



A.D. MDLXII

UNIVERSITÀ DEGLI STUDI DI SASSARI  
DIPARTIMENTO DI SCIENZE BIOMEDICHE  

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SCUOLA DI DOTTORATO IN  
SCIENZE BIOMOLECOLARI E BIOTECNOLOGICHE

Indirizzo in

Proteomica, Metabolomica, Biochimica Clinica e Biologia Molecolare Clinica

XXIII CICLO

Coordinatore Prof. Bruno Masala

# **Clusterin/Apo J protein expression in plasma samples of an elderly Sardinian population**

Tutor:

Prof. Luca Deiana

Tesi della

Dott.ssa Angela Baralla

Anno Accademico 2009-2010



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## *List of abbreviations*

2DE	Two dimensional electrophoresis
ABESF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AkeA	A kent'annos
ANS	1-anilino-8-naphtalene sulfonate
AP-1	Activator protein 1
AP-2	Activator protein 2
ASAP	Alternative splicing annotation project
A $\beta$	Amyloid $\beta$ -peptide
CCL64	Epithelial cell line from mink lung
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLI	Complement cytolysis inhibitor
CLU	clusterin
CRES	Cyclic AMP response element
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EQ	Equilibration buffer
HSC70	Heat shock cognate protein 70
HSE	Heat shock response element
LC MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
LDLR	LDL receptor family
LSD	Least significant different method
MEK/ERK	Extracellular signal regulated kinase (ERK) mitogen-activated protein kinase pathway
nCLU	Nuclear clusterin
NF1	Nuclear factor 1
NGF	Nerve growth factor
NLS	Nuclear localization signal
PC12	Pheochromocytoma cell line from adrenal medulla
PI3 kinase	Phosphatidyl inositol 3 kinase
pnCLU	Precursor nuclear clusterin
PSA	Prostate specific antigen
psCLU	Precursor secreted clusterin
PTMs	Post translational modifications
sCLU	Secreted clusterin
SDS	Sodium Dodecyl Sulphate
sHSPs	Small heat shock proteins
SP1	Selective promoter factor 1
SP40	Secreted protein 40
SSP	Standard spot number
TCA	Trichloroacetic acid
TGF $\beta$	Transforming growth factor $\beta$
UTR	Untranslated region
VDLD	Very low density lipoprotein

# **ABSTRACT**



## *Abstract*

Clusterin (CLU) is a glycoprotein with a nearly ubiquitous tissue distribution that has been reported to be implicated in several physiological processes as well as in many pathological conditions including ageing, diabetes, atherosclerosis, degenerative diseases and tumorigenesis.

In this work we studied the changes in the CLU protein expression in plasma samples of a Sardinian population divided by age into four groups.

We applied a proteomic approach using 2D-PAGE-MS analyses, western immunoblotting and ELISA to perform comparisons between the four groups. The typical train of spots of the Clusterin protein in a 2D plasma map was examined and according to statistical analyses. Five spots were detected as being differentially expressed within the four groups.

Regarding total Clusterin the results agree with the data shown by literature but only values up to 90 years old have been reported for Clusterin protein expression. This work extends to older people showing that after 90 a decrease in plasma Clusterin levels seems to occur especially in centenarians.

Considering that Clusterin is a sensitive biosensor of oxidative stress it could be in accordance with the fact that centenarians, considered as a model of successful ageing, are characterized by low levels of ROS (radical oxygen species).

Chapter 1

# **INTRODUCTION**

## Clusterin (CLU)/ApoJ

### A brief introduction

Clusterin (CLU) is a glycoprotein with a nearly ubiquitous tissue distribution that has been reported to be implicated in several physiological processes such as sperm maturation, lipid transportation, complement inhibition, tissue remodelling, membrane recycling, cell-cell and cell-substratum interaction, stabilization of stressed proteins in a folding-competent state and promotion or inhibition of apoptosis.

Clusterin, known by several other names, has recently drawn much attention because of its association with cancer promotion and metastasis (S. Pucci *et al.*, 2004, M. Redondo *et al.*, 2000, B. Shannan *et al.*, 2006). First discovered as serum apolipoprotein J with chaperoning properties for protein stabilization, CLU also can exist within the cell to function in either proapoptotic or prosurvival processes. This diverse set of functions can be attributed to the existence of two alternatively spliced forms of the CLU gene that encode secretory CLU (sCLU) or nuclear CLU (nCLU). The sCLU form seems to be cytoprotective (K.N. Chi *et al.*, 2008) while nCLU migrates to the nucleus on cytotoxic stress to trigger cell death (A.E. Caccamo *et al.*, 2005, K.S. Leskov *et al.*, 2003). It interacts with the DNA double-strand break repair antigen Ku70, blocking its function and causing cell death (K.S. Leskov, *et al.*, 2003).

Clusterin/Apolipoprotein J (ApoJ) was firstly identified in ram *rete testis* fluid in 1983 as a secreted glycoprotein enhancing cell aggregation in vitro (named thus as Clusterin) (O. Blaschuk *et al.*, 1983). In humans, it was firstly purified from serum and the cloned gene was named CLI (complement cytolysis inhibitor) (D.E. Jenne and J. Tschopp, 1989), SP-40,40 (secreted protein 40,40) (L. Kirschbaum *et al.*, 1989) or ApoJ (H.V. de Silva *et al.*, 1990a) due to similarities with other known apolipoproteins.

The ApoJ gene is differentially regulated by cytokines, growth factors and stress inducing agents and it has been functionally implicated in many pathological conditions including ageing, diabetes, atherosclerosis, degenerative diseases and tumorigenesis.

The ApoJ protein sequence among mammalian species is highly conserved with a degree of ~70–85% and until now no functional ApoJ polymorphisms in humans have been found (B. Tycko *et al.*, 1996) suggesting that the protein has evolved in vertebrates to carry out a function of fundamental biological importance (I.P. Trougakos and E.S. Gonos, 2002).

Although about ten years of work on this protein, there is still a lack of information about the complex regulation of its expression.

## The Human Clusterin gene

The *CLU* gene is a single 9-exon expressed at very different levels in almost all major tissues in mammals. *CLU* gene maps on chromosome 8 proximal to the lipoprotein lipase gene locus (Fink et al., 1993). The nine exons are of variable size, ranging from 126 to 412 bp and spanning a region of 17,877 bp (Wong et al., 1994). Before 2006, it was believed that a unique transcript of about 1.9 kb was the product of the transcription of the *CLU* gene, now it is known that there are three ATG start sites in frame with each other giving rise to three Clusterin isoforms called Isoform 1, 2 and 11036 (F. Rizzi et al., 2009).

The *CLU* gene promoter is highly conserved and contains many regulatory elements (AP-1, AP-2 and Sp1, NF1) that may regulate the complex tissue-specific control of the gene (Y. Herault et al., 1992, D. Michel et al., 1997, P. Wong et al., 1994). The conserved AP-1 site was identified as being responsive to transforming growth factor-beta (TGF $\beta$ ) (G. Jin and P.H. Howe, 1997) and to both nerve growth factor (NGF) and epidermal growth factor (EGF) (C. Gutacker et al., 1999) in CCL64 and PC12 cells, respectively. It was also identified a conserved domain of 14-bp (D. Michel, et al., 1997) that the authors called CLE element. This domain appears to be strictly related to the heat shock response elements (HSE), with only one base difference from the ideal HSE. It was, thus, proposed that this element by acting in synergy with the AP-1 element could make the ApoJ gene promoters particularly sensitive to environmental changes.

Supportively to this assumption, ApoJ gene appears to be responsive to a variety of both intrinsic or extrinsic factors including oncogenes, transcription and growth factors, cytokines and most stress inducing agents including oxidants, hyperoxia, proteotoxic stress, heavy metals, UVA, UVB, ionizing radiation (IR), heat shock, chemotherapeutic drugs (S.E. Jones and C. Jomary, 2002, L.V. July *et al.*, 2002, I.P. Trougakos and E.S. Gonos, 2006) and stress or apoptosis inducing agents, on a cell-type specific basis (I.P. Trougakos and E.S. Gonos, 2002).

Glucocorticoid/androgen-like response elements and cyclic AMP response elements (CREs) are also present in the 5'-flanking region of the gene (F. Rizzi, *et al.*, 2009).

According to the ASAP (Alternative Splicing Annotation Project) for the *CLU* UniGene cluster there are three transcripts that contain 9 exons, 8 introns and a terminal 3'-UTR. All this transcript have a unique exon 1 and share the remaining sequence from exon 2 to exon 9. Concerning Isoform 1 , its mRNA is predicted to produce a protein of 501 amino acids with a molecular weight of 57.8 kDa. A computational prediction of its subcellular localization by the PSORT program (P. Horton *et al.*, 2007) suggests that the protein product should have a prevalent cytoplasmic/nuclear localization. Isoform 2, has an alternative untranslated exon 1; the first available ATG is located on exon 2 just upstream of a functional endoplasmic reticulum (ER) localization leader sequence thus suggesting that this isoform is destined to secretion producing a

protein of 449 aa. The exon I of Isoform 11036 is located on the *CLU* gene sequence just between the exon I of Isoforms 1 and 2. This sequence is not given as a RefSeq in the GeneBank database, but appears to be one of the most probable splicing products of the *CLU* gene. Isoform 11036 is supposed to produce a protein of 460 aa with a prevalent nuclear localization (P. Horton, *et al.*, 2007). Moreover, all transcripts have a third ATG site in exon 3 in frame with the others ATG. Translation from this ATG would produce a shorter form of the CLU protein which is supposed to localize to the nucleus (M. Scaltriti *et al.*, 2004). Different studies showed that these isoforms can be differentially expressed in tissues. As an example, Andersen *et al.* demonstrated that Isoform 2 was downregulated in cancer cells compared to normal tissue, while Isoform 11036 was found to be expressed at very low level in normal and transformed colonic cells (C.L. Andersen *et al.*, 2007). Cochrane *et al.* found that both the two isoforms 1 and 2 are expressed in prostate cancer cell line but with a different regulation modulated by androgens (D.R. Cochrane *et al.*, 2007). Isoform 2 is upregulated by androgens, while Isoform 1 is downregulated.

It is still unclear whether transcription of each mRNA is driven by a unique promoter or by different promoters.

## The Clusterin protein forms

Three protein forms related to each other have been described with distinct subcellular and extracellular localization that may have different functions (A.E. Caccamo, *et al.*, 2005, A.E. Caccamo *et al.*, 2004, A.E. Caccamo *et al.*, 2003, R.M. Moretti *et al.*, 2007, S. Pucci, *et al.*, 2004, I.P. Trougakos *et al.*, 2005, C.R. Yang *et al.*, 2000, Q. Zhang *et al.*, 2006).

The secreted form of CLU (sCLU of 75-80 kDa) is a glycosylated heterodimer present in almost all biological fluids. Translation of sCLU starts from the ATG in exon 2 and produces a protein that is then targeted to the ER by an initial leader peptide. An intracytoplasmic precursor of 60-64 kDa enter the ER where it is glycosylated and proteolytically cleaved at an internal site in two distinct monomers with limited homology: the  $\alpha$  (34-36kDa) and  $\beta$  (36-39 kDa) chains linked together through five disulfide bonds resulting in the mature heterodimeric ApoJ protein of about 70–80 kDa.

