Urinary proteomics and the role of orosomucoid (ORM) in vascularization of bladder cancer

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To my family and

nephew Yusuf Heja

Ever tried. ever failed.
Try again, fail again.
Fail better…
Samuel Beckett

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Summary

2-dimensional gel electrophoresis (2-DE) is currently the method of choice for separation of complex protein mixtures such as in cell and tissue extracts or body fluids including urine or serum. Human urine plays a central role in clinical diagnostics of diseases such as cancer and inflammation. Researchers and scientists are working on the development of a map of the human proteome but in the literature there are only a few investigations about urinary proteomics and their behaviour in the case of bladder tumor. The first aim of this study was to view the whole proteins present in urine and to predict their functions. For this aim, fourtyfive urine samples from patients with bladder cancer of different stages, patients on follow-up and healthy volunteers were analysed by 2-DE. At the beginning of this study the 2-DE protein pattern of human urine was almost unknown because of lack of appropriate preparation of urine samples prior to the 2-DE. Thus, the study focused on methods for preparation of urine samples providing the best preservation of urinary proteins. After experimental optimization of 2-DE, usable 2-D protein patterns of urine were analysed to identify proteins related to bladder cancer using subsequent mass spectrometric analyses and/or conventional immunoblotting and immunohistochemical methods. In comparison to other cancer types, the bladder carcinoma is the seventh causing death in man and ninth in women among malignant tumors. The growth and metastasis as well as the transformation of superficial non-invasive tumors to an invasive tumor phenotype are closely associated with activation of angiogenesis resulting in neovascularisation of tumor tissue. This process is regulated by a net balance between angiogenic activators and inhibitors.

In the present study, orosomucoid (ORM) and human zinc-alpha-2-glycoprotein (ZAG) have been identified to be increased in 2-DE of urine samples of patients with bladder cancer in comparison to the urine samples of healthy volunteers. Immunohistochemical results let assume that in addition to cancer cells also a part of the tissue resident inflammatory cells and endothelial cells of tumor associated blood vessels may serve as source for this increase of ORM in urine samples of patient with bladder cancer. ORM is an acute phase protein and increased in acute infection, inflammation, and cancer. Recent studies show that ORM forms a complex with the active form of plasminogen activator inhibitor-1 (PAI-1) in thymosin β4 (Tβ4)-activated but not in quiescent endothelial cells. Therefore it was aimed to determine a potential role of ORM up-regulation in angiogenesis. The findings presented in this

study and results reported by Sorensson showed, that, in addition to the cancer cells, human vascular endothelial cells (HDMECs) produce ORM endogenously. For functional characterization of ORM in vascular endothelial cells, ORM-gene overexpression and ORM-gene silencing were perfromed. Employing the supernatants of HDMECs-ORM and HDMECs-ORM-siRNA in *in-vitro* angiogenesis assays, it was found that ORM supports the VEGF-induced endothelial tube formation. This supportive effect of ORM was potentiated by co-treatment of HDMECs with VEGF, ORM and anti-PAI-1 antibody.

This study demonstrates for the first time that ORM is increased in urine of patients with bladder cancer and acts pro-angiogenic supporting the tube forming effects of VEGF. This supportative effect was significantly increased by additive blockage of PAI-1.

In summary, the interaction between ORM and PAI-1 and anti-PAI-1 system seems to be essentially involved in the VEGF-mediated angiogenesis and probably in the vascularisation of urinary bladder cancer.

Zusammenfassung

Bei der 2D-Elektrophorese (2-DE) handelt es sich zurzeit um die Methode der Wahl für die Trennung komplexer Proteinzusammensetzungen von Zell- und Gewebeextrakten sowie Körperflüssigkeiten wie Urin oder Serum. Die Untersuchung von Urinproben stellt eine zentrale Rolle in der Diagnostik von Krankheiten wie Krebs und Entzündungen dar. Wissenschaftler arbeiten an der Entwicklung eines humanen Gesamtproteomenmusters, in der Literatur sind jedoch nur wenige Untersuchungen zu Urinproteomics und ihrem Muster beim Harnblasenkarzinom beschrieben. Die Zielsetzung dieser Arbeit war die Untersuchung des Proteinmusters im Urin von Harnblasenkarzinompatienten im Vergleich zu normalen Patienten. Dazu wurden 45 Urinproben von Harnblasenkarzinompatienten, Follow-up-Patienten und gesunden Probanden mittels der 2D-Elektrophorese analysiert. Zu Beginn dieser Experimente war das 2-DE Proteinmuster in Urinproben aufgrund fehlender angemessener Vorbehandlung der Urinproben für die 2D-Elektrophorese kaum bekannt. Daher waren die Untersuchungen zunächst auf Methoden zur Aufbereitung der Urinproben, die die bestmögliche Proteinerhaltung gewährleisten, fokussiert. Nach experimenteller Optimierung der 2D-Elektrophorese wurden 2D-Proteinmuster der Urinproben analysiert, um mittels massensprektrometrischer Analysen, Western Blot-Analysen und Immunhistochemie Proteine zu identifizieren, die mit Harnblasenkarzinomen assoziiert sind. Beim Harnblasenkarzinom handelt es sich um die siebthäufigste Krebstodesursache beim Mann und die neunthäufigste Krebstodesursache bei der Frau. Harnblasentumorwachstum- und metastasierung sowie der Übergang von einem oberflächlichen, nicht-invasiven in einen invasiven Tumorphänotyp sind stark verknüpft mit der Aktivierung der Angiogenese und somit der Tumorvaskularisierung. Dieser Prozess wird geregelt durch ein Gleichgewicht zwischen angiogenetischen Aktivatoren und Inhibitoren.

