of the identified positive acute phase proteins show a slight increase and all negative acute phase proteins show a slight decrease during aging. Although no individual parameters are determined for these proteins in our experiments, these results are in line with data from others (Aillaud et al., 1986; Ernst and Resch, 1993; Ishikawa et al., 1998; Mackness et al., 1997; Ritchie et al., 1998; Ritchie et al., 1999; Ritchie et al., 2000; Ritchie et al., 2004a; Tofler et al., 2005; Trougakos and Gonos, 2002; Witte et al., 1993). Interestingly, alpha-1-antitrypsin forms an exception to this observation. The most abundant differentially expressed

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 3A). Data from others using immuno-assays (Ritchie et al., 2000) show no or at most a very slight increase in alpha-1-antitrypsin expression after the age of 55. An explanation for this apparent contradiction is the use of different detection techniques in the different experiments. Immuno-assays provide information on total protein level expression, while 2D-DIGE provides information about separate protein isoforms that reveal differential expression. The differential expression of several acute phase proteins, suggests that during aging a minor acute phase response is triggered. There are several indications that with increasing age circumstances arise that stimulate this more pro-inflammatory immune state, known as inflamm-Aging. Several changes in the adaptive and innate immune system arise with increasing age, which contribute to this disturbed immune state (Grubeck-Loebenstein and Wick, 2002). One of the changes concerns the alteration of important functions of members of the adaptive immune response, such as T-cells. The expression of some very important cytokines needed for T cell clonal expansion, including IL-2, are decreased with aging (Gillis et al., 1981). This contributes to the dysfunction of the adaptive immune response and the increased incidence of infections and several age-related diseases, such as auto-immune disorders, cancers and atherosclerosis (Castle, 2000a; Castle, 2000b; Fulop et al., 2005; Wick, 2000). Also some specific functions of the innate immune response are altered (Fulop et al., 2004), possibly caused by the imbalance in the adaptive immune response. One of these changes is the production of pro-inflammatory cytokines TNFα, IL-1 and IL-6. Indeed, the increased presence of these primary pro-inflammatory cytokines in plasma has been described(Bruunsgaard et al., 2003; Ershler et al., 1993; Fagiolo et al., 1993; Ferrucci et al., 2005; Pedersen et al., 2000) and could be an explanation for the differential expression of the acute phase proteins found in the present study as these cytokines are known to induce an acute phase response.Furthermore, during life LPS and other bacterial breakdown products that for a large extent seem to originate from the normal bacterial flora of the gut might accumulate in the organism (Winchurch et al., 1982). Moreover, different protein modifications, such as carbonylation and glycation of plasma proteins, and endothelial and blood cell proteins, have been shown to

accumulate during aging (Goswami and Koner, 2002; Jana et al., 2002; Wautier and Schmidt, 2004). With an increase in pathological events upon aging, such as atherosclerotic plaque formation and an increase in the amount of cell mortality and thus the release of intracellular proteins in the circulation (Ji et al., 2002; Seifert et al., 1990), these, among others, could induce this slightly increased and constitutive activation of the immune response.

Another indication for the disturbed function of the innate and adaptive immune response are the observed differences in expression of several CO3 fragments, especially of C3dg and C3c (Figures 3B and 4). This protein is a positive 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 key roles in several steps of the adaptive immune response, and thus is considered to be the link between these separate immune response systems (Sahu and Lambris, 2001). The expression of CO3 is controlled by several different factors. During infection of an individual, CO3 can form complexes (e.g. with immunoglobulins), which are removed from circulation. Furthermore, cytokines, IL-1 and IL-6, produced during inflammation induce the hepatic production of CO3. The overall ratio between production and removal of CO3 determines the overall expression levels of CO3 in the blood. This ratio could even result in normal circulating levels of CO3 during an infection (Ritchie et al., 2004b). From literature, there are conflicting data concerning the expression levels of this protein during aging. CO3 levels were either reported to be unchanged (Bellavia et al., 1999; Oyeyinka and Salimonu, 1999; Ritchie et al., 2004b) or increased (Nagaki et al., 1980) during aging. This study clearly shows that not total CO3 levels, but CO3 protein fragments could be important determinants. The increased presence of the CO3 fragments C3dg and C3c are indicative of an increased activation of the complement system at old age, which could be caused by accumulation of illegal post-translationally modified proteins during aging, such as glycation or by increased atherosclerotic plaque formation.

As most immunological based assays that are used for measuring CO3 levels are not suitable for detecting differences in protein fragments or post-translational modified forms of CO3, this could explain the conflicting results found by others.

To increase the information that can be extracted from a single gene product, methods have to be developed that enable the accurate analysis of protein

isoforms. This could be accomplished by isoform specific monoclonal antibodies or the application of mass spectrometric techniques.

Ultimately, the described expression differences with aging could be used for developing methods for determining the biological age of an individual over the chronological age, which could facilitate in monitoring the effects of intervention studies to slow down the aging process. Therefore, distinction must be made between differences induced by pathologies or infections and aging or age-associated pathologies. In combination with the detection of proteins known to be highly induced upon an infection, but mildly induced with increasing age, such as C-reactive protein and several cytokines (Ferrucci et al., 2005), the above described differences together could be used for the development of such an approach.

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