Additional reported post-translational modifications include sulfation (J. Urban *et al.*, 1987), iodination and mannose-6 phosphorylation (P. Lemansky *et al.*, 1999), while predicted putative motifs on the protein sequence include sites for phosphorylation and three nuclear localization signals (NLS) located in both the  $\alpha$  and  $\beta$  chains (K.S. Leskov, *et al.*, 2003). A second form of CLU protein is directed to the nucleus; this protein is detected as a 49kDa nonglycosylated precursor pnCLU (predicted molecular weight 48.8kDa) in the cytosol and a 55kDa glycosylated protein (referred to as nCLU)

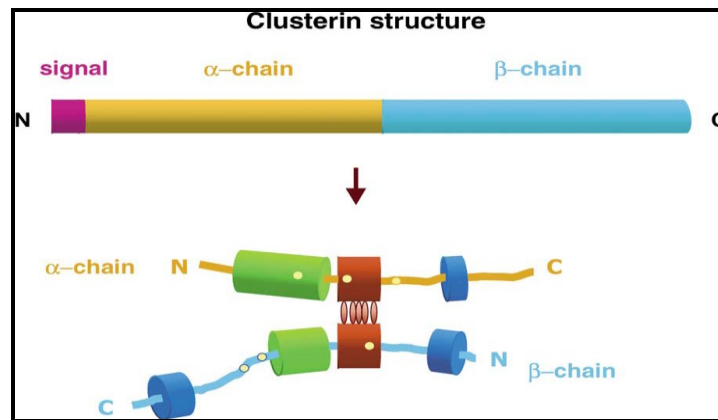


in the nucleus whose enhanced expression is usually associated to cell death (A.E. Caccamo, *et al.*, 2005, A.E. Caccamo, *et al.*, 2004, A.E. Caccamo, *et al.*, 2003, K.S. Leskov, *et al.*, 2003, R.M. Moretti, *et al.*, 2007, I.P. Trougakos *et al.*, 2009).

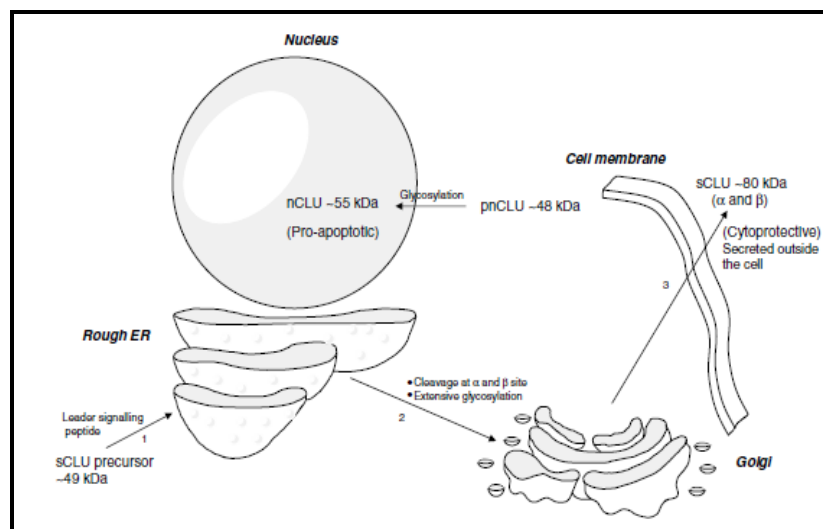
Analysis of the protein's secondary structure revealed an enrichment in  $\alpha$  helical domains (H.V. de Silva, *et al.*, 1990a, H.V. de Silva *et al.*, 1990b), while as proposed recently (R.W. Bailey *et al.*, 2001) the promiscuous ApoJ binding activity with various dissimilar molecules may rely on three long regions of natively disordered or molten globule-like structures with a flexible nature containing putative amphipathic  $\alpha$  helices.

At physiological pH, clusterin exists as a mixture of dimers and tetramers of the disulfide-linked  $\alpha$ - $\beta$  heterodimer "monomer". When exposed to mildly acidic conditions, these oligomers dissociate to form  $\alpha$ - $\beta$  monomers which show enhanced binding to the hydrophobic probe 1-anilino-8-naphtalenesulfonate (ANS), indicating significant exposure to hydrophobic surfaces (A. Wyatt *et al.*, 2009).

ApoJ protein is constitutively secreted by a number of cell types including epithelial and neuronal cells, while in cells featuring a regulated exocytotic pathway its secretion depends on the appropriate exogenous stimulus (F. Bonavita *et al.*, 2003, I.P. Trougakos, *et al.*, 2009). Using different assays, clusterin levels in human serum has been reported to range from 35 to 105 $\mu$ g/ml (B.F. Murphy *et al.*, 1988) and 111 $\pm$ 50  $\mu$ g/ml (O. Blaschuk, *et al.*, 1983).while it is about 10 times higher in human seminal plasma (B.F. Murphy, *et al.*, 1988) (N.H. Choi *et al.*, 1990)



**Figure 1.** The precursor polypeptide chain (top) is cleaved proteolytically to remove the 22-mer secretory signal peptide (magenta) and subsequently between residues 227/228 to generate  $\alpha$  and  $\beta$  chains. These are assembled in anti-parallel to give a heterodimeric molecule (bottom) in which the cysteine-rich centers (red) are linked by five disulfide bridges (red ellipses) and are flanked by two predicted coiled-coil  $\alpha$ -helices (green) and three predicted amphipathic helices (dark blue). The six sites of N-linked glycosylation are indicated as yellow spots (S.E. Jones and C. Jomary, 2002).



**Figure 2:** sCLU precursor is transported into the rough ER by leader signalling peptide (1), and then undergoes cleavage and extensive glycosylation while being transported to the Golgi apparatus (2). The result is a secreted protein of ~80 kDa with five disulphide bonds between the  $\alpha$  and  $\beta$  subunit that is secreted outside the cell (3). pnCLU does not undergo any cleavage nor extensive glycosylation, and resides in the cytoplasm of unstressed cells. It becomes the mature form (~55 kDa) once it is transported to the nucleus (S.E. Jones and C. Jomary, 2002)

## Biological functions

### A sensitive biosensor of oxidative stress

CLU has been implicated in a number of physiological processes as well as in many pathological conditions including ageing, diabetes, atherosclerosis, degenerative diseases and tumorigenesis. The link between these otherwise unrelated pathologies is that they are characterized by increased oxidative injury. Oxidative stress occurs due to overproduction of oxygen-free radicals that exceed the cell's antioxidant capacity. Oxygen-free radicals can be both beneficial and deleterious to the cells, in fact, low physiological amounts of oxidants may act as a secondary messenger in intracellular signalling cascades promoting normal cell homeostasis and proliferation, while abnormally high levels of ROS may result in deregulation of redox-sensitive signalling pathways, and extensive cell damage leading to responses which include arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, reversible growth arrest, senescence, or cellular death (G.S. Omenn, 2006, W. Yan *et al.*, 2009). Protein modifications refer to protein oxidation, formation of peroxy radicals (G.S. Omenn, 2005), conjugation with lipid peroxidation products, glycation, and glycoxidation (G.S. Omenn, 2004).

The presence in the CLU promoter of the regulatory elements AP-1 and HSF1

that are both modulated by oxidants and oxidation injury make the CLU gene a sensi-

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tive biosensor to environmental changes and in particular to the effects of free radicals and their derivatives. Many oxidants induce an immediate sCLU mRNA and protein upregulation in human diploid fibroblasts (L. Ehrman *et al.*, 1972) and CLU was found to be induced in retinal pigment epithelial cells after subtoxic oxidative stress via TGF- $\beta$  release (N.L. Anderson and N.G. Anderson, 2002) and in human neuroblastoma cells under conditions of increased production of ROS and lipid peroxidation (P. Ghosh *et al.*, 2001). Moreover sCLU concentration increased in human serum with exposure to acute in vivo oxidative stress induced by heavy metals (P. Cantos Gómez, 2009), while adult onset of caloric restriction that retarded age related oxidative damages resulted in CLU suppression in kidneys of rats (D. Howitt and D. Cramer, 2008). In another study, immunohistochemical analysis showed that CLU is constantly associated with altered elastic fibers in aged human skin binding elastin and inhibiting UV-induced aggregation of this protein (S.P. Schacht and J.E. Aspelmeier, 2005).

There is some controversy about sCLU role during cell death execution due to opposing reported functions (I.P. Trougakos and E.S. Gonos, 2002). In certain cellular types sCLU may enhance apoptosis after cell exposure to stress or apoptosis inducing agents (S. Gangemi *et al.*, 2003, G. Passeri *et al.*, 2003, M. Scaltriti, *et al.*, 2004, I.P. Trougakos, *et al.*, 2005) and high amount of CLU may become cytotoxic when accumulated intracellularly (R.A. Rabini *et al.*, 2003).

On the other hand, in  $CLU^{-/-}$  mice, sCLU was found to confer protection from autoimmune myocardial damage (R. Coppola *et al.*, 2003), permanent focal cerebral ischemia (J. Delay *et al.*, 1954), heat-shock mediated in the testis (R.W. Bailey *et al.*, 2002) and progressive age-related glomerulopathy (M.E. Rosenberg *et al.*, 2002). Moreover, sCLU biosynthesis inhibition by antisense oligonucleotides or small interfering RNA (siRNA) resulted in significant cell sensitization to apoptosis induced by oxidative stress (I. Viard *et al.*, 1999). Other reports indicate that even in the absence of external stress sCLU knock down reduces cell proliferation and increases the rates of spontaneous apoptosis in normal or cancer mammalian cell types (S.W. Kang *et al.*, 2000, M.R. Schlabach *et al.*, 2008). Recent findings indicate that sCLU inhibits apoptosis by specifically interacting with conformation altered activated Bax in the mitochondria (H. Zhang *et al.*, 2005).

These findings indicate that sCLU may have an important function even in the absence of stress having a vital housekeeping role in the maintenance of cellular proteome stability (I.P. Trougakos and E.S. Gonos, 2009).

nCLU contrary to sCLU has been correlated to a pro-death function that is mediated through a nuclear localization and Ku70 binding after exposure to IR (S. Siegel and N.J. Castellan, 1988, I.P. Trougakos *et al.*, 2002) or due to its ability to dismantle the actin cytoskeleton (L. Gaspari *et al.*, 2003).

## Implications of CLU in ageing and age related diseases

Ageing is a consequence of complicated interactions between genetic factors along with the accumulation of a variety of stochastic changes over time due to a progressive failure of homeostasis that promotes multiple biochemical, molecular and cellular changes which lead to increased disability, morbidity, and to death (T.B. Kirkwood, 2002). So ageing is a multifactorial process modulated by the interplay among genetic and environmental factors (R. Holliday, 1998, N. Lane, 2003). Increased levels of ROS can lead to deleterious by-products. This increasingly damaging process can be responsible for degenerative diseases and ageing. During *in vivo* ageing CLU gene expression was found to increase from gestation to adults in humans (P. Wong, *et al.*, 1994), in the human pituitary gland (T. Ishikawa *et al.*, 2006), in the rat ventral prostate (K.M. Lau *et al.*, 2003), in human glial cultures (N.V. Patel *et al.*, 2004) and in human lymphocytes (I.P. Trougakos *et al.*, 2006). In serum sCLU levels seems to increase during *in vivo* ageing at least in males (I.P. Trougakos, *et al.*, 2002). Considering that ageing is a risk factor for many severe diseases including cancer, vascular diseases and neurodegeneration it is not surprising that CLU has been implicated in all these pathological conditions.

During vascular damage sCLU was found to accumulate in the human serum of diabetes type II patients or during myocardial infarction (I.P. Trougakos, *et al.*, 2002). sCLU accumulates in the artery wall during atherosclerosis suggesting that can have a

protective role against the oxidative stress associated with the development of the disease (B. Mackness *et al.*, 1997).

CLU mRNA synthesis is particularly evident in the normal adult brain of some mammalian species and during neurodegenerative conditions there is a significant increase of its expression. sCLU was found to associate with amyloid  $\beta$ -peptide in senile and diffuse plaques of Alzheimer Disease (E. Kida *et al.*, 1995). Yerbury *et al.* In 2007 suggested that the proamyloidogenic effects of CLU may be explained considering the CLU:client protein ratio, in particular when the substrate protein is present in high concentration, CLU coincorporates with it into insoluble aggregates, while when CLU is present in higher levels it potently inhibits amyloid formation conferring cytoprotection (J.J. Yerbury *et al.*, 2007). CLU expression seems to be correlated with an anti-inflammatory function, for example, it was found to exert a cytoprotective function in lung during leukocyte-induced injury (A.R. Heller *et al.*, 2003) in inflammatory exocrine pancreas (V. Savkovic *et al.*, 2007) and in glomerulonephritis (M.P. Rastaldi *et al.*, 2006). Finally, CLU has been functionally implicated in all aspects of tumorigenesis including tumor formation and progression, metastasis and chemoresistance acquisition (B. Shannan, *et al.*, 2006, I.P. Trougakos and E.S. Gonos, 2002).