In der vorliegenden Arbeit wurden Orosomucoid (ORM) und humanes Zink-alpha-2-Glykoprotein (ZAG) anhand der 2D-Elektrophorese als im Urin von Harnblasenkarzinompatienten im Vergleich zu normalen Urinproben erhöhte Markerproteine identifiziert. Immunhistochemische Untersuchungen lassen vermuten, dass zusätzlich zu Tumorzellen auch ein Teil der gewebaansässigen inflammatorischen Zellen und Endothelzellen tumorassoziierter Blutgefäße die Quelle für den ORM-Anstieg in Urinproben von Harnblasenkarzinompatienten darstellen könnten. Bei ORM handelt es sich um ein Akute-Phase-Protein, welches bei akuten

Infektionen, Entzündungen und Tumorerkrankungen erhöht ist. In neueren Publikationen konnte gezeigt werden, dass ORM einen Komplex mit der aktiven Form von Plasminogen-Aktivator-Inhibitor-1 (PAI-1) in Thymosin- β4 (T β4)-aktivierten, aber nicht in ruhenden Endothelzellen eingeht. Unsere weitergehenden Untersuchungen zielten auf die Ermittlung einer möglichen Rolle von ORM in der Angiogenese. Sowohl die hier präsentierten Ergebnisse als auch Daten der Arbeitsgruppe Sorensson zeigen, dass neben Tumorzellen auch humane vaskuläre Endothelzellen (HDMECs) ORM endogen exprimieren. Zur funktionellen Charakterisierung von ORM in Endothelzellen wurde ORM überexprimiert und gesilenct. Anhand von *in-vitro*-Angiogeneseassays mit Überständen ORM-überexprimierender und –gesilencter HDMECs konnte gezeigt werden, dass ORM die VEGF-induzierte endotheliale Tubeformierung steigert. Diese unterstützende Wirkung von ORM konnte potentziert werden durch simultane Behandlung der HDMECS mit VEGF, ORM und PAI-1-Antikörper.

Die Daten zeigen erstmalig, dass ORM erhöht ist in Urinproben von Harnblasenkarzinompatienten und proangiogenetisch wirkt durch die Verstärkung des tubeformierenden Effektes von VEGF. Dieser unterstützende Effekt konnte signifikant gesteigert werden durch zusätzliche Blockade von PAI-1.

Zusammenfassend scheint die Interaktion zwischen ORM und PAI-1 und dem Anti-PAI-1-System entscheidend involviert zu sein in die VEGF-vermittelte Angiogenese und möglicherweise in die Vaskularisierung des Harnblasenkarzinoms.

Table of Abbreviations

2-DE	2 dimensional gel electrophoresis
ABC	Avidin-biotin complex
AJCC	American Joint Committee on Cancer
APS	Ammonium per sulphate
BLAST	Basic local alignment search tool
BM	Basement membrane
CBB	Coomassie blue brillant
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DAB	3,3'-Diaminobenzidin-tetrahydrochloride
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylesulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleosid triphosphate
DTT	Dithiothreitol
EC	Endothelial cells
E.coli	Escherichia coli
EDTA	Ethylendiamine tetraacetate
EMBL	European Molecular Biology Laboratory
FCS	Fetal calf serum
FGF	Fibroblast growth factor
h,min,sec	Hour, minute,second
HDMECs	Human dermal microvascular endothelial cells
HUVEC	Human umbilical cord vein endothelial cells
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kb	Kilobasepairs
kDa	Kilodalton
l	Liter
LB	Luria broth
M	Mol/ Liter
mA	Miliamper

MALDI	Matrix assisted laser desorpsion/ ionization
MALDI-TOF	Matrix-assisted laser desorpsion/ ionization-time of flight
MEM	Minimum essential medium
mg	Milligram (=10^{-3}g)
ml	Milliliter (=10^{-3}l)
µg	Microgram (=10^{-6}g)
µl	Microliter (=10^{-6}l)
mM	Millimol/ Liter
mmol	Millimol (=10^{-3}mol)
mRNA	Messenger RNA
MS	Mass spectrometry
Mu	Mass unit
m/z	Mass to charge ratio
MVB	Multivesicular body
NCBI	National Center for Biotechnology Information
ng	Nanogram
OD	Optical density
ORM	Orosomucoid
PAI	Plasminogen activator inhibitor
PAP	Peroxidase-antiperoxidase
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gelelectrophoresis
siRNA	Small interfering RNA
TAE	Tris-acetate-EDTA
TBP	Tributyle phosphin

TBS-T	Tris buffered saline with tween
TE	Tris-EDTA
TEMED	Tetramethylethylendiamin
TFA	Trifluoroacetic acid buffer
TFB	Transformation Buffer
TRIS	Tris(hydroxymethyl)aminomethane
U	Units
UV	Ultra violate
V	Volt
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vh	Volt-hour
WHO	World Health Organization
Wt	Wild-type
ZAG	Human zinc-alpha-2-gylcoprotein

1 Introduction

The field of proteomics has become an increasingly important part of life sciences, especially after completion of the human genome sequence. Proteome analysis includes separation, identification, and quantitation of proteins from biological samples with the purpose of revealing the function of living cells. Applications range from prognosis of many types of cancer over drug development to monitoring of environmental pollution.

