doi:10.4149/neo_2010_05_488

Detecting soluble Clusterin in in-vitro and in-vivo models of prostate cancer

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Received February 2, 2010

PSA, the only relevant marker for prostate cancer, has a low predictive value; moreover its low threshold leads to unnecessary biopsies with associated complications. Identification of prognostic factors is an important goal in prostate cancer. In the search for new markers, clusterin, has some potential as it is closely linked with cancer progression and resistance to apoptosis.

We looked at the expression of secreted clusterin (sCLU) in prostate cells to determine correlations with progression and drug resistance. The plasmatic expression of sCLU was also investigated in order to use it as a potential marker for prostate cancer. sCLU expression was studied using Western blotting on cultured prostate cells, PWR-1E, PC3 and PC3 Docetaxel resistant cells in the cytosol and culture medium. An inhouse ELISA test was developed to determine sCLU expression in culture media and plasma samples. A patient cohort was identified from the Prostate Cancer Research Consortium Bio-Resource and plasmatic expression of sCLU was studied using western blotting and the inhouse ELISA test.

Only the fully processed form of sCLU was identified in the medium of cells with increased expression associated with increased progression of disease and resistance to docetaxel. Plasmatic expression of sCLU was significantly higher in the plasma of patients with high grade prostate cancer with extracapsular extension than in the plasma of prostate cancer patients without extracapsular extension.

Plasmatic sCLU may be an effective prognostic marker of prostate cancer and needs to be tested in a multimarker approach.

Key words: Clusterin, prostate cancer, prognostic, biological marker

Prostate cancer represents a significant health issue for European men [1] which is confounded by the lack of suitable markers for the presence or extent of disease which could offer reliable informations for appropriate treatment strategies. The discovery of more accurate and appropriate biological markers is required.

Previous studies from our laboratory [2] have identified panels of blood markers which correlate and predict the presence of prostate cancer and its pathological features such as Gleason grade and extracapsular extension. These markers could be very helpful in the diagnosis and prognostication of prostate cancer and offer important information for appropriate treatment strategies. One such protein identified was clusterin which was differentially expressed in the blood and urine of men with different Gleason grades of prostate cancer. Previous studies have identified clusterin as an important protein contributing to the progression of prostate cancer from an androgen dependent to independent phenotype [3].

Clusterin is situated at 8p12-21 and translates for two different proteins. The mature 70-80 kDa secreted protein is processed and glycosylated [4] in the Golgi apparatus and the endoplasmic reticulum and composed of a α and β chain held together by five disulfides bonds which has been associated with cell survival [5, 6]. The second isoform is produced through an alternative splicing of the mRNA or a differential initiating site, resulting in a shorter protein with a single chain which facilitates its migration to the nucleus where it has been shown to promote apoptosis through a number of pathways [7–9].

Based on our previous proteomic biomarker discovery work [2] we hypothesise that secreted Clusterin in the blood of men with prostate cancer could help to identify men with low and high risk disease. To date, only cellular levels of clusterin have been detected at the level of the gene or protein, as part of this study we have developed an in-house ELISA test using a commercially available antibody confirmed to detect the secreted isoform of Clusterin.

Materials and methods

Cell culture. PC3 androgen independent prostate cancer cells and human non-neoplastic PwR-1E prostate epithelial cell line was purchased from the American Type Tissue Collection. The PwR-1E cells were cultured in Keratinocyte serum-free medium supplemented with 2.5 µg epidermal growth factor, 25 mg bovine pituitary extract, 50 units/ml penicillin and 50 µg/ml streptomycin. PC-3 cells were cultured to confluence in RPMI (Roswell Park Memorial Institute medium) with 10% FBS (Foetal Bovine Serum) . A flask of the PC-3 cell was aged to match the PC-3 D12 cells which we selected following repeated treatment with Docetaxel (12µM) for the course of six month to yield the Docetaxel resistant phenotype. To generate the media sample all cells were cultured in 0% FBS, in order to avoid artefacts which would prevent the correct reading of the western blot, and their respective conditioned medium generated over 24 hours was collected.