In all these pathological conditions the main mission of CLU would be to cease the deleterious effects of oxidative stress. If this is true, than CLU gene expression and protein upregulation during ageing should reflect the general “oxidative status” of the

subject rather than its chronological age (I.P. Trougakos and E.S. Gonos, 2009). In fact, Franceschi et al., showed that although CLU gene expression levels in lymphocyte samples are higher in old donors compared to their younger counterparts, in similar samples from centenarians (a model of successful ageing characterized by low ROS load), CLU gene expression levels were significantly lower than those found in the old donors (C. Franceschi and M. Bonafe, 2003).

### **Chaperone activity of CLU**

Molecular chaperons are a large family of ubiquitous proteins that appeared early in evolution to form a defensive system in cells by sequestering damaged proteins and preventing their aggregation. Much of the current understanding of molecular chaperons centres on the heat-shock protein (HSP) family and their action include: 1) ensure correct folding of newly synthesized polypeptides 2) unfold and refold polypeptides during translocation across membranes, 3) assemble and disassemble macromolecular complexes, or 4) resolubilise or facilitate the degradation of partially folded and/or aggregated proteins.

sCLU shares functional similarities with HSPs and similar expression pattern, but, the presence of different regulatory elements in the CLU gene promoter makes CLU gene more sensitive than HSPs in various environmental insults.

Humphreys et al., 1999 provided for the first time evidence to the hypothesis that Clusterin may function as a molecular chaperone (D.T. Humphreys *et al.*, 1999)

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and further studies highlighted Clusterin's ability to inhibit the stress induced aggregation and precipitation of a quantity of unrelated target proteins (S. Poon *et al.*, 2000, S. Poon *et al.*, 2002). Clusterin, like sHSPs binds to stressed target proteins forming high-molecular weight complexes, even if it is not clear how this interaction occurs. Although its ability to stabilize aggregation-prone proteins, Clusterin is unable to restore the stress-induced loss of enzyme activity of alcohol dehydrogenase, catalase and glutathione-S-transferase because it is unable to refold these enzymes alone but it can maintain stressed proteins in a state that is competent for subsequent refolding by heat-shock cognate protein 70 (HSC70) (S. Poon, *et al.*, 2000).

Different reports described the ability of clusterin to inhibit the *in vitro* fibrillar aggregation of the amyloid  $\beta$  (A $\beta$ ) peptide, the major component of senile plaques associated with Alzheimer's disease (E. Matsubara *et al.*, 1995). However Clusterin has been found associated with all disease-associated extracellular protein deposits tested to date, including amyloid deposits (J.J. Yerbury, *et al.*, 2007). The presence of clusterin in these deposits, as stated in the previous paragraph, may indicate its incorporation into insoluble aggregates under conditions in which the capacity of extracellular protein folding mechanisms have been exceeded.

A supposed main difference between sCLU and sHSPs (small heat shock proteins) seemed to be their differential localization. sHSPs localize mainly in the cytosol and nucleus (A.P. Arrigo *et al.*, 2007) whereas sCLU, because of the ER targeting hy-

drophobic signal peptide were expected to reside in the membranous compartments of the cell as well as in the extracellular milieu (M. Calero *et al.*, 2005, B. Shannan, *et al.*, 2006). Due to this different localization Trougakos *et al.*, suggested that sCLU may represent the only chaperone that confers proteome stability both extra and intracellularly (I.P. Trougakos and E.S. Gonos, 2009).

### **sCLU involved in signalling pathways for cell survival**

CLU gene expression is upregulated in tumors of various origins (H.J. Ahn *et al.*, 2008, B. Shannan, *et al.*, 2006). It is further increased under conditions known to induce apoptosis, such as hormone withdrawal in estrogen-dependent breast tumor or in androgen-dependent prostate cancer models (A. Brandstrom *et al.*, 1994) and other conditions involving cell death, such as tissue remodelling and injury (M.G. Bandyk *et al.*, 1990). sCLU has now been clearly found to be cytoprotective in the presence of apoptosis and necrosis (H. Miyake *et al.*, 2000, J.A. Sensibar *et al.*, 1995, B. Shannan, *et al.*, 2006). A possible way by which sCLU exerts its protective function is binding to a cellular receptor. The first identified sCLU receptor is Megalin (M.M. Bartl *et al.*, 2001) that belongs to the LDL receptor family (LDLR) of structurally related membrane receptors. These receptors bind preferentially lipoproteins and various proteins like protease inhibitors (D.K. Strickland *et al.*, 1995, D.K. Strickland and S. Ranganathan, 2003), and internalize them by endocytosis.

Ligand binding to these receptors (VLDL receptor, the apoE receptor 2, LRP1 and megalin) can promote signal transduction and it has been demonstrated that sCLU can interact with all of them. Ammar and Closset in 2008 evidenced that sCLU binding to its receptor megalin activated the P13 kinase /Akt pathway and produced multiple protein phosphorylation (H. Ammar and J.L. Closset, 2008). Moreover, protection by sCLU induced signalling was not blocked when inhibiting P13 kinase/Akt pathway, suggesting that additional pathways can be involved in the cell protective function of sCLU (G. Klock et al., 2009). Shim et al. showed that sCLU stimulates astrocyte proliferation by modulating EGF receptor signalling activity, resulting in MEK/ERK stimulation (Y.J. Shim et al., 2009). However, the potential signaling functions of sCLU remain still to be clarified.

## Ageing and Longevity

### **Complex trait where environmental, genetic and stochastic factors are involved**

Ageing and longevity are complex phenomena influenced by multiple genetic and environmental factors (N. Barzilai and A.R. Shuldiner, 2001, P. Hamet and J. Tremblay, 2003, T. Perls and D. Terry, 2003). Genes or the environment can promote longevity by either protecting individuals from age-related illnesses or by slowing down the ageing process (N. Barzilai and A.R. Shuldiner, 2001, T. Perls *et al.*, 2002). Environmental factors that may have a strong influence on the lifespan include physical activity (T.H. Lam *et al.*, 2004), smoking (D.H. Taylor, Jr. *et al.*, 2002), diet (F. Seccarecchia *et al.*, 2003), and also caloric restriction in animals (L.K. Heilbronn and E. Ravussin, 2003, L.H. Mehta and G.S. Roth, 2009). A strong relationship between genetics and longevity is suggested by studies of life expectancies of subjects who have reached a very old age and their offspring (T. Perls, *et al.*, 2002). Different studies indicate that gene polymorphisms can contribute to human longevity through different mechanisms, including the immune systems and the metabolic systems (G. Atzmon *et al.*, 2006, N. Barzilai *et al.*, 2003, C. Franceschi *et al.*, 2005) and recently RNA editing genes have been reported to be important regulators of ageing in humans (P. Sebastiani *et al.*, 2009).

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Moreover, proteomic analyses represent a great potential to study cellular and tissue protein alterations in various disease states and ageing (H.J. Hwang *et al.*, 2009, E. Schiffer *et al.*, 2009, F. Sun and V. Cavalli). However, proteomic studies on human longevity are still limited.

Centenarians represent a group of individuals in the population that escaped neonatal mortality, antibiotic era illnesses, and avoided or postponed the most severe age-related diseases and their fatal outcomes (S. Salvioli *et al.*, 2008). For this reason, these individuals have been an extraordinary model to study human longevity and healthy ageing. Moreover, it should be highlighted that within this group of exceptional individuals, a consistent part of them (about one third) are in good health (C. Franceschi *et al.*, 2007, S. Mariotti *et al.*, 1993).

Among the many factors involved in human longevity, one that might be cited is the gender. It is well established that females have a longer life expectancy than males (about 4 years in developed countries). Among very old people, females outnumber males of the same age. In particular, when centenarians are considered, the male/female (M/F) ratio has been reported to range between 1:4 and 1:7 (A.M. Menard *et al.*, 1992, P. Sammallahhti *et al.*, 1996). However, a surprising 1:2 ratio has been reported for Sardinian centenarians (L. Deiana *et al.*, 1999). This Sardinian data has been much discussed, and researchers of the field attributed it to either the peculiarity of the Sardinian population or environmental effects (G. Passarino *et al.*, 2002).

Although ageing processes may be biologically modulated in many tissues, longevity is also determined by escaping from common killing diseases: cardiovascular disease, cancer, and infection are the leading causes of death in adults. Thus, in addition to possibly finding genes and proteins that modulate the ageing process, research on human longevity is also likely to identify genes that protect from these diseases. Consequently, the discovery of longevity-associated genes and proteins will lead to fundamental understandings of the molecular basis of several important diseases having a profound impact on morbidity, mortality, and enhancing the quality of life in the elderly population (N. Barzilai and A.R. Shuldiner, 2001).

### **Theories of ageing**

Theories formulated to explain ageing processes have been grouped into two principal categories: the “programmed” and “error” theories of ageing (B.T. Weinert and P.S. Timiras, 2003). According to the “programmed” theories, ageing depends on regulation of genes sequentially switching on and off signals to the nervous, endocrine, and immune systems responsible for maintenance of homeostasis and for activation of defence responses. The “error” theories identify environmental insults to living organisms that induce progressive damage at various levels (e.g., mitochondrial DNA damage, oxygen radicals accumulation, cross-linking) (B.T. Weinert and P.S. Timiras, 2003).

Between these two categories we can identify evolutionary (L.A. Gavrilov and N.S. Gavrilova, 2002, G. Rose *et al.*, 2007), molecular, cellular and system-based theories (B.T. Weinert and P.S. Timiras, 2003).

The evolutionary theories include the mutation accumulation theory that suggests that there are purely deleterious mutations that have no effect earlier in life but exert their effects later. These mutations would tend to accumulate in the population because of their minimal effects on fitness. Evolutionary theories argue that ageing results from a decline in the force of natural selection. Because evolution acts primarily to maximize reproductive fitness in an individual, longevity is a trait to be selected only if it is beneficial for fitness. The essential concept in these theories is that the balance of resources invested in longevity vs. reproductive fitness determines the lifespan (B.T. Weinert and P.S. Timiras, 2003).

Molecular theories of ageing proposes that senescence results from changes in gene expression (gene regulation theory) (M.S. Kanungo, 1975). System-based theories of ageing explain it in terms of alterations in neuroendocrine functions controlling homeostasis, which result in physiological changes (neuroendocrine theory), by increased susceptibility to infectious and autoimmune diseases due to a decline in immune function (immunological theory), or by assuming a fixed metabolic potential for living organisms (rate of living theory) (B.T. Weinert and P.S. Timiras, 2003).

Cellular theories include the cellular senescence-telomere and free radical theories. Cellular senescence has been described as limiting cell divisions in cell cultures. This has been ascribed to the loss of telomeres from the chromosome endings or the loss of telomerase function (C. Martin-Ruiz *et al.*, 2004, K. Masutomi *et al.*, 2003). The free radical theory, suggests that ageing is the result of cumulative effects of oxidative damage to various cellular structures such as DNA, proteins and lipids (A.P. Wickens, 2001).