The study of genes cannot provide much information on the properties of proteins, because the molecules responsible for cellular functions (e.g. signal transduction) are proteins. Proteins may impose more than 200 different types of post-translational modification, including phosphorylation, glycosylation, acetylation, deamination, farnesylation, myristolation, palmitoylation, and proteolysis (Krishna and Wold, 1993). Such a wide range of modifications cannot be predicted purely from DNA sequences. Only through the study of proteins themselves their characteristics and functions can be expounded. From the data obtained from studies of the genome project, an estimated number of proteins encoded by the genome are known. However, it is difficult to predict the actual numbers of proteins encoded based on genomic data, for a number of reason (Eisenberg et al., 2000). Firstly, the exon-intron cannot be accurately predicted from genomic DNA (Dunham et al., 1999) i.e. genomic information needs to be integrated with data obtained from protein studies to confirm the presence of a specific gene. Secondly, alternative splicing of a transcript can yield more than one protein product (Newman, 1998). Therefore, the direct analysis of mRNA or genome does not reflect the exact number of protein products in a cell. Thirdly, as a result of compartmentalization and translocation, the same protein can be found with different properties and functions in different locations (Fig. 1) (Colledge and Scott, 1999).

These problems can only be solved by proteomics, which can directly identify the proteins and provide the genomic information by the appropriate integration of genomic and proteomic data (Fig. 2). Scientists worldwide are applying proteomic technology to solve problems which cannot be resolved by traditional methods.

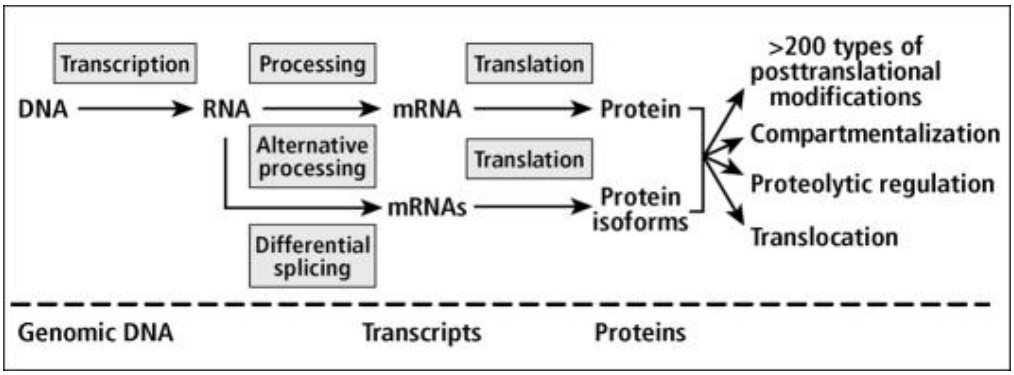


Figure 1: The flow of genomic information to protein products. Different epigenetic processes leading to physically and/or functionally variable forms of protein isoforms from a single gene sequence. The concept of one-gene to one-protein is over-simplified since an RNA can be differentially spliced and can produce various protein products.The protein can be exposed to more than 200 different types of post-translational modifications. Compartmentalization and translocation result in the same protein can be found with different properties and functions in different locations (Lau et al., 2003).

1.1 Proteomics

The term proteome refers to all the proteins expressed by a genome, and thus proteomics involves the identification of proteins in the body and the determination of their role in physiological and pathological processes.