Western blot. The cytosol protein fractions were extracted from the cellular fraction using NP-40 extraction buffer (20 mM Hepes -4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid - pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA - ethylenediaminetetraacetic acid-, 0.1 mM EGTA - ethylene glycol tetraacetic acid- , 1% NP-40 -nonyl phenoxylpolyethoxylethanol-, 1 mM NaF and 1 mM Na_3VO_4, plus cocktail of protease inhibitors) and centrifuged to remove the membrane fraction collecting the supernatant as the cytosol fraction. The culture media was concentrated with centricon plus-70 10 kDa spin filters resulting in the concentrated media fraction (Media). Plasma was processed using the 2D gel cleaning kit protein (2-D Clean-Up Kit 50 samples –Amersham bioscience-) and Urine was concentrated using the centricon tubes (Amicon Ultra 15ml 10K 24pk, 5 minutes at 4000g in a refrigerated centrifuge at 4 degrees Celsius). Protein fractions were assayed for protein concentrations using the Bradford method and 20µg of protein prepared for loading by boiling in loading dye with or without β-mercaptoethanol (30mM TRIS - tris(hydroxymethyl)aminomethane- , 12.5% glycerol, 1% SDS - Sodium Dodecyl Sulphate- , 12.2mM β-mercaptoethanol, 0.05% bromophenol blue). The samples were loaded onto an 8% SDS PAGE - polyacrylamide gel electrophoresis- gel and then transferred on Immobilin P (Millipore) membranes. Membranes were stained with the primary antibody C41 D (Upstate) (specific for the alpha subunit of clusterin) and incubated overnight at 1/1000 concentration and the membranes were then washed in TBST - Tris-Buffered Saline Tween-20- (50 mls of 1M TRIS, 5,85g NaCl, in 1 litre of deionised water, add 1ml of tween 20) before incubating with horseradish peroxidase-conjugated secondary anti-mouse antibody at 1/5000 concentration for one hour. Signals were detected using ECL™ (Amersham Biosciences).

ELISA. We used 96 well plates (NUNC maxisorp ref: 439454). The samples were loaded in triplicates using 0.45µl of diluted plasma (1/100) in 100µl of Vollers buffer (15mM of carbonate anhydrous Na_2CO_3: 1,5885 g/l and 34,8mM of bicarbonate $NaHCO_3$: 2,9232 g/l diluted in deionised water) per well. Standard curve was elaborated using recombinant Clusterin (Prospec Catalog No. CYT-278) in increasing concentration (rCLU solution: 0,5 µg of rCLU diluted in 1ml of vollers buffer, add 5µl of the solution in the first well, 10 in the second well and progressively increasing the amount of rCLU by 5µl per well. The dilution is made by completion with Vollers buffer to reach 100µl in each well). The samples were incubated overnight at 4°C temperature. The primary antibody (C41 D) was incubated at 37°C (1/1000 concentration) for 3 hours and the secondary at 37°C for one hour. The result was read after 30 minute incubation with 100µl of ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) - solution.

Patient cohort. Pre-operative plasma samples were available from the Prostate Cancer Research Consortium Bio-Resource where all patients were appropriately consented and all studies were approved by the local Hospital Research Ethics committee of the corresponding hospital where the samples were collected. The Bioresource Information Management Systems Database was searched for patients with Gleason 5 (3+2) without extracapsular extension (LG -Low Grade group- , n=11) and Gleason 7 (4+3) with extracapsular extension (HG –High Grade group- , n=11). Clinical data of age (LG = median age 58.81; standard deviation 4.42 and range 52-68, HG = median age 62.81 +/- 4.23 range 65-70), average PSA (LG PSA = 7.9 +/- 5.3 ng/ml verses HG PSA = 10.3 +/- 4.9 ng/ml).

The initial control group was composed of 13 patients suffering from BPH - Benign prostatic hyperplasia - all of whom took benefit from a prostatectomy or a transurethral resection of the prostate. Their histology was systematically benign and they all had a PSA follow up of at least 36 months. The median age of this population was 70.2 years old at the time of intervention with a standard deviation of 9.19 years making that population not comparable to the LG and HG group.