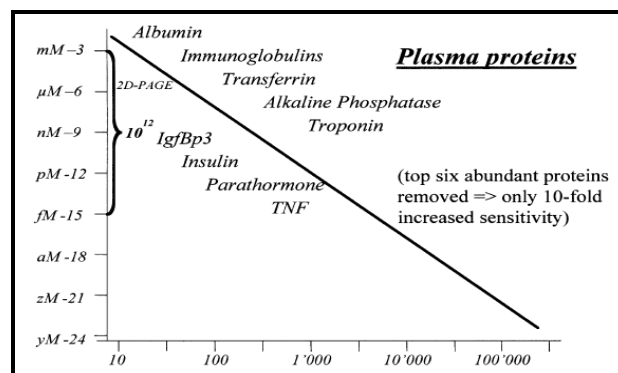


## The human plasma: complexity of the sample

Human plasma and serum are the preferred specimens for non-invasive studies of normal and disease-associated proteins in the circulation and arising from cells throughout the body. Blood plasma contains several different water-soluble components, among them the blood plasma proteins. In principle, any protein present in the body can become at least temporarily a blood plasma protein depending on the actual state of the body, e.g. especially in the case of a pathological situation. A protein that is usually not present in blood plasma may accumulate in blood plasma and function as a characteristic diagnostic marker for a certain disease. Most plasma proteins carry post translational modifications (PTMs), either permanently or temporarily, and the vast majority are glycoproteins, thus ensuring a reasonable solubility. Blood plasma proteins exhibit a wide variety of functions and have different structural properties; moreover they cover a very wide concentration range of at least 10 orders of magnitude (see Fig.3).

The most abundant by far, albumin being 40 mg/mL, while cytokines and kallikreins (including prostate-specific antigen [PSA]) are at about 1 ng/mL, arriving to much lower concentrations with proteins of tissue origin. From 40 mg/mL to 4 pg/mL is 10 orders of magnitude. The most common technology for fractionating and identifying

proteins, 2D gel electrophoresis (2DE), has a range of detection of not more than 3 orders of magnitude. Other methods have similar or slightly larger ranges. Plasma has a far larger dynamic ranges than tissue or other body-fluid specimens and albumin accounts for approx 50% of the total mass of proteins in the plasma. Another several proteins account for about 40% of total protein mass (P. Ping *et al.*, 2005). The most abundant 22 proteins are estimated to account for 99% of total protein mass (N.L. Anderson and N.G. Anderson, 2002). Table 2 lists particularly abundant and readily detected proteins, with their usual concentrations. Abundance is an extremely important variable, not only because more abundant proteins are more readily detected, but also because the peptides and peptide ions from these proteins compete against peptides from other proteins in identification by mass spectrometry (MS). Thus, several methods have been developed for depletion of highly abundant proteins in plasma.



**Figure 3:** Dynamic range of proteins in plasma (V. Thongboonkerd, 2007), **fM** femtomolar, **pM** picomolar, **nM** nanomolar, **μM** micromolar, **mM** millimolar.

When blood is collected, many more changes in proteins may occur due to proteolytic enzymes (proteases) and other enzymes that are active in the blood sample during handling and processing. Plasma is protected from clotting by use of one of three different anticoagulants: sodium citrate, K<sub>2</sub>-EDTA, or lithium heparin. Each has its own characteristics; citrate and EDTA have desirable features of antiprotease activity. Some investigators add a protease inhibitor cocktail containing both peptide and small molecule inhibitors (15). The peptide(s) may compete directly with and interfere with the detection of peptides in the mass spectrometer, whereas such small molecules as ABESF, a sulfonyl fluoride, have been shown to form covalent bonds with proteins and thereby shift the isoelectric point (pI) of the protein (16). Omenn et al. suggested that plasma samples are preferable to serum samples because there is less degradation *ex vivo* and much less variability than arises in the protease-rich process of clotting. Clotting is unpredictable, owing to influences of temperature, time, and medications, which are difficult to standardize. These authors also recommend the use of EDTA over heparinized plasma because heparin acts through activation of antithrombin III, whereas citrate and EDTA inhibit coagulation and other enzymatic processes by chelate formation with ion dependent enzymes, but the use of EDTA is preferable because citrate introduces a 10 to 15% dilution effect. Moreover they did not recommend inclusion of protease inhibitors in the collection tubes or buffers because they can alter

analyses as shown the small molecule inhibitor ABESF which forms covalent bonds with proteins that alter the mobility of the protein (16).

Chapter 2

## **OBJECTIVES**

The aims of the present study were to compare the typical train of spots of the Clusterin protein in 2D plasma maps between four groups of people divided by chronological age, to perform a semi-quantitative analysis of the expression of the secreted form of Clusterin with western blotting as well as to compare total Clusterin concentration values in the plasma samples of the four groups.

The Sardinian people analysed were recruited through the AkeA project that studies longevity in Sardinia and includes plasma samples of people spanning from 20 to 100 years and more and so another aim was to investigate Clusterin expression in people at a very old age.

Chapter 3

## **METHODS**

## Samples

Plasma samples for this study were recruited from the AKeA project (the name 'AKeA' is derived from an expression in the Sardinian language that means 'health and life for 100 years!') that studies the Sardinian centenarians (L. Deiana, *et al.*, 1999).

For 2D analyses we selected a group of 7 centenarians, 12 aged 90-99, 12 aged 80-89 and 10 controls aged 20-50. While for ELISA validation we increased the number of samples in this way: 18 centenarians (10M, 9F), 17 aged 90-99 (9M, 8F), 15 aged 80-89 (8M, 8F) and 18 controls aged 20-50 (9M, 10F).

None of the participants in this study were in an acute care situation. The control subjects were 10 healthy volunteers aged 20-50 years (mean age 35) without diseases or relevant history, such as vascular disease, stroke, diabetes or cancer.

All patients gave informed consent prior to collection of blood samples.

## Plasma sample

For plasma separation, whole blood (7.5 mL) was collected in EDTA-coated tubes, mixed immediately and incubated for about one hour to allow the samples to be transported to the laboratories. The red cell fraction was then separated by centrifugation at 2500 g at 4 °C for 15 min and the clear plasma supernatant was stored in aliquots frozen at -80 °C.



## Removal of albumin from serum samples

Abundant proteins, such as albumin, hide the detection of many low-abundance proteins, some of which might potentially be relevant to particular disease states.

For protein extraction we used the modified TCA (trichloroacetic acid)/acetone precipitation method by Chen et al., which showed evidence of efficient removal of albumin in mouse serum. Levine (M. Levine, 1954) and Delaville (M. Delaville *et al.*, 1954) have reported that human and bovine serum albumins after TCA precipitations are soluble in organic solvents, such as methanol, ethanol, and acetone. Chen et al. tested the effect of adding different volumes of 10% TCA/acetone to aliquots of human serum concluding that most of the abundant albumin was effectively removed from the remaining serum proteins when adding four volumes of 10% TCA/acetone. We simply applied the same method to plasma samples with a satisfactory result in the gels obtained through bidimensional electrophoresis.

A 20  $\mu$ L sample of human plasma was precipitated by adding four volumes of ice-cold acetone containing 10% w/v TCA and immediately mixed by gentle vortexing. The mixture was incubated at -20°C for 90 min and centrifuged at 15.000 g at 4°C for 20 min. The supernatant was removed and collected. After that, 1 mL of ice-cold acetone was added to wash the precipitate. The sample was incubated on ice for 15 min and centrifuged as above. The acetone-containing supernatant was removed and the

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precipitate was resolubilised with sonication in sample buffer containing the chaotrope 8M Urea to prevent protein aggregation and the detergent CHAPS 4% to increase the solubility of certain proteins.

### **Protein quantification**

The protein concentrations of the plasma samples were determined through the DC protein assay (BioRad) according to the manufacturer's instructions.

The DC Protein Assay uses a modified Lowry assay based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent where the color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine. Proteins reduce the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thus producing one or more of several possible reduced species which have a characteristic blue colour with a maximum absorbance at 750 nm. Standard curve was constructed using albumin as standard sample in a range between 0.2 and 2mg/ml. In order to obtain concentrations of the buffer compounds compatible with the assay and the one of the sample inside the range of the curve constructed we diluted samples in two steps, a first dilution 1:2.5 of the sample in buffer containing Urea 8M and CHAPS 4%, and a second dilution 1:4 with water.

## Two dimensional SDS PAGE

### Isoelectrofocusing (IEF)

After protein extraction and quantification the first dimension of the 2D-SDS PAGE was performed on isoelectric focussing (IEF) strips with pH 4-7 (IPG strips 4-7, 17 cm from BioRad). At first, the strips were rehydrated for 20 hours in rehydration solution (Urea 8M, Thiourea 2M, 4% CHAPS, Destreak reagent, 10mM DTT, 1% carrier ampholyte 4-6 and 0.05% bromophenol blue) and covered with mineral oil. After rehydration, 1000 µg of proteins were applied onto IPG strips and proteins were separated in the first dimension according to the following protocol: 250 V for 2 h, 500 V for 2 h, 750 V for 2 h, 1000 V for 2 h, 5000 V for 2 h and 8000 V for 50.000 Volthours, for a total of 80.000 Vh. After isoelectric focusing the IPG strips were equilibrated in a two step equilibration protocol:

The equilibration buffer (EQ) contained 50 mM Tris-Cl pH 8.8, 6M urea, 20% (v/v) glycerol and 2% (w/v) SDS. For the first equilibration step 2% DTT were added to the EQ buffer. For the second step 2.5% iodoacetamide and 0.005% bromphenolblue were added. Equilibration steps were done at room temperature for 15 min each.

### SDS-PAGE

To separate proteins according to their size denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed. Strips were embedded into 5% agarose on top of SDS polyacrylamide gels. The anionic detergent sodium do-

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decyl sulfate (SDS) denatures the proteins and provides the protein with a negative net charge. For the second dimension 5-15% gradient SDS acrylamide/bisacrilamide gels were used. The gradient gels were produced using the Gradient Former Model 495 and the Multicasting Chamber from BioRad. Using this apparatus we were able to produce ten gels simultaneously with a high reproducibility. The gels were run on a Protean Multicell (BioRad) where six gels at a time can be run. The instrument is connected to a cooling bath to avoid excessive heating of the buffer. The lower chamber was filled with buffer containing Tris 0.125M, Glycine 0.96M, SDS 1%, while the upper chamber had the same composition but with the SDS concentration doubled (2%). This modification was suggested by the work of Werner et al. who proved that in order to minimize gel smearing the concentration of SDS in the cathodic buffer needs to be increased. After completion of the run, gels were stained in Coomassie staining solution that can detect protein spots with concentrations between 3 ng – 15 ng (Westermeier and Marouga 2005) which is typically enough protein to obtain mass spectrometry results using MALDI-TOF MS (matrix-assisted laser desorption ionisation time of flight mass spectrometry). Coomassie staining is cheap, quantitative and reproducible. The staining solution used was composed of 0.05% coomassie Blue R-250 dissolved in 50% methanol, 10% acetic acid and H<sub>2</sub>OmilliQ. Gels were then washed with H<sub>2</sub>OmilliQ and destained in destaining solution contained the same components of the staining solution except for the dye. Gels were scanned using a GS-800 densitometer from BioRad.

## Image analysis

Images were analysed using the PDQuest 2D Analysis Software (BioRad). Before performing the analysis we cropped the images to the area containing the train of spots of interest (the one of clusterin) and then we performed the image analysis following the steps summarized below:

- *Scanning*: gels are turned into pixel data and then each map is turned into a series of pixels describe by their optical density value (OD).
- *Filtering*: this step eliminates noise, background effect.
- *Automated spot detection*: identification of spots present in each gel independently, then spots are located on the gel image fitted by ideal gaussian distributions and quantified by the sum of the OD values within each gaussian distribution.
- *Matching of protein profiles*: 2D gels are then edited and matched to one another in a “match set”, to achieve this, landmarks are used, consisting of reference spots used by PDQuest to align and position the match set members for matching.
- *Normalization*: is used to compensate gel to gel variations due to sample preparation and loading, staining and de-staining procedures.

- Spots were first detected and matched automatically to a master gel selected by the software and then we corrected manually the errors in the editing and matching steps.
- Spot volumes were normalised with the option “total quantity in valid spot” where the raw quantity of each spot in a member gel is divided by the total quantity of all the spots in that gel that have been included in the Master . The quantity table was exported to a spreadsheet .xls file and submitted to statistical analyses. Features that displayed statistically significant changes in their normalised spot volume ( $p$  value  $\leq 0.05$ ; ANOVA), were selected for protein identification.