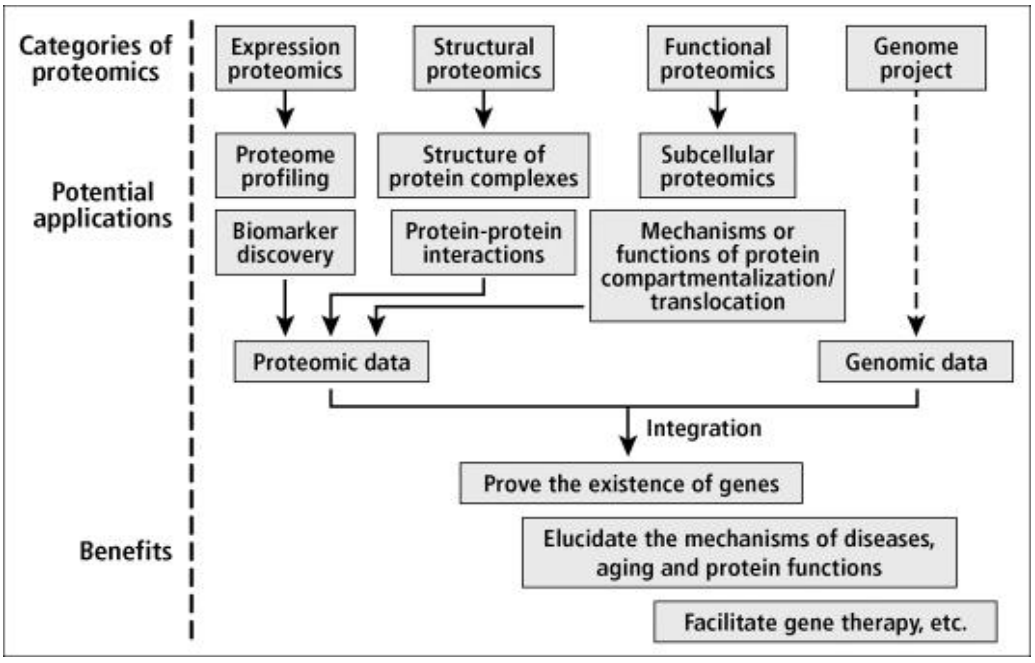


Figure 2: Proteomics and genomics integration. Representation of categories, potential applications of proteomics, and the benefits of integrating proteomic and genomic data (Lau et al., 2003).

The studies of proteomics give a chance for the characterization of all or selected proteins expressed within a given cell and can outline the flow of information within that protein network (Petricoin et al., 2004). It is estimated that more than 500,000 proteins comprise the human proteome, derived from ~35,000 genes in the human genome (Banks et al., 2000; Stein, 2004). Proteomics offer more complexity but potentially more specificity than examining genes alone. The study of proteomics can generally be divided into two categories: (*i*) the characterization of protein expression and (*ii*) the characterization of protein function.

Expression proteomics evaluate cellular protein production encoded by those genes active in a cell and present in the target organ. It exposes the differential expression of proteins between healthy and diseased states. The technologies applied to expression proteomics allow investigation of protein expression in multiple sources including tissue as well as serum and urine.

Functional proteomics evaluate the activation state of and/or interactions between proteins and can be used to map the extensive network of signaling pathways in a cell. Mapping protein-protein interactions can be useful due to discovery of new binding interactions which may give insight into new proteins that are involved in cancer as well as novel oncogenes or provide evidence of common downstream events shared by two distinct signaling networks. Further information of how various pathways intersect can help in developing therapeutic interventions directed against these pathway targets (Cai et al., 2004).

There are several major technologies used in proteomics. The first technology is necessary to separate proteins from each other. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is currently the method of choice for separation of complex protein mixtures such as in cell and tissue extracts or body fluids.

1.2 Technology of proteomics: 2-Dimensional Gelelectrophoresis (2-DE)

2-DE can provide protein databases for qualitative and quantitative analyses of protein expression in a wide range of biochemical, biological and biomedical investigations (Celis et al., 1989). This technique separates proteins in two steps, according to two independent properties: the first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). In this way, complex mixtures

consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined.

1.2.1 Sample preparation

Appropriate sample preparation is essential for good 2–DE results. The optimal procedure must be determined empirically for each sample type. An ideal process should result in complete solubilisation, disaggregation, denaturation, and reduction of the proteins in the sample. It is important to have a clear idea of what is desired in the final 2-DE result when developing a sample preparation strategy. Different treatments and conditions are required to solubilise different types of protein samples; some proteins are naturally found in complexes with membranes, nucleic acids, or other proteins; some proteins form various non-specific aggregates; and some proteins precipitate when removed from their natural environment. The effectiveness of solubilisation depends on the choice of cell disruption method, protein concentration and dissolution methods, choice of detergents, and composition of the sample solution. The composition of the sample solution is particularly critical for 2-DE, because solubilisation treatments for the first-dimension separation must not effect isoelectric point (pI), nor leave the sample in a highly conductive solution. In general, concentrated urea as well as one or more detergents is used.

1.2.2 Protein extraction

The extraction of proteins from a cell requires the disruption of the cell membrane and the solubilization of the proteins contained in the cell and the cell membrane. An extraction buffer should extract all proteins, denature them, and exclude nucleic acids and cell debris. The most successful and widely used solubilising buffers contain chaotropic agents that will disrupt the hydrogen bonding, a combination of several detergents that prevents hydrophobic interactions between hydrophobic side chains and helps solubilize the proteins, and a reducing agent that breaks the disulfide bridges initially formed between two cysteine residues (Chevallet et al., 1998; Tastet et al., 2003). Such detergents will achieve cell disruption, removal of interfering agents, and solubilization and denaturing of proteins (Gorg et al., 2004; Herbert, 1999).

1.2.2.1 Chaotropic agent

The use of urea and thiourea (Fig. 3A,B) increase the solubility of proteins by disrupting the hydrogen bonding (Rabilloud et al., 1997). Urea solubilises and denaturates proteins, unfolding them to expose internal ionisable amino acids. Commonly 8 M urea is used, but the concentration can be increased to 9 or 9.8 M. Using thiourea in addition to urea improves solubilisation, particularly of hydrophobic membrane proteins (Molloy, 2000; Rabilloud, 1998).