Results

Cellular and secreted expression of Clusterin in prostate cancer cell lines. The initial 449 amino-acid polypeptide chain of Clusterin undergoes a number of post translation modifications, firstly the leader sequence is cleaved off in the endoplasmic reticulum [8,10]. The resulting amino acid is then transported to the Golgi apparatus where the five disulfides bonds are formed followed by cleavage at amino acid 227/228 resulting in the α and β chain held together by the five disulfide bonds. The resulting protein then undergoes glycosylation in the trans Golgi network at predefined N-glycosylation sites on both chains [4].

Previous studies in our laboratory have demonstrated that the C41 D antibody is the most appropriate antibody to detect the pre-processed and processed forms of Clusterin following analysis of a number of different antibodies. Figures 1 and 2 demonstrate that Clusterin detected in the cytosolic fraction is a 55kDa -kilodalton- pre-processed form which cannot be dissociated into the α and β chain following boiling with β-

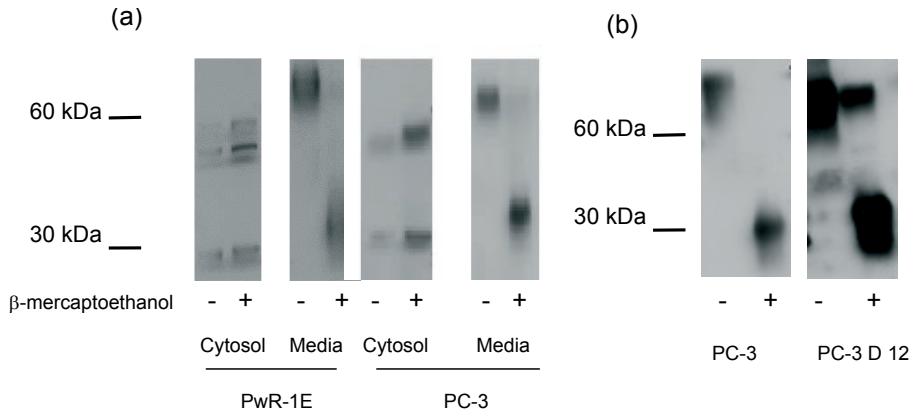

Figure 1 : Cytosol (a) and secreted (b) Clusterin expression in PwR-1E and PC-3 cells – Western analysis. PwR-1E and PC-3 cells were grown to 80% confluence and incubated in 0% FBS for 24 hours after which the cytosolic protein fraction (Cytosol) was extracted using NP-40 from the cells and a media sample collected and concentrated (Media). Cytosol and Media fractions were assayed for protein concentration and 20µg of each protein was boiled with or without β-mercaptoethanol. Resulting protein as assessed for sCLU with the C41 antibody but western blotting. Blots represent 1 of 4 independent experiments.

Figure 1 a: Cytosol and secreted Clusterin expression in PwR-1E and PC-3 cells – Western analysis

Figure 1 b: Secreted Clusterin expression in PC-3 and PC-3D12 prostate cancer cells – Western analysis.

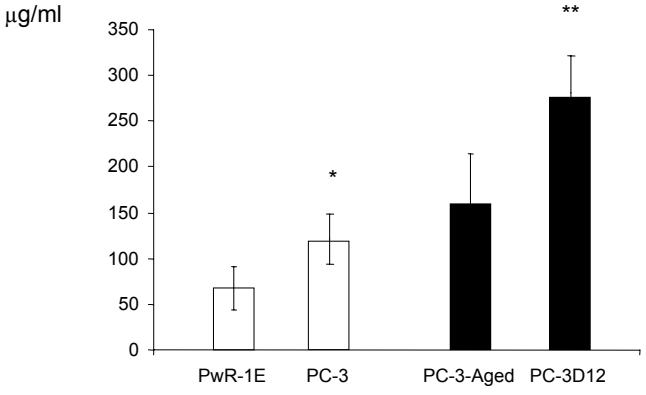

Figure 2: Secreted Clusterin expression in PwR-1E, PC-3 and PC-3D12 prostate cancer cells – ELISA analysis. Cells were grown to 80% confluence and a 24 hour culture media collected. Culture media was collected and concentrated with centricon plus-70 10 kDa spin filters (Media). The Media fractions were assayed for protein concentration and 20µg of each protein was assessed for Clusterin expression using the in-house Clusterin ELISA using the C41 antibody. n=3 independent experiments.