### **Statistical analyses**

All the statistical analyses were performed using the software SPSS v.17. For 2D spot intensity comparisons we performed and compared a parametric test, The One Way ANOVA followed by “Fisher’s least significant difference method” (LSD) and a non parametric one The Kruskal Wallis test which doesn’t assume a normal distribution of the samples. While the results of the ELISA experiment were evaluated using only the ANOVA test.

## Western blot

For western blot analyses the same extracts (proteins in urea and CHAPS) used for 2D PAGE were analysed. The samples were boiled in Laemmli buffer (TrisHCl 1M pH6.8, glycerol, 10% SDS, 0.5% bromophenol blue) for 4 minutes at 95°C and then loaded on a 12% acrylamide gel. After SDS PAGE the gel was transferred to a nitrocellulose membrane using the Mini Trans Blot system from BioRad. The gel size was measured and two Whatman filter papers and one nitrocellulose membrane of the same size were prepared. The filter papers and nitrocellulose were soaked in transfer buffer (25mM Trizma base, 200mM glycine in H<sub>2</sub>O milliQ). Then the soaked nitrocellulose and the polyacrylamide gel were placed on to one of the filter papers and covered with the second filter paper. This stack was placed between two sponge cloths before they were placed into the holding device of the blot chamber. Blotting was performed at 350mA for 1 hour. During the blotting procedure, the entire apparatus was placed in a box with ice to avoid heating of the buffer. After disassembling, the membrane was soaked in blocking buffer containing 3% albumin in PBS-T (PBS 10X, 20% (v/v) methanol 0.05% (v/v) and Tween-20 in ddH<sub>2</sub>O) for 1 hour at room temperature. It was then shortly rinsed with washing buffer (PBS-T) and the primary antibody (dilution 1:500) was applied in the same buffer overnight at 4°C. The blots were washed 3 times for 10 min in washing buffer before the secondary horseradish peroxidase labelled goat anti-rabbit antibodies was applied in the same buffer at a dilution of 1:2000 for 80 minutes at RT. The membranes were washed five times in PBS-T before the blots were devel-

oped using the ECL Immunoblotting Detection Kit from Amersham. An excess of the applied developing solution was removed and the membrane was covered with saran wrap before detection with the Versa Doc device from BioRad. The exposure time was set at 180 sec and then the image acquired for densitometric studies.

### **Mass spectrometric analysis of proteins**

Mass spectrometry is the most common, high throughput method for identifying proteins from 2D gels. It accurately weighs peptides after digestion with a suitable enzyme and the masses of these peptides can then be matched against the theoretical peptide masses of known proteins for identification. In the present work, selected spots were cut from gels, and sent to Porto Conte Ricerche for identification. The spots were digested with trypsin enzyme and analysed with a nano (HP-1200) LC-MS/MS ion trap mass spectrometer (XCT Ultra 6340) from Agilent Technologies (Palo Alto, CA). LC-MS/MS combines the separation capabilities of liquid chromatography with the accurate mass analysis of mass spectrometry. The database searches were performed using MASCOT available at [www.matrixscience.com](http://www.matrixscience.com). We set the mass tolerance at 300 ppm, 0.6Da MS/MS tolerance and allowed a maximum of one missed cleavage site per peptide.



## **ELISA**

A competitive ELISA kit (AdipoGen, Korea) for human clusterin was used following manufacturer instructions.

## **REAGENTS**

IPG strips pH 4-7 17cm, were purchased from BioRad Laboratories (Hercules, CA,USA), Ampholyte 4-6, Dithiothreitol (DTT), urea and thiourea from Sigma-Aldrich (St. Louis, MO, USA). Goat Polyclonal antibody Anti-clusterin- $\alpha$  (human) (C-18) sc-6419 and HRP-conjugated anti-goat IgG were purchased from Santa Cruz Biotechnology (CA, USA), Magic Mark XP western protein standard from Invitrogen (Carlsbad, CA, USA), ELISA kit from AdipoGen (Korea). All other reagents were of the highest quality available.

Chapter 4

# **RESULTS**

## 2D-PAGE comparisons

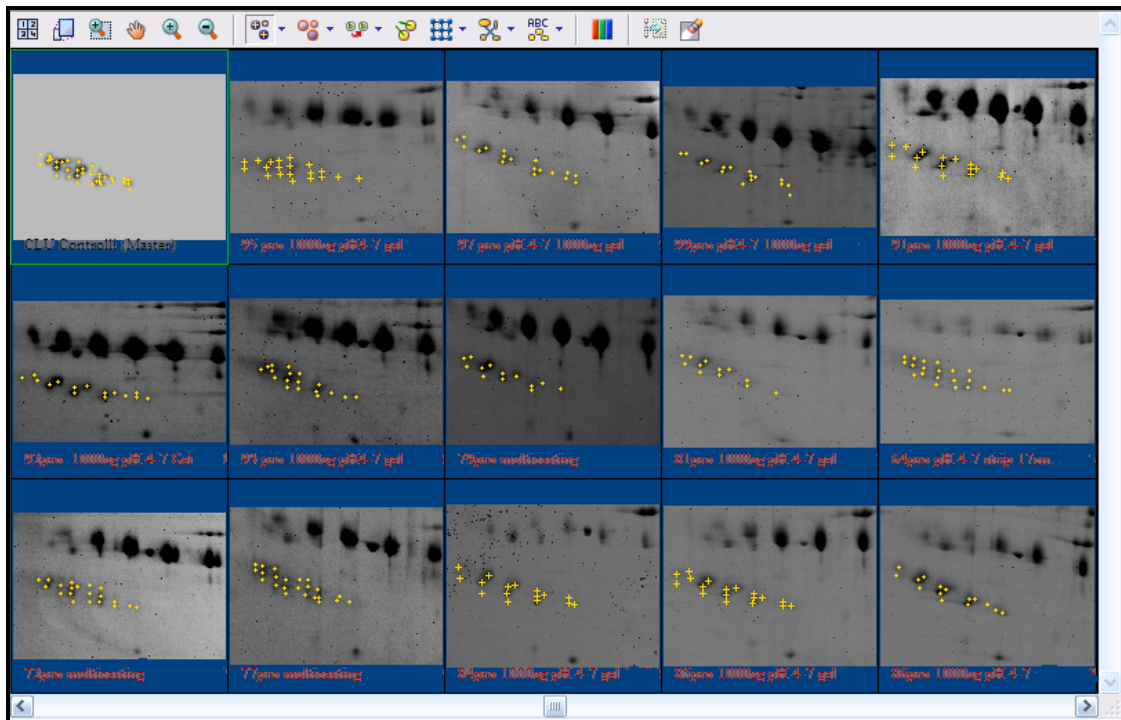
To explore the changes in the protein expression of the CLU protein a 2D-PAGE study was performed. After preparation of plasma samples with the method of Celis *et al.* for the removal of great part of albumin, a 2DE protein map in the pH range 4-7 was prepared for each sample. In the typical 2D plasma map available on SWISS 2D-PAGE from ExPASy Proteomic Server ([www.expasy.org](http://www.expasy.org)) Clusterin appears as a train of spots whose isoelectric points extend from 4.65 to 5.65 and their molecular weight are in the range between 32KDa and 40KDa positioned under the train of haptoglobin  $\beta$  chain spots. The presence of this train of spots is generally caused by a variability in sialylation of the different protein glycoforms (P. Ghosh, *et al.*, 2001) The fig. 5 shows a typical 2D plasma map obtained in our laboratory through the method described in materials and methods with a zoom (fig. 6) on the clusterin spots. The figure 5 also shows the typical position of most abundant plasma proteins.

The Gels were analyzed using the PDQuest 2D Analysis basic software and the images cropped in the area of interest (containing the train of clusterin spots) before performing the image analysis. We realized that this step had a positive and a negative aspect, the positive one was that it allowed us to have a better visualization of all the spots on the computer screen and to better perform the editing and matching of the spots in the sample group without a lot of zooming on the images, while the negative

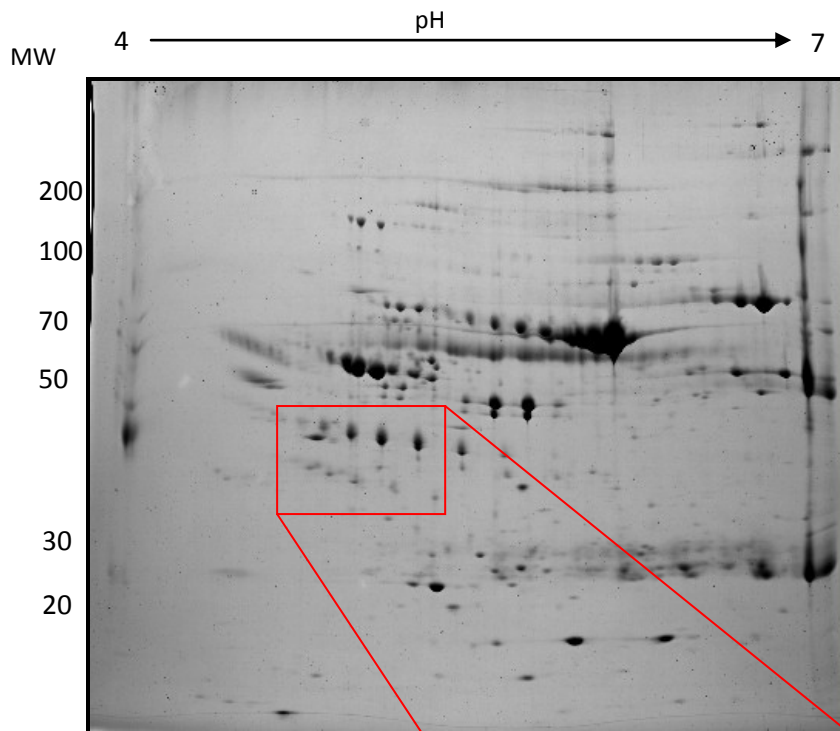
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one is that we couldn't perform the normalization "total density in gel image" because cropping the image we have lost the total intensity of it. For this reason we used the "total quantity" in valid spot normalisation.

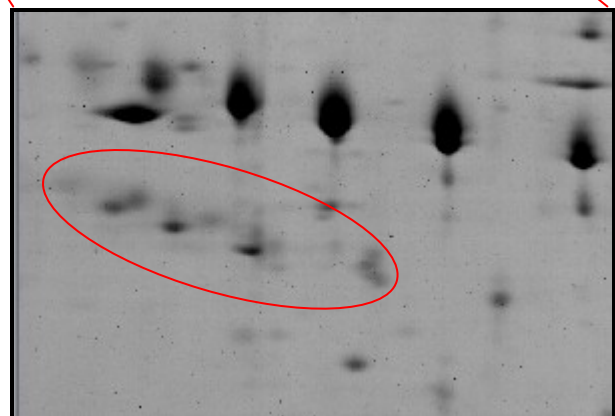
The software used is not designed to compare more than 15 images at a time per experiment so we decided to use a single experiment "matchset" for each group, the spot intensities were normalised and the quantity table exported for future analysis. For each "matchset" (4 groups) we had different SSP (standard spot number) numbers assigned by the software, to overcome this problem we annotated all the spots manually with visual inspection in order to have the same number for matched spots between the four groups. At this point we had 4 comparable spot quantity tables with which we performed all the statistical analysis with the software SPSS statistics v.17. The fig. 4 shows a typical screen of a matchset in PDQuest.



**Figure 4:** Matchset example created by PDQuest software, the yellow crosses indicate the clusterin spots and the image on the left represents the master gel which contains all the spots that appear in each of the sample images.



**Figure 5:** Typical gel image obtained in our lab for plasma proteins after removal of a great part of albumin in a gradient gel 5-15%.