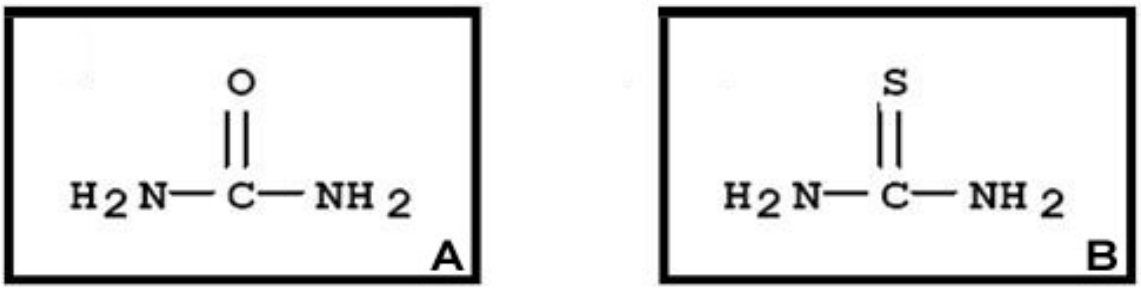


Figure 3: Structure of Urea and Thiourea. Two chaotropic reagents, urea (A) and thiourea (B) used for the protein extraction. The presence of these two reagents in the lysis buffer allows for the solubilization of non-soluble proteins by disrupting the hydrogen bonding that are formed between the oxygen of the carbonyl group of an amino acid with the hydrogen of an amino group of another amino acid.

1.2.2.2 Detergents

Detergents prevent hydrophobic interactions between hydrophobic side chains from occurring and allow for better solubility of proteins. SDS is considered the most efficient detergent for the solubilisation of proteins (Boucherie et al., 1995; Harder et al., 1999), but because of its ionic nature, it may interfere with the first dimension of 2-DE and it has been reported to cause horizontal streaks on the gel (Gorg et al., 2004). Non-ionic detergents like OG (Octylglucopyranoside) and zwitterionic reagents like CHAPS (3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate) are being favoured over SDS use (Kersten et al., 2002; Rabilloud et al., 1997; Rabilloud et al., 1999) (Fig. 4A-C). A combination of several non-ionic and zwitterionic detergents should be used because no one detergent is perfect for the solubilization of most of the hydrophobic proteins (Schuck et al., 2003).

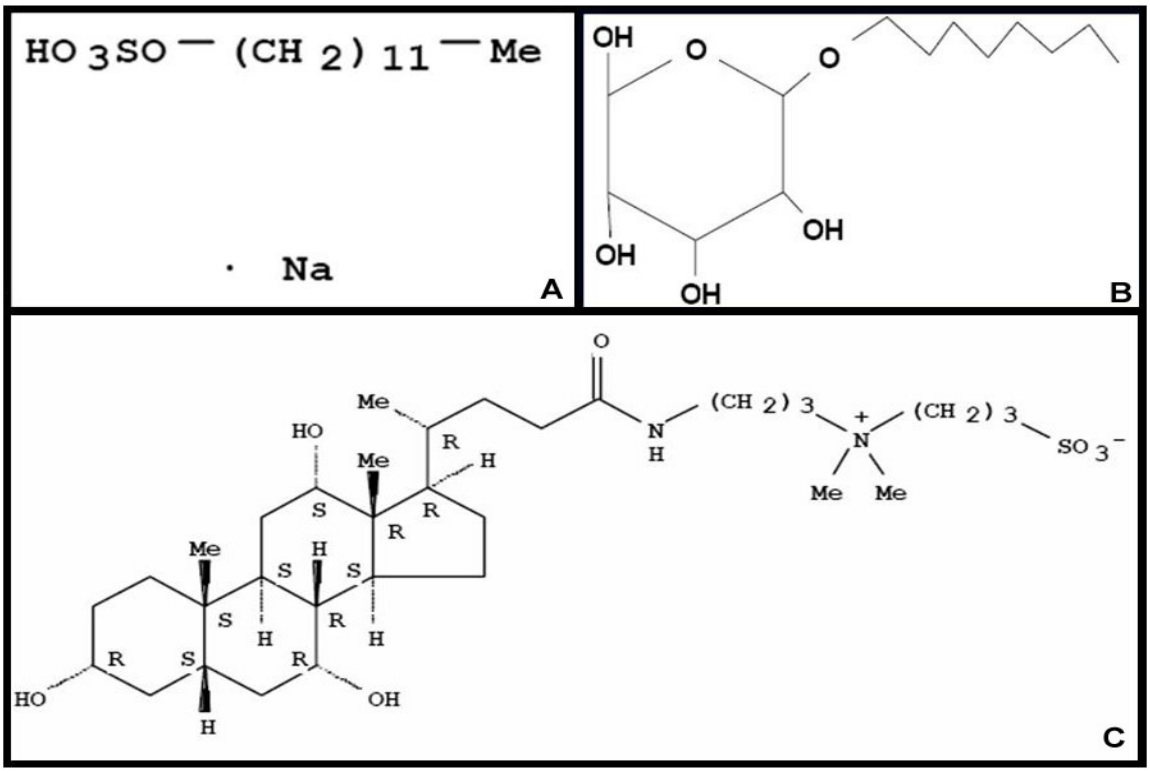


Figure 4: Structure of detergents used in solubilisation of proteins. (A) Sodium Dodecyl Sulfate (SDS), an anion detergent; (B) Octyl glucopyranoside (OG) a non-ionic detergent; and (C) 3-[(3-cholamidopropyl)dimethylamino]–1– propanesulfonate (CHAPS) a zwitterionic detergent.

1.2.2.3 Reducing agents

To obtain well resolved spots, disulfide bridges should be cleaved to achieve total denaturation of the protein. This can be done using a reducing agent like Dithiothreitol (DTT) (Fig. 5).