Statistical analysis was carried between the PwR-1E and PC-3 using the students t-test, *p<0.05 verses PwR-1E and between PC-3 aged and PC-3D12 using the students t-test, **p<0.05 verse PC-3 aged.

mercaptoethanol indicating that cleavage at the 227/228 amino acids has not yet occurred. However, clusterin detected in the media is a 70kDa protein indicating its glycosylation and boiling in β-mercaptoethanol, which would dissociate the disulfide bonds, results in the detection of a 35kDa band which would represent the α chain.

Figures 1 demonstrates no significant difference in the cytosolic pre-processed form of Clusterin or the secreted glycosylated form of Clusterin in the media between the androgen resistant PC-3 cancer cells and benign PwR-1E prostate epithelial cells using western blotting. However, there is a significantly detected increase in both the cytosolic pre-processed form of Clusterin and its secreted glycosylated form in the media between the parental age-matched PC-3 cells and the Docetaxel resistant PC-3 D12 cells.

As western blotting is only a semi-quantitative method for the assessment of protein expression we developed an in-house ELISA using the C41 D antibody to detect soluble forms of Clusterin. Figure 2 demonstrates the detection of Clusterin in the media of the PwR-1E, PC-3, PC-3 aged and PC-3D12 cells. There was a significant increase in Clusterin between the PwR-1E and PC-3 cells which was not detected by western blotting. There was also a significant increase in clusterin expression between the PC-3 aged and PC-3D12 cells which was in line with the western blots.

Detection of Clusterin in human plasma and urine samples. Having demonstrated that the C41 D antibody could work in a western and ELISA detection system and was able to detect the fully processed form of Clusterin in the media of cell cultures, we next wanted to use these techniques to detect Clusterin in human plasma and urine samples. Plasma and urine samples were collected from a healthy control volunteer processed as in the methods section. The fully processed and glycosylated form of Clusterin was detected by western blotting which was confirmed following boiling with β-mercaptoethanol (Figure 3a). We also assessed Clusterin from patients with low risk and high risk prostate cancer by western blotting and again only the fully processed from of Clusterin was identified with no significant detectable difference between the two cancer patients (Figure 3b) with confirmed protein loading.

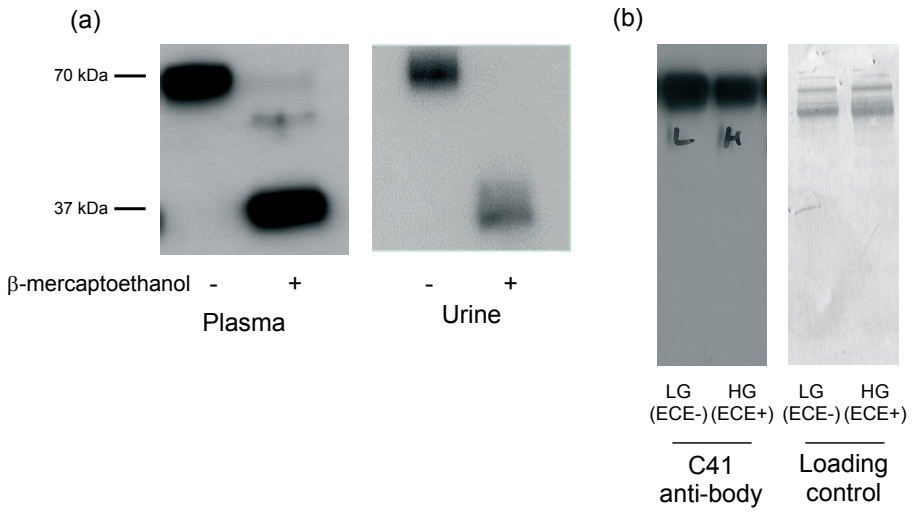

Figure 3: Soluble Clusterin expression in human Plasma and Urine human samples – Western analysis. (a) Plasma and Urine was collected from a single health control volunteer. Plasma was processed using the 2D gel cleanup kit and Urine was concentrated using the centricon tubes. Plasma and Urine protein was prepared for loading by boiling in loading dye with and without the β-mercaptoethanol and assessed for sCLU by western blotting (b) Plasma was collected from two patient groups: LG(ECE-) and HG(ECE+) and processed using the 2D gel cleanup kit. Plasma protein was prepared for loading by boiling in loading dye and assessed for sCLU by western blotting.