**Figure 6:** Zoom on the position of clusterin spots (inside the red ellipse).

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To analyse the differential expression of clusterin spots between groups of gels we decided to use two statistical tests that are alternatively used for proteomic analyses, a parametric and a non parametric one. The One-Way ANOVA parametric test, can be used for comparing means when there are more than two levels of a single factor (in our case “the age”: 4 groups). However, the ANOVA doesn’t identify which means are different, methods for investigating this issue are called multiple comparisons methods, in this work we used the “Fisher’s least significant difference method” (LSD) to identify which means were different. The ANOVA test is a very powerful parametric test but the data must respect the restrictive assumptions (normal distribution of the dataset, homogeneity of variance and independent samples). Although some empirical evidence illustrates that slight deviations in meeting the assumptions underlying parametric tests may not have drastic effects on the obtained probability levels, there is no general agreement as to what is a “slight” deviation (S. Siegel and N.J. Castellan, 1988).

In total we had 44 variables to analyse, obviously the clusterin spots are not so numerous but this number represents all the spots present in all samples of all the groups, having a mean of 30 spots in each master gel created by the the group experiment. So while the matched spots to all gels were only five or six in some groups, the unique spots present in each gel of an experiment (matchset) increased the number to analyse to 44.

For the reasons cited above, before applying the ANOVA test we performed the Levene Test for Homogeneity of Variances and found that on the 44 variables (our clusterin spots from all the groups) there were 9 spots that didn't pass the test. In ANOVA table (table 1) only the spots with a p-value  $\leq 0.05$  are listed eliminating those that didn't pass the test for homogeneity of variance.

We further investigated our data performing a non parametric test the Kruskal-Wallis Test (table 2) which doesn't assume a normal distribution of the samples, to see if there was a similarity between the spots identified.

The spots identified by the ANOVA test were 10, while those identified by the Kruskal-Wallis Test were 21. We can notice that not all the spots identified by the two tests were the same; the spots unique for each test are highlighted in blue in the respective tables. The figure 7 below shows the positions of the spots identified by the ANOVA analysis on the master gel images of each group.

ANOVA						LSD	
Spot		Sum of Squares	df	Mean Square	F	Sig.	Group difference
<b>V3</b>	Between Groups	2,479	3	0,826	3,524	0,023	<b>50 vs 100</b> <b>80 vs 100</b> <b>90 vs 100</b>
	Within Groups	9,849	42	0,234			
	Total	12,328	45				
<b>V7</b>	Between Groups	4,363	3	1,454	10,16 5	0,000	<b>50 vs 100</b> <b>80 vs 100</b> <b>90 vs 100</b>

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	Within Groups	6,009	42	0,143			
	Total	10,372	45				
							<b>50 vs 90</b>
<b>V12</b>	Between Groups	18,222	3	6,074	3,926	0,015	<b>80 vs 90</b>
							<b>90 vs 100</b>
	Within Groups	64,987	42	1,547			
	Total	83,209	45				
							<b>50 vs 90</b>
<b>V13</b>	Between Groups	50,030	3	16,677	4,396	0,009	<b>80 vs 90</b>
							<b>90 vs 100</b>
	Within Groups	159,323	42	3,793			
	Total	209,352	45				
							<b>50 vs 100</b>
<b>V2307</b>	Between Groups	151,762	3	50,587	4,558	0,007	<b>80 vs 100</b>
							<b>90 vs 100</b>
	Within Groups	466,132	42	11,098			
	Total	617,894	45				
							<b>50 vs 80</b>
<b>V3305</b>	Between Groups	34,614	3	11,538	3,076	0,038	<b>80 vs 90</b>
							<b>80 vs 100</b>
	Within Groups	157,565	42	3,752			
	Total	192,179	45				
							<b>50 vs 80</b>
<b>V4204</b>	Between Groups	275,594	3	91,865	3,211	0,032	<b>50 vs 90</b>
							<b>80 vs 100</b>
	Within Groups	1201,678	42	28,611			<b>90 vs 100</b>
	Total	1477,272	45				
							<b>50 vs 80</b>
<b>V4206</b>	Between Groups	175,554	3	58,518	5,192	0,004	<b>50 vs 100</b>
							<b>90 vs 80</b>
							<b>80 vs 100</b>
	Within Groups	473,377	42	11,271			
	Total	648,932	45				
<b>V5104</b>	Between Groups	558,166	3	186,055	5,330	0,003	<b>50 vs 90</b>

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							80 vs 90
							90 vs 100
	Within Groups	1466,224	42	34,910			
	Total	2024,390	45				
							50 vs 90
<b>V5109</b>	Between Groups	16,115	3	5,372	3,953	0,014	80 vs 90
	Within Groups	57,078	42	1,359			90 vs 100
	Total	73,192	45				
							50 vs 80
<b>V6106</b>	Between Groups	18,475	3	6,158	4,388	0,009	80 vs 90
	Within Groups	58,950	42	1,404			80 vs 100
	Total	77,425	45				
							50 vs 80
<b>V6108</b>	Between Groups	36,866	3	12,289	3,479	0,024	50 vs 90
	Within Groups	148,332	42	3,532			90 vs 80
	Total	185,197	45				80 vs 100
							50 vs 90
<b>V7106</b>	Between Groups	50,424	3	16,808	4,396	0,009	80 vs 90
	Within Groups	160,580	42	3,823			90 vs 100
	Total	211,004	45				

**Table 1** ANOVA table showing the spots that had a p-value  $\leq 0.05$  between groups, the last column on the left shows the groups identified by the LSD test; 50 stays for the sample group aged 20-50, 80 for 80-89, 90 for 90-99 and 100 for  $\geq 100$ . Spots highlighted in blue represent those spots not revealed by the analysis using the Kruskal-Wallis test.

Kruskal Wallis test							
	V3	V7	V11	V12	V13	V16	V602
Chi-Square	9,711	25,957	9,711	19,005	15,483	14,371	12,112
df	3	3	3	3	3	3	3
Asymp. Sig.	0,021	0,000	0,021	0,000	0,001	0,002	0,007

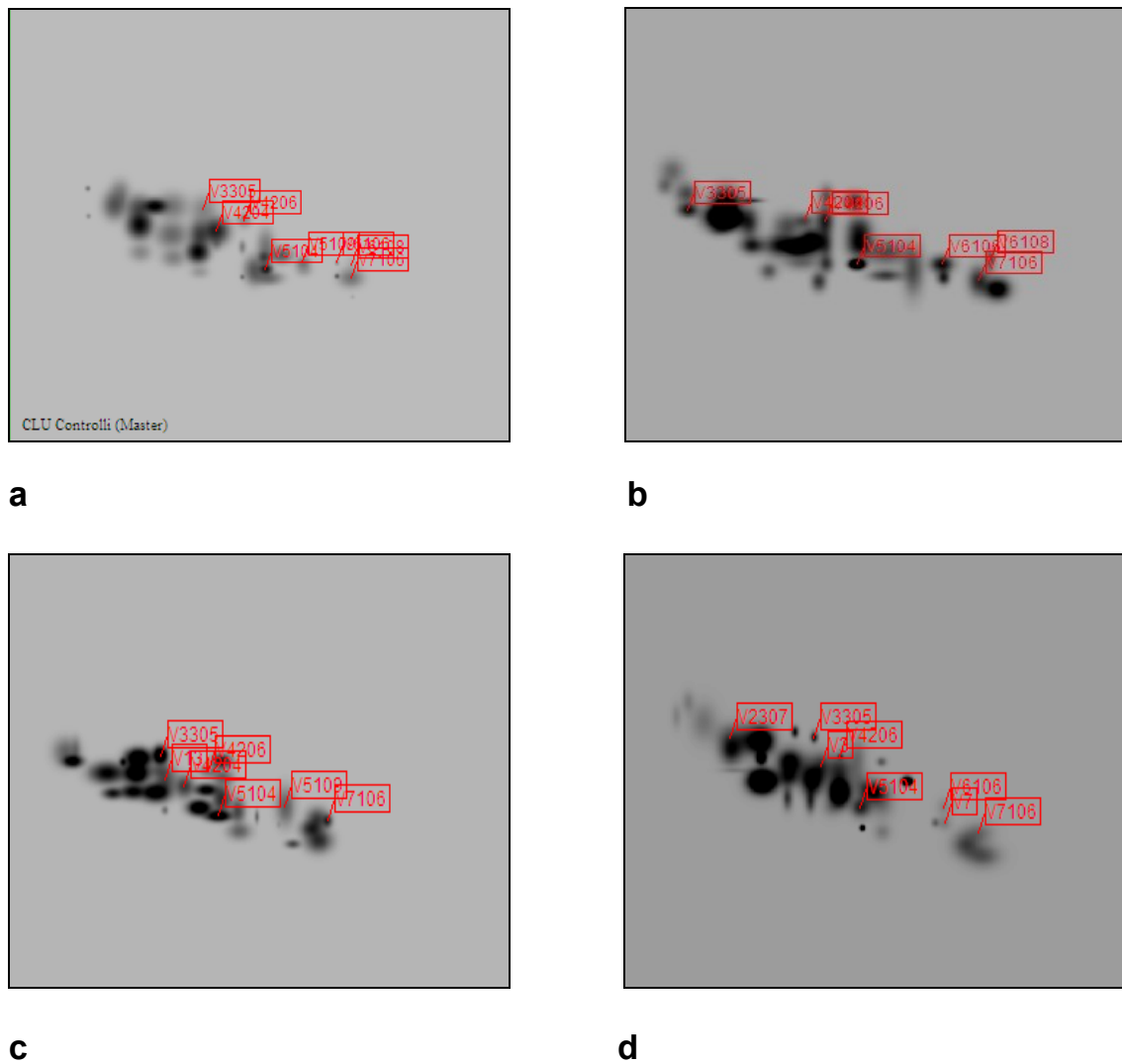
  

	V1307	V2101	V2307	V3101	V3501	V4203	V4105
Chi-Square	15,331	13,706	10,926	8,645	8,883	18,838	10,268
df	3	3	3	3	3	3	3
Asymp. Sig.	0,002	0,003	0,012	0,034	0,031	0,000	0,016

	V4204	V5104	V5106	V6107	V6108	V7106	V6106
Chi-Square	9,247	8,162	8,902	8,485	12,152	19,490	10,943
df	3	3	3	3	3	3	3
Asymp. Sig.	0,026	0,043	0,031	0,037	0,007	0,000	0,012

**Table 2** Kruskal Wallis table results, the spots highlighted in blue represents those spots not revealed by the analysis using ANOVA.



**Figure 7:** Master gels of the four groups with the spots identified by the ANOVA test highlighted in red, **a** 20-50 group, **b** 80-89 group, **c** 90-99group, **d**  $\geq 100$  group.

## Mass spectrometry and protein identification

Of the spots highlighted by the statistical analyses only six were easily separated when cutting the gel because some of them were too overlapped for a good separation; for this reason we sent to identification only five of them. The identifications were made using a nano (HP-1200) LC-MS/MS ion trap mass spectrometer. Peptide mass fingerprints of the peptides were identified by database search using MASCOT available at [www.matrixscience.com](http://www.matrixscience.com) with the parameters indicated in materials and methods. The table 3 shows how identification confirmed that those spots were Clusterin with a reasonable high score.