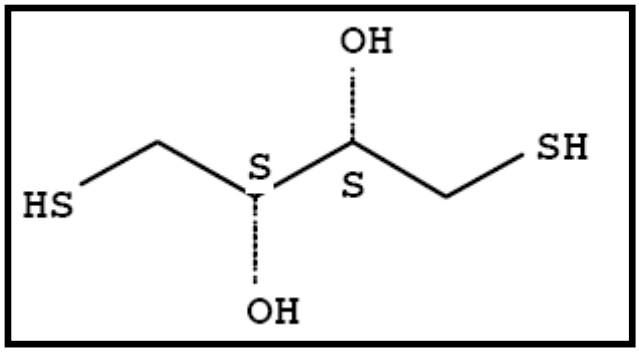


Figure 5: Structure of dithiothreitol (DTT). A reducing agent used to cleave the disulfide bridges that can form between two cysteine residues to achieve complete denaturation of proteins and increase their solubility.

However, DTT is a weak acid which will migrate to the basic region of the IPG strip when the sample is loaded for isoelectric focusing (IEF) and will only reduce proteins

in that region, necessitating the introduction of excess DTT at the cathode (Hoving et al., 2002). Tributyl phosphine (TBP) (Fig. 6) was reported as an alternative reducing agent (Herbert et al., 1998).

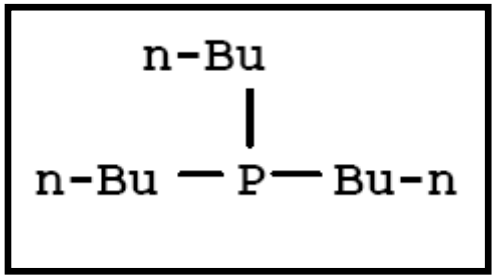


Figure 6: Structure of tributylphosphine (TBP). A reducing agent used in 2-DE process.

1.2.3 First dimension: Isoelectric focusing (IEF)

IEF is an electrophoretic method that separates proteins according to their isoelectric points (pI). Proteins are amphoteric molecules; they carry either positive, negative, or zero net charge, depending on the pH of their surroundings (Fig. 7). The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and aminoand carboxyl-termini. The isoelectric point (pI) is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI. If the net charge of a protein is plotted versus the pH of its environment, the resulting curve intersects the x-axis at the isoelectric point (Fig. 7). The presence of a pH gradient is critical to the IEF technique. In a pH gradient, under the influence of an electric field, a protein moves to the position in the gradient where its net charge is zero. A protein with a positive net charge migrates toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge migrates toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back.

This is the focusing effect of IEF, which concentrates proteins at their isoelectric points and allows proteins to be separated on the basis of very small charge differences.

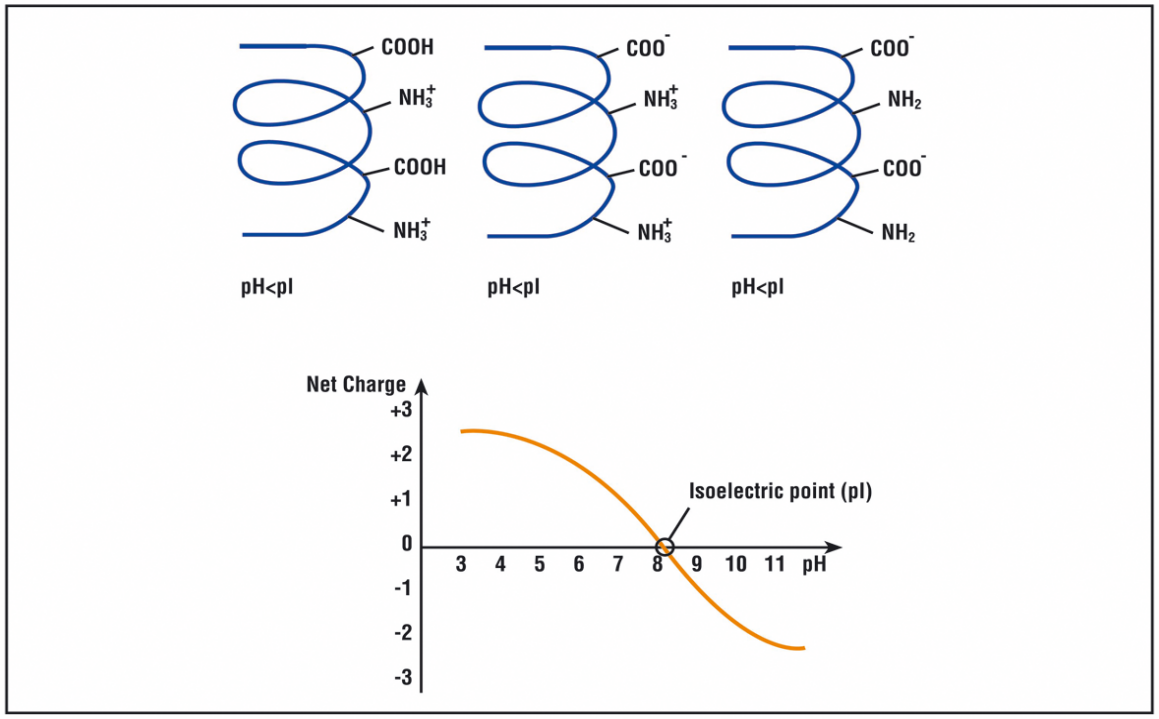


Figure 7: Structures of protein as amphoteric molecules, isoelectric point of proteins.