There was also no difference in the mass of the Clusterin protein across the patients.

Detection of Clusterin in human plasma from prostate cancer patients. As western blotting is only semi-quantitative next we used the in-house ELISA to assess Clusterin expression across the two prostate cancer patient cohorts. Figure 4 demonstrates a significant (p=0.012) increase in Clusterin between the patients with low risk and no extracapsular extension and the patients with high risk disease with extracapsular extension.

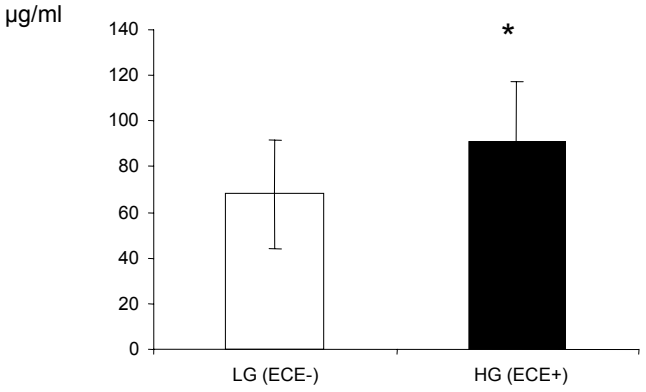

Figure 4: Soluble Clusterin expression in patients with low and high grade prostate cancer – ELISA analysis. Plasma samples collected from 11 patients with low grade Gleason 5 (3+2) without extra-capsular extension [LG(ECE-)] and 11 patients with high grade Gleason 7 (4+3) with extra-capsular extension [HG(ECE+)] were analysis by ELISA. Plasma was processed using the 2D gel cleaning kit protein assayed for protein concentration and 20µg of each protein was assessed for Clusterin expression . Statistical analysis was carried out using the student t-test and *p=0.012.

Discussion

Clusterin, in its secreted form, is a ubiquitously expressed protein. Its primary function is to act as a molecular chaperone, protecting the cell by binding to misfolded proteins and preventing against cellular dysfunction. [11]. The α-helixes situated at both ends of the α- and β-chains of the protein define the active binding portion of sCLU [10].

sCLU is associated with cancer cell survival by binding to the activated complement or death receptors such as TRAIL [12] preventing the activation of the cell death pathway. More recently it has been shown that sCLU binds to Paclitaxel [13] and prevents its action. sCLU has also been shown to impair indirectly the action of Docetaxel through the activation of STAT1 [14], an effect which can be reversed when the Clusterin gene is knocked down. sCLU may also mediate its anti-apoptotic effects intracellularly [15] and can prevents BAX activation and mitochondrial membrane disruption [6, 16].

Normally, sCLU, is fully processed as a glycolysed protein and is detected extracellularly [4, 17]. Our current study demonstrated that only the fully processed and glycosylated form of sCLU was detected in the medium of cell cultures. This was clearly demonstrated by incubation with β-mercaptoethanol were sCLU in the medium can be reduced to a 30kDa protein, following breakage of the five disulphide bonds. Previous studies have however detected sCLU intracellularly. The study by Pucci et al. in colon cancer cells has demonstrated the expression of sCLU intracellularly in more undifferentiated cancer cells [18], opposite results were showen in the study by Scaltriti et al. who found that in prostate cancer clusterin expression was confined to the border of the tumor where the

stroma was disrupted by the cancer cells. In that study, the authors stress that the use of different antibodies could change the results of the experiments and that variation in the affinity for certain isoforms of clusterin may be at the origins of such observations [19].

Following our confirmation that the C41 D antibody could detect sCLU in the medium of prostate cancer cells we next wanted to develop an ELISA with the C41 D antibody to quantitatively measure sCLU. Previous studies have measured sCLU in bladder cancer [20, 21] and as a marker of renal failure using a commercially available ELISA kit [22] but there was no evidence presented that this antibody detects the fully processed sCLU protein.