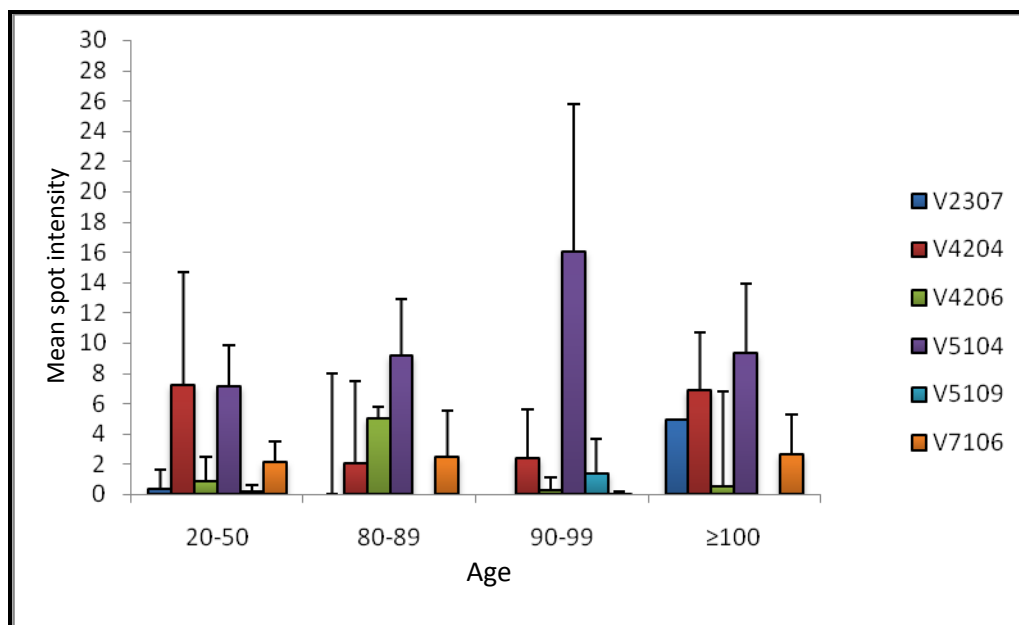
Spot	p-value	Protein	UniProt accession N°	Mass	pI	Score	Matches	Sequences
V2307	0,007	CLUS_HUMAN	P10909	52461	5.89	117	12 (7)	9 (4)
V4204	0,032	CLUS_HUMAN	P10909	52461	5.89	355	27 (17)	13 (11)
V4206	0,004	CLUS_HUMAN	P10909	52461	5.89	205	15 (13)	12 (10)
V5104	0,003	CLUS_HUMAN	P10909	52461	5.89	168	12 (9)	9 (7)
V5109	0,014	CLUS_HUMAN	P10909	52461	5.89	208	14 (10)	12 (8)
V7106	0,009	CLUS_HUMAN	P10909	52461	5.89	126	9 (5)	7 (5)

**Table 3:** Mass spectrometric identification of spots using the Mascot search engine; **matches** spectral counts, **sequences** unique peptides.

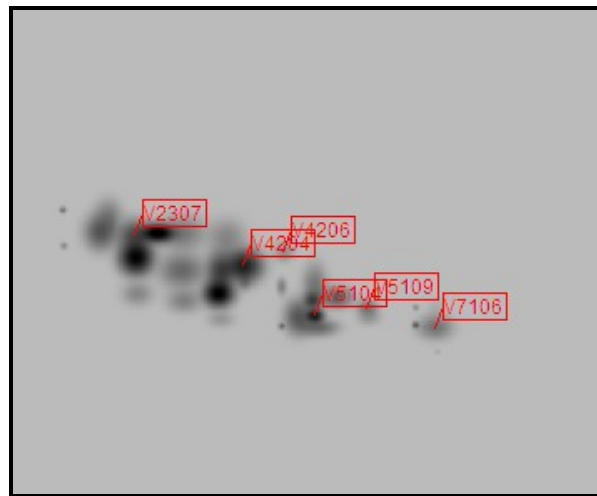
Analysing the unique peptides retrieved by the mascot search we found that they were composed of peptides coming from both the  $\alpha$  and the  $\beta$  chain sequences of the clusterin protein, suggesting that the spots analysed are a mixture of both chains.

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The histogram in fig 8 shows the mean spot intensity variation between groups using the normalised quantity values obtained with the software PDQuest, while fig. 9 shows where these spots are positioned in the cluster of a representative master gel.



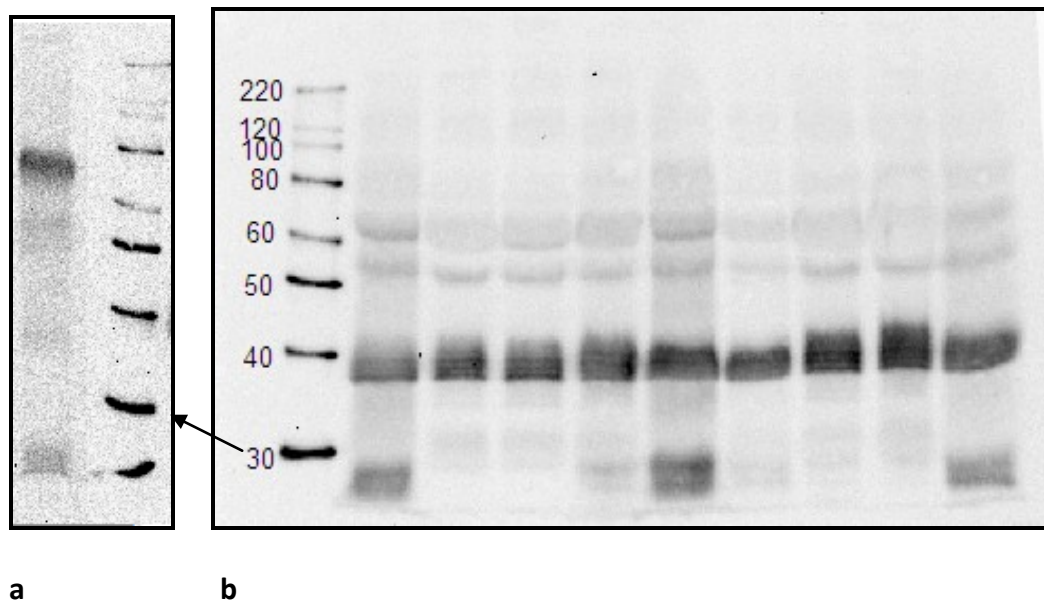
**Figure 8:** intensity variation of the six spots identified by mass spectrometry in the four groups. On the y axis the intensity values are the ones exported from the analysis with PDQuest, after normalization, while on the x axis the four groups analysed. Error bars represent standard deviation.



**Figure 9:** Representative master gel with the spots identified highlighted in red.

## Western blot analyses

For western blot analyses we used an anti-CLU polyclonal antibody. The fig. 10 shows the bands detected by this antibody on a 1-D gel when 50 $\mu$ g of total protein extracts were loaded per well. On the left we show a sample under non reducing conditions, while on the right a representative gel of some of the samples investigated, under reducing conditions.



**Figure 10:** Immunodetection of CLU by the polyclonal antibody, **a** under non reducing conditions, **b** under reducing conditions.

Native analysis (fig. 10 a) revealed a smeared band ranging from  $\sim 70$  to  $85$  kDa, this mass range fits with the expected molecular mass for the sCLU corresponding to the glycosylated heterodimer. Under reducing conditions (fig.10b) we found a set of bands of about  $40$  kDa which corresponds to the protein of  $\sim 75$ - $80$  kDa after being reduced producing the  $\alpha$  and  $\beta$  chains which have similar masses of about  $25$  kDa that when glycosylated increase to  $\sim 40$  kDa.

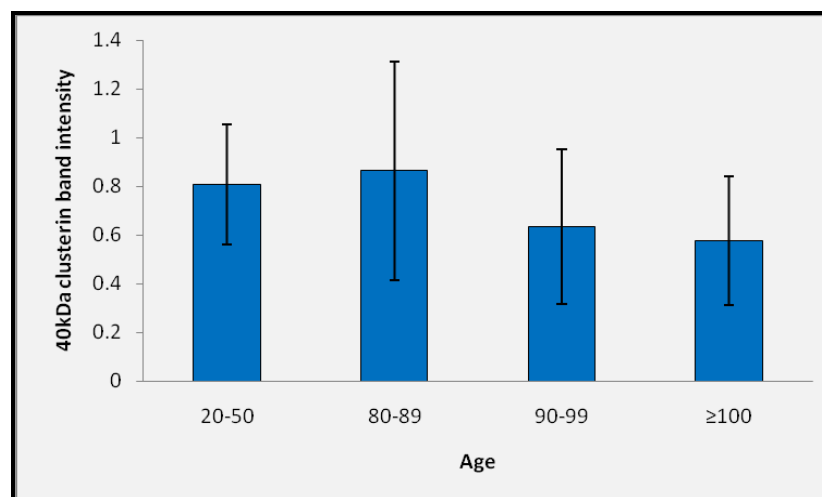
In this gel other bands are visible, of  $55$ ,  $61$  and  $28$  kDa. The size of  $55$  kDa fits with the nuclear form of clusterin while the band of  $61$  kDa with the precursor form of the secreted clusterin. Moreover the band of  $28$  kDa corresponds to one of the chains



( $\alpha$  or  $\beta$ ) not glycosylated and they appeared only in some of the samples analysed. Probably some samples had clusterin proteins with a lower degree of glycosidation and their  $\alpha$  and/or  $\beta$  chains shifted to a lower molecular weight value.

The clusterin spots analysed with the 2D-PAGE corresponded to the smeared band of  $\sim 40$ kDa and our analysis of quantification concentrated on this band.

We must take into account that the western blot technique is not completely reliable for quantification because the transfer process could introduce artifacts altering the ratio between bands but surely it can give an idea of what the general behaviour is. Below is the histogram representing the mean intensity of the  $\sim 40$ kDa band with increasing age. It seems that a slight decrease occurs in centenarians although not statistically significant.



**Figure 11** The mean percentage of volume contribution of the  $\sim 40$ kDa band evaluated for each group with the software Quantity One, the bars represent standard deviations.

## ELISA

We wanted to quantify with a more reliable technique the clusterin concentration in our plasma samples, using an available commercial kit for human clusterin. For these analyses we increased the number of samples in order to have robust statistical analysis. We found significative differences between the groups 80-89 versus  $\geq 100$  and 90-99 vs  $\geq 100$ . The table below shows the ANOVA result and the LSD test for the identification of the groups that contribute to the significative value of the ANOVA test.

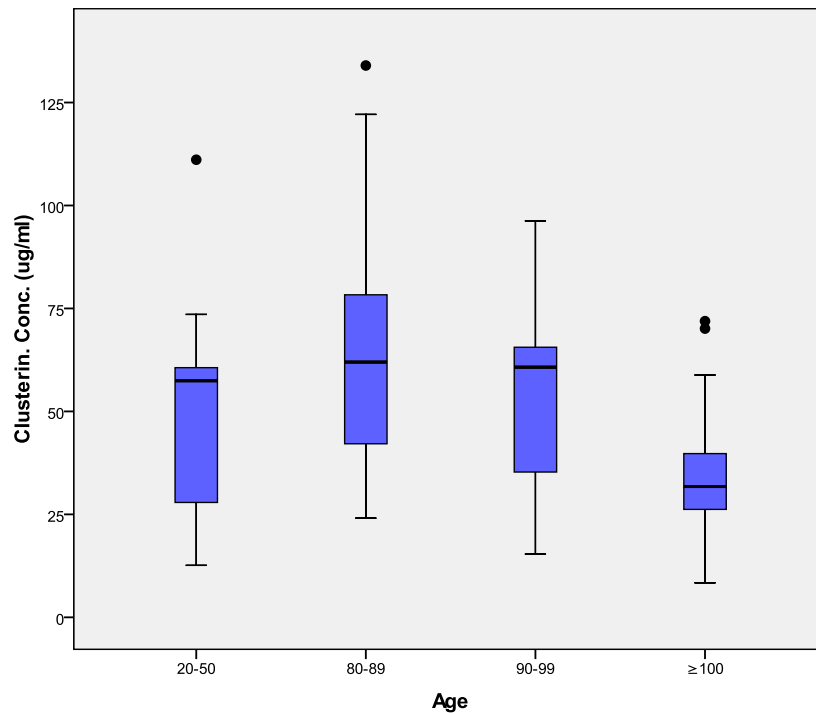
	ANOVA					LSD
	Sum of Squares	df	Mean Square	F	Sig.	
						<b>80 vs 100 (0.0003)</b> <b>90 vs 100 (0.019)</b>
Between Groups	9439,028	3	3146,343	5,030	0,003	
Within Groups	40035,193	64	625,550			
Total	49474,221	67				

**Table 4:** ANOVA table showing the spots that had a p-value  $\leq 0.05$  between groups, the last column on the left shows the groups identified by the LSD test with the p-values in brackets.