The resolution is determined by the slope of the pH gradient and the electric field strength. IEF is therefore performed at high voltages (typically in excess of 1000 V). When the proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically below 1 mA). IEF of a given sample in a given electrophoresis system is generally performed for a constant number of Volt-hours (Volt-hour (Vh) being the integral of the volts applied over the time).

IEF performed under denaturing conditions gives the highest resolution and the cleanest results. Complete denaturation and solubilization is achieved with a mixture of urea and detergent, ensuring that each protein is present in only one configuration and aggregation and intermolecular interaction is minimized.

An immobilized pH gradient (IPG) is created by covalently incorporating a gradient of acidic and basic buffering groups into a polyacrylamide gel at the time it is cast. The general structure of Immobiline reagents is:

CH2 = CH–C–NH–RO

R = weakly acidic or basic buffering group.

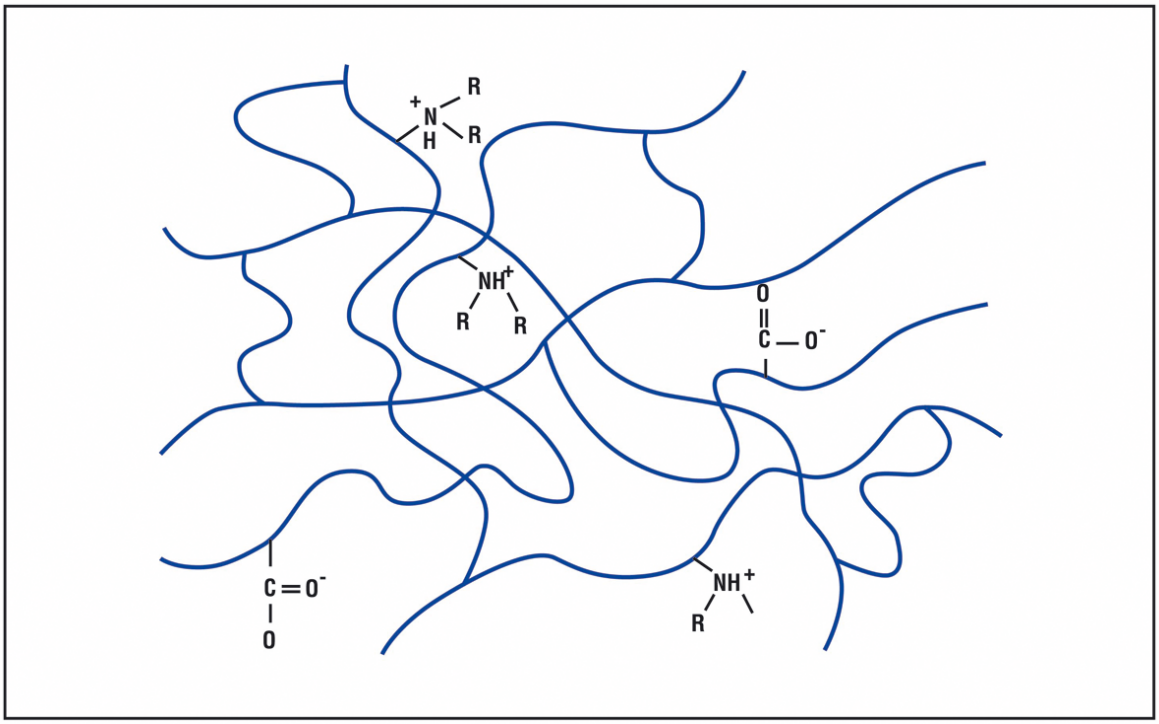


Figure 8: Structure of polyacrylamide gel matrix. Graphical representation of the polyacrylamide matrix with attached buffering groups.

Immobilized pH gradients are formed using two solutions, one containing a relatively acidic mixture of acrylamido buffers and the other containing a relatively basic mixture. The concentrations of the various buffers in the two solutions define the range and shape of the pH gradient produced. Both solutions contain acrylamide monomers and catalysts. During polymerization, the acrylamide portion of the buffers copolymerizes with the acrylamide and bisacrylamide monomers to form a polyacrylamide gel. The graphical representation of polyacrylamide matrix with attached buffering groups is shown in figure 8.

The IEF is the most critical step of the 2-DE process. The proteins should be solubilised without charged detergents, usually in high concentrated urea solution, reducing agents and chaotrophs. To obtain high quality data it is essential to achieve low ionic strength conditions before the IEF it self. Since different types of samples differ in their ion content, it is necessary to adjust the IEF buffer and the electrical profile to each type of sample.

1.2.4 Second dimension: SDS-PAGE

In the second dimension, the protein molecules are separated vertically according to their molecular weight. Sodium dodecyl sulfate treatment denatures proteins (that is, it unfolds them into long, straight molecules) and coats all proteins essentially in proportion to their mass.