Using our in-house developed ELISA for sCLU we are able to quantify for the first time sCLU in the medium of the prostate cancer cells and demonstrate an increase in the expression of sCLU between the benign PwR-1E prostate epithelial cells versus the androgen independent PC-3 prostate cancer cells in which the expression was not shown using the semi-quantitative western blotting technique. We also demonstrated using the in-house ELISA an increase in sCLU in the medium of Docetaxel resistant PC-3 cells versus the age-matched controls as we had shown by western blotting.

Next we wanted to demonstrate that the ELISA could detect sCLU in the plasma of a control man and patients with different grades of prostate cancer which was confirmed by western blotting. In the patient study we demonstrated that plasma from the HG group had a significantly higher level of sCLU than plasma from patients in the LG group. It has been demonstrated before that sCLU in the sera of prostate cancer patients would not change regarding the Gleason grade of the tumor [23]. These are the first findings to demonstrate an increase in plasma sCLU levels correlated with extracapsular extension and demonstrate sCLU as a potential plasma biomarker for prostate cancer. Currently, PSA is the only diagnostic tool for prostate cancer [1] but is limited by it specificity to prostate cancer and cannot predict aggressiveness of disease such as increased Gleason grade or extracapsular extension. The ability of sCLU to distinguish between organ confined and non-organ confined disease would have significant implication for determining the suitability of a patient for surgical treatment options.

In conclusion, our findings show that sCLU in its fully functional form is secreted from prostate cancer cells which can be detected by western blotting but more sensitively by an in-house ELISA that we have developed. We have also demonstrated that using both Western blotting and the in-house ELISA we can detect sCLU in the plasma and urine of normal and prostate cancer patients and that there is an increase in sCLU in the plasma of patients with higher grade disease with extracapsular extension as compared to patients with lower grade disease without extracapsular extension. We propose that plasma levels of sCLU may be a novel prognostication marker for pathological features of prostate cancer but will most likely have to be combined with other protein markers to more accurately predict disease and its outcome.

Acknowledgements. This project was funded by a grant from the Irish Cancer Society as part of the Prostate Cancer Research Consortium. Frederic Girard was funded by the European Association of Urology for a one year Scholarship program

References

[1] HEIDENREICH A, AUS G, BOLLA M, JONIAU S, MATVEEV VB, et al. European Association of Urology. EAU guidelines on prostate cancer. Eur Urol. 2008; 53: 68–80 doi:10.1016/j.eururo.2007.09.002

[2] BYRNE JC, DOWNES MR, O'DONOGHUE N, O'KEANE C, O'NEILL A et al. 2D-DIGE as a strategy to identify serum markers for the progression of prostate cancer. J Proteome Res. 2009; 8: 942–57. doi:10.1021/pr800570s

[3] COCHRANE DR, WANG Z, MURAMAKI M, GLEAVE ME, NELSON CC. Differential regulation of clusterin and its isoforms by androgens in prostate cells. J Biol Chem. 2007; 282(4): 2278–87. doi:10.1074/jbc.M608162200

[4] KAPRON JT, HILLIARD GM, LAKINS JN, TENNISWOOD MP, WEST KA. Identification and characterization of glycosylation sites in human serum clusterin. Protein Sci. 1997; 6: 2120–33. doi:10.1002/pro.5560061007 143570

[5] AMMAR H, CLOSSET JL. Clusterin activates survival through the PI-3 kinase/Akt pathway. J. Biol. Chem. 2008; 283: 12851–12861. doi:10.1074/jbc.M800403200

[6] ZHANG H, KIM JK, EDWARDS CA, XU Z, TAICHMAN R et al. Clusterin inhibits[apoptosis by interacting with activated bax. Nature Cell Biology 2005; 7: 909–915. doi:10.1038/ncb1291

[7] YANG CR, LESKOV K, HOSLEY-EBERLEIN K, CRISWELL T, PINK JJ et al. Nuclear clusterin/XIP8, an x-ray-induced Ku70-binding protein that signals cell death. Cell Biologie 2000; 97: 5907–5912.