The table 5 shows the values obtained in the ELISA experiment and the fig. 12 its graphical representation.

Age	Sex	Mean	N	Std. Deviation	Median	Minimum	Maximum
50	Male	53.93638	8	30.229678	58.50250	12.624	111.110
	Female	47.10333	9	18.179783	52.36100	26.284	73.557
	Total	50.31888	17	24.029432	57.42100	12.624	111.110
80	Male	60.59575	8	32.795181	55.77550	26.494	133.986
	Female	75.20629	7	36.878539	77.53000	24.083	122.121
	Total	67.41400	15	34.315525	61.98800	24.083	133.986
90	Male	51.61378	9	23.101631	60.76200	15.336	80.385
	Female	58.08988	8	25.011504	60.41400	19.862	96.273
	Total	54.66135	17	23.486882	60.76200	15.336	96.273
100	Male	34.57950	10	14.114774	31.69700	18.021	70.108
	Female	37.69100	8	20.452230	36.36400	11.612	71.913
	Total	35.96239	18	16.740452	32.21950	11.612	71.913
Total	Male	49.33074	35	26.231899	42.76600	12.624	133.986
	Female	53.64441	32	27.792398	58.56300	11.612	122.121
	Total	51.39100	67	26.870007	50.59100	11.612	133.986

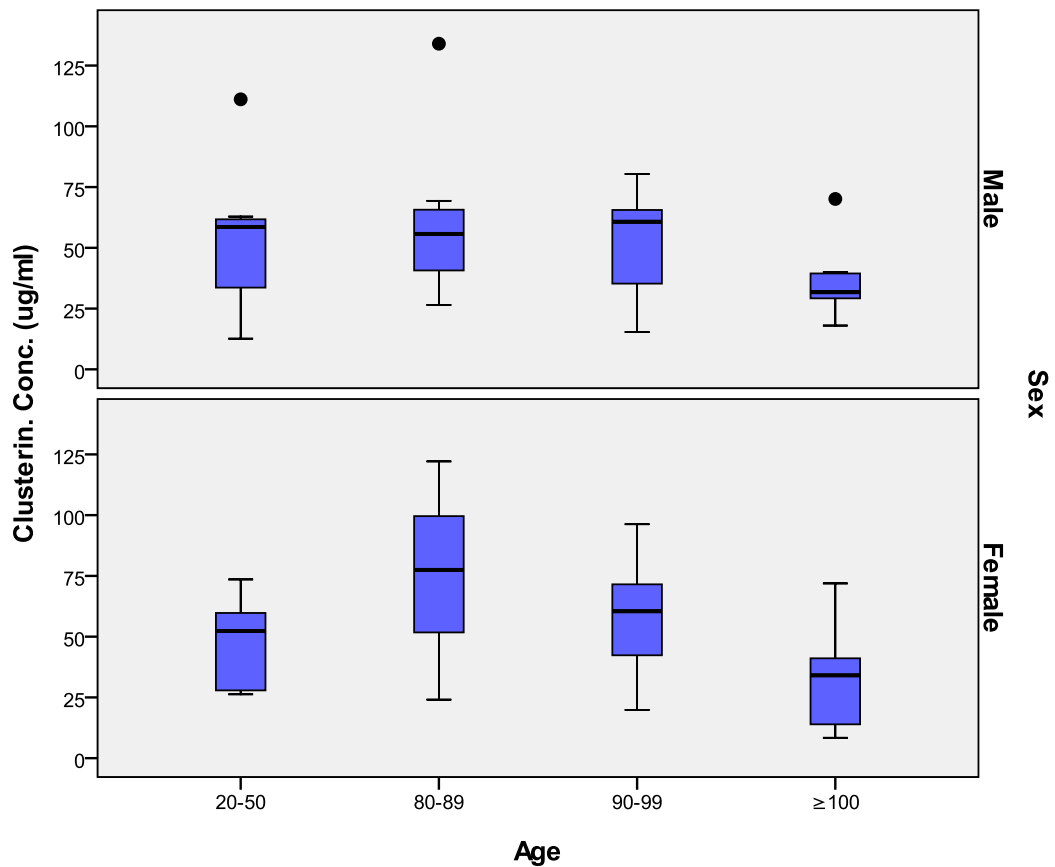
**Table 5:** Clusterin concentration values in plasma samples with the ELISA assay.



**Figure 12:** Box plots of the clusterin concentration from ELISA experiment showing the distribution of data values in the four groups. The bar inside the box indicates the median of distribution, the box spanning the interquartile range, and the external bars represents the lowest and highest data points. The black dots are outliers

As a previous work by Trougakos *et al.*, reported an increase in CLU serum levels with age in males but not in females (I.P. Trougakos, *et al.*, 2002) we wanted to evaluate if the same behaviour was detectable with our plasma samples.

The box plot in fig.13 shows how the clusterin concentration values vary with age in males and females separately.



**Figure 13:** Box plots of the clusterin concentration from ELISA experiment in the four groups in separated panels for males and females.

The ANOVA analysis (tables 7, 8) showed that for females we have significant differences between groups aged 20-50 vs 80-89 and between 80-89 vs  $\geq 100$ , while for males we have a significant difference only between the group aged 80-89 vs  $\geq 100$ .

ANOVA						LSD
Adj. Conc.	Sum of Squares	df	Mean Square	F	Sig.	
Between Groups	7056,622	3	2352,207	3,614	0,025	<b>50 vs 80 (0.037)</b> <b>80 vs 100 (0.004)</b>
Within Groups	18874,746	29	650,853			
Total	25931,368	32				

**Table 6:** ANOVA analyses of the ELISA results only for females, the last column on the left shows the groups identified by the LSD test with the p-values in brackets.

ANOVA						LSD
Adj. Conc.	Sum of Squares	df	Mean Square	F	Sig.	
Between Groups	3407,800	3	1135,933	1,762	0,175	<b>80 vs 100 (0.039)</b>
Within Groups	19988,026	31	644,775			
Total	23395,825	34				

**Table 7:** ANOVA analyses of the ELISA results only for males, the last column on the left shows the groups identified by the LSD test with the p-values in brackets.

Chapter 5

**DISCUSSION**

CLU gene expression was found to increase during in vivo ageing from gestation to adults in humans in different tissues like the human pituitary gland (T. Ishikawa, *et al.*, 2006), in the rat ventral prostate (K.M. Lau, *et al.*, 2003), in human glial cultures (N.V. Patel, *et al.*, 2004) and in human lymphocytes (I.P. Trougakos, *et al.*, 2006).

Previous work of Trougakos *et al.*, have reported an increase in CLU serum levels with age in males but not in females with the maximum age being 90 years old (I.P. Trougakos, *et al.*, 2002).

In a paper by Franceschi *et al.*, it was reported that CLU gene expression levels increase during ageing but decrease in centenarians. To examine the changes in the CLU protein expression we performed a comparative 2D-PAGE study with subjects coming from the AkeA Project grouped into 4 classes with ages in the range 20-50, 80-89, 90-99 and  $\geq 100$ . In the 2D-gels clusterin appears as a train of spots whose isoelectric point is in the range between 4.65 and 5.65 and molecular weight between 32KDa and 40KDa. With the removal of ~60% of albumin with a modified TCA precipitation method we were able to resolve a cluster composed of ~16 spots in the position indicated by the reference gel map present in SWISS 2D-PAGE database, but considering unique spots coming from each single gel from all the groups this number increased to 44.



The two statistical tests used revealed a group of 10 significant spots using the parametric test ANOVA and 21 spots using the non parametric Kruskal Wallis test. We decided to analyse our data with both tests because even if parametric tests are the ones widely used, they must respect restrictive assumptions like a normal distribution of the data and homogeneity of variance, which is not always the case in spot intensity values. The non parametric test was able to detect more spots whose values vary significantly between groups but some of the spots identified by the ANOVA were not identified by the Kruskal Wallis. In this work we had a small number of variables to compare but the different result obtained when using the two tests might suggest that both of them should be used in a first attempt to highlight differentially expressed proteins because small variations that can be detected by one test could not be highlighted by the other. Of the spots founded by the statistical tests only the ones that were easily separated when cutting the gel were sent to mass identification (6 spots) while for the others that were overlapped between spots or were too faint, probably a better separation should be considered for future analyses with enrichment methods targeting this protein. LC-MS/MS analyses confirmed the identity of these spots as being Clusterin. Fig. 8 shows how the intensity of each spot varies with age. The spot V2307 is present only in centenarians, spot V5109 is present only in the group aged 90-99 and at a very low value in group 20-50, spot V4204 seems to decrease with age and then increase in centenarians, spot V5104 increase until 99 years old and then decrease in centenarians, while spot V7106 seems to be present at comparable levels

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in all the groups except for the 90-99 group, spot V4206 increase until 89 and then decrease in the other two groups.

Considering that a notable feature of secreted clusterin is its heavy glycosylation, for future analyses, it will be interesting to investigate the types of oligosaccharides attached to the clusterin peptides for each spot in the cluster.

In the western blot analyses using a polyclonal antibody we detected not only the expected band at ~40kDa which is the result of cleavage of the disulfide bonds of the mature secreted protein sCLU, but also a band at ~55kDa and ~61kDa. These bands fit with the mass of the nuclear form of CLU (nCLU) and with the intracellular precursor protein (psCLU) respectively. Why these isoforms appear in our plasma samples is unclear, it can be that some cells have been broken during preparation of the samples releasing these proteins which from literature should reside inside the cell. Further investigation should be necessary to better identify the nature of these bands, even because they were repeatedly found in all samples analysed. It is also visible a band of ~28kDa that fits with the polypeptidic backbone of the  $\alpha$  or  $\beta$  chain not glycosylated, interestingly they appeared only in some of the samples analysed. A deglycosylation assay should be performed to determine whether this band is glycosylated.

The quantitative determination of CLU in plasma samples was evaluated using a commercial kit. The boxplot in figure 12 and the p-values in table 4 shows how clusterin level significantly differ between the group aged 80-89 and the group of centenarians ( $p=0.0003$ ) and between the group aged 90-99 and centenarians ( $p=0.019$ ).

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These results agree with the data shown by literature by the work of Trougakos *et al.*, but they stopped investigating at 90 years old, while our work spanned over older people showing a decrease after 90 years especially in centenarians. One work taking into account centenarians is a paper by Franceschi *et al.* that evaluated the mRNA levels of CLU from lymphocytes finding a decrease in centenarians.

The ELISA experiment agrees in some way with the western blot analyses of the 40 kDa band but the differences between the two assays can be attributed to the presence of other isoforms in our samples that we didn't consider in the western blot quantification because we first must be sure that these bands are clusterin, in the future we would use monoclonal antibody to increase specificity. In fig.13 we separated males from females in the analyses to see if there was any difference in the clusterin concentration with age.

From table 6 we can see that the clusterin mean values are a bit higher in females than in males in all groups except for the group 20-50. If we look at the general behaviour no great differences are visible on the basis of sex, an increase in clusterin values until 90 years old is visible and then a decrease in older and centenarian people. We can even see that the difference between the groups aged 80-89 and  $\geq 100$  is bigger in females ( $p= 0.004$ ) if compared with males ( $p=0.039$ ).

As stated in the introduction the CLU gene seems to be a sensitive biosensor of environmental insults and particularly oxidative stress which is the driving force of most age-related diseases (including cancer) where CLU has functionally being impli-

cated. So if the clusterin function is to cease the damaging effects of oxidative stress, its upregulation should reflect the “general oxidative status” of the subject rather than its chronological age (I.P. Trougakos and E.S. Gonos, 2009). Centenarians are considered as a model of successful ageing characterised by low ROS (reactive oxygen species) load (C. Franceschi and M. Bonafe, 2003) and this can be the explanation why we found lower level of this protein in plasma. A larger sample size should be investigated to confirm the behaviour found in our samples and a comparison with centenarians of other countries should also be interesting to analyse to see if they share the same trend in plasma clusterin concentration values during ageing.

Chapter 6

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