[8] LESKOV KS, DMITRY Y, BOOTHMAN, KINSELLA TJ, BOOTHMAN DA. Synthesis and Functional Analyses of Nuclear Clusterin, a Cell Death Protein. The Journal of Biological Chemistry 2003; 278: 11590–11600. doi:10.1074/jbc.M209233200

[9] CACCAMO AE, DESENZANI S, BELLONI L, BORGHETTI AF, BETTUZZI S. Nuclear clusterin accumulation during heat shock response: implications for cell survival and thermo-tolerance induction in immortalized and prostate cancer cells. J Cell Physiol. 2006; 207: 208–19. doi:10.1002/jcp.20561

[10] ROBERT W, BAILEY A, DUNKER K, GARNER EC, GRISWOLD MD. Clusterin, a[binding protein with a molten globule-like region. Biochemistry 2001; 40: 11828–840. doi:10.1021/bi010135x

[11] TROUGAKOS IP, GONOS ES. Clusterin/Apolipoprotein J in human aging and cancer. The international journal of biochemistry & Cell biology 2002; 34: 1430–1448. doi:10.1016/S1357-2725(02)00041-9

[12] YOO J, PARK SS, LEE YJ. Pretreatment of docetaxel enhances TRAIL-mediates apoptosis in prostate cancer cells. J Cell Biochem. 2008; 104: 1636–46. doi:10.1002/jcb.21729

[13] PARK DC, YEO SG, WILSON MR, YERBURY JJ, KWONG J. Clusterin interacts with Paclitaxel and confer Paclitaxel resistance in Ovarian cancer. Neoplasia. 2008; 10: 964–72.

[14] PATTERSON SG, WEI S, CHEN X, SALLMAN DA, GILVARY DL. Novel role of STAT1 in the development of docetaxel resistance in prostate tumor cells. Oncogene 2006; 6113–6122.

[15] NIZARD P, TETLEY S, LE DRÉAN Y, WATRIN T, LE GOFF P. Stress-Induced retro translocation of Clusterin /ApoJ into the Cytosol. Traffic 2007; 8: 554–565. doi:10.1111/j.1600-0854.2007.00549.x

[16] MARTINEAU JC, GREEN DR. Breaking the mitochiondrial barrier. Nature reviews, Molecular cell biol. 2001; 2: 63–67. doi:10.1038/35048069

[17] STEWART EM, AQUILINA JA, EASTERBROOK-SMITH SB, MURPHY-DURLAND D, JACOBSEN C. Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. Biochemistry 2007; 46: 1412–22.

[18] PUCCI S, BONANNO E, PICHIORRI F, ANGELONI C, SPAGNOLI LG. Modulation of different clusterin isoforms in human colon tumorigenesis. Oncogene 2004; 23: 2298–304. doi:10.1038/sj.onc.1207404

[19] SCALTRITI M, BRAUSI M, AMOROSI A, CAPORALI A, D'ARCA D et al. Clusterin (SGP-2, ApoJ) expression is down-regulated in low- and high-grade human prostate cancer.Int J Cancer. 2004 Jan 1; 108: 23–30. doi:10.1002/ijc.11496

[20] KRÜGER S, MAHNKEN A, KAUSCH I, FELLER AC. Value of clusterin immunoreactivity as a predictive factor in muscle-invasive urothelial bladder carcinoma. Urology 2006; 67: 105–109. doi:10.1016/j.urology.2005.04.043

[21] STEJSKAL D, FIALA RR. Evaluation of serum and urine clusterin as a potential tumor marker for urinary bladder cancer. Neoplasma. 2006; 53: 343–6.

[22] SOLICHOVAA P, KARPISEK M, OCHMANOVA R, HANULOVA Z, HUMENANSKA V et al. Urinary clusterin concentrations A possible marker of nephropathy ? Pilot Study Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2007; 151: 233–236.

[23] MORRISSEY C, LAKINS J, MOQUIN A, HUSSAIN M, TENNISWOOD M. An antigen capture assay for the measurement of serum clusterin concentrations. Biochem Biophys Methods. 2001 Mar 28; 48: 13–21. doi:10.1016/S0165-022X(00)00137-8

[24] BROWN JA, RODIN DM, HARISINGHANI M, DAHL DM. Impact of preoperative endorectal MRI stage classification on neurovascular bundle sparing aggressiveness and the radical[prostatectomy positive margin rate. Urol Oncol. 2009; 27: 174–9. doi:10.1016/j.urolonc.2008.04.009