The pores of the second dimension gel sift proteins according to size because dodecyl sulphate coats all proteins essentially in proportion to their mass. The coating of the protein in negatively-charged SDS allows the proteins to migrate as ellipsoids with a uniform negative charge-to-mass ratio, with mobility related logarithmically to mass. Proteins are therefore separated horizontally based on their isoelectric point and vertically based on their molecular mass.

SDS is an anionic detergent, that, when in solution in water, forms globular micelles composed of 70–80 molecules with the dodecyl hydrocarbon moiety in the core and the sulfate head groups in the hydrophilic shell. SDS and proteins form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (Ibel et al., 1990). The result of the necklace structure is that large amounts of SDS are incorporated in the SDS-protein complex in a ratio of approximately 1.4 g SDS/g protein. SDS masks the charge of the proteins themselves and the formed anionic complexes have a roughly constant net negative charge per unit mass. Besides SDS a reducing agent such as dithiothreitol (DTT) is also added to break any -S-S-linkages present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein. In fact, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide complex.

The most commonly used buffer system for second-dimension SDS-PAGE is the tris-glycine system described by Laemmli (Laemmli, 1970). This buffer system separates proteins at high pH, which confers the advantage of minimal protein aggregation and clean separation even at relatively heavy protein loads. The Laemmli buffer system has the disadvantage of a limited gel shelflife.

1.2.5 Equilibration

The equilibration step saturates the IPG strip with the SDS buffer system required for the second-dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye. An additional equilibration step replaces the reductant with iodoacetamide. Equilibration introduces reagents essential for the second-dimension separation.

Urea (6 M) together with glycerol reduces the effects of electroendosmosis by increasing the viscosity of the buffer (Gorg et al., 1985). Electroendosmosis is due to the presence of fixed charges on the IPG strip in the electric field and can interfere with protein transfer from the IPG strip to the second-dimension gel.

Glycerol (30%) together with urea reduces electroendosmosis and improves transfer of protein from the first to the second-dimension (Gorg et al., 1985).

DTT preserves the fully reduced state of denatured, unalkylated proteins.

Sodium dodecyl sulfate (SDS) denatures proteins and forms negatively charged protein-SDS complexes. The amount of SDS bound to a protein, and therefore the additional negative charge, is directly proportional to the mass of the protein. Thus, electrophoresis of proteins through a sifting gel in the presence of SDS separates proteins on the basis of molecular mass.

Iodoacetamide alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis. Protein reoxidation during electrophoresis can result in streaking and other artifacts. Iodoacetamide also alkylates residual DTT to prevent point streaking and other silver-staining artifacts. Iodoacetamide is introduced in a second equilibration step. The second equilibration with iodoacetamide is also used to minimize unwanted reactions of cysteine residues (i.e. when mass spectrometry is to be performed on the separated proteins).

Tracking dye (bromophenol blue) allows monitoring of electrophoresis.

1.2.6 Identification of proteins

After first and second dimension the proteins are detected by color, immunoanalysis, or prebound fluorescent dyes, unique spots are quantified by their staining intensity. Spots of interest are excised from gel and subjected to protease digestion, and peptide fragments can be examined using Mass Spectrometry. Mass spectrometry measures the mass-to-charge ratio (m/z) of ionized proteins as they migrate through an electric or a magnetic field. Proteins are analyzed and identified based on unique

spectrometric signatures. These features allow identification of structural features such as phosphorylation or methylation. Trypsin digestion of proteins yields peptides that are amenable to MS sequencing. Coupling MS with MALDI is another method by which proteins can be identified.

1.3 Urinary proteomics

1.3.1 Formation of urine

Urine is formed in the kidney by ultrafiltration from the plasma to eliminate waste products, for instance urea and metabolites. Although the kidney accounts for only 0.5% of total body mass, a large volume of plasma (350-400 ml/100 g tissue/min) flows into the kidney, generating a large amount of ultrafiltrate (150-180 l/day) under normal physiologic conditions. Components in the ultrafiltrate such as water, glucose, amino acids, and inorganic salts are selectively reabsorbed, and less than 1% of ultrafiltrate is excreted as urine. Serum proteins are filtered based on their sizes and charges at the glomeruli (Haraldsson and Sorensson, 2004).

After passing through glomeruli, abundant serum proteins such as albumin, immunoglobulin light chain, transferrin, vitamin D binding protein, myoglobin, and receptor-associated protein are reabsorbed, mainly by endocytic receptors, megalin, and cubilin in proximal renal tubules (Christensen and Gburek, 2004; Maunsbach, 1997). Thus, protein concentration in normal urine is very low (less than 100 mg/l when urine output is 1.5 l/day), and normal protein excretion is less than 150 mg/day. This is about a factor 1000 less compared with other body fluids such as plasma. Excretion of more than 150 mg/day protein is defined as proteinuria and is indicative of glomerular or reabsorption dysfunction.

1.3.2 Sources of urinary proteins

Urinary proteins include soluble proteins and protein components of solid phase elements of urine (Tab. 1). Solid phase elements consist of “sediments” that can be precipitated at low centrifugation speeds and “exosomes” that are of very low density and sediment only with ultracentrifugation. Prefractionation of these components can be useful as a means of enriching for markers of particular types of disease. A study of urine collected from normal human adult subjects indicated that, of the total urinary protein excreted, 48% was contained in sediments, 49% was soluble, and the remaining 3% was in exosomes (Zhou et al., 2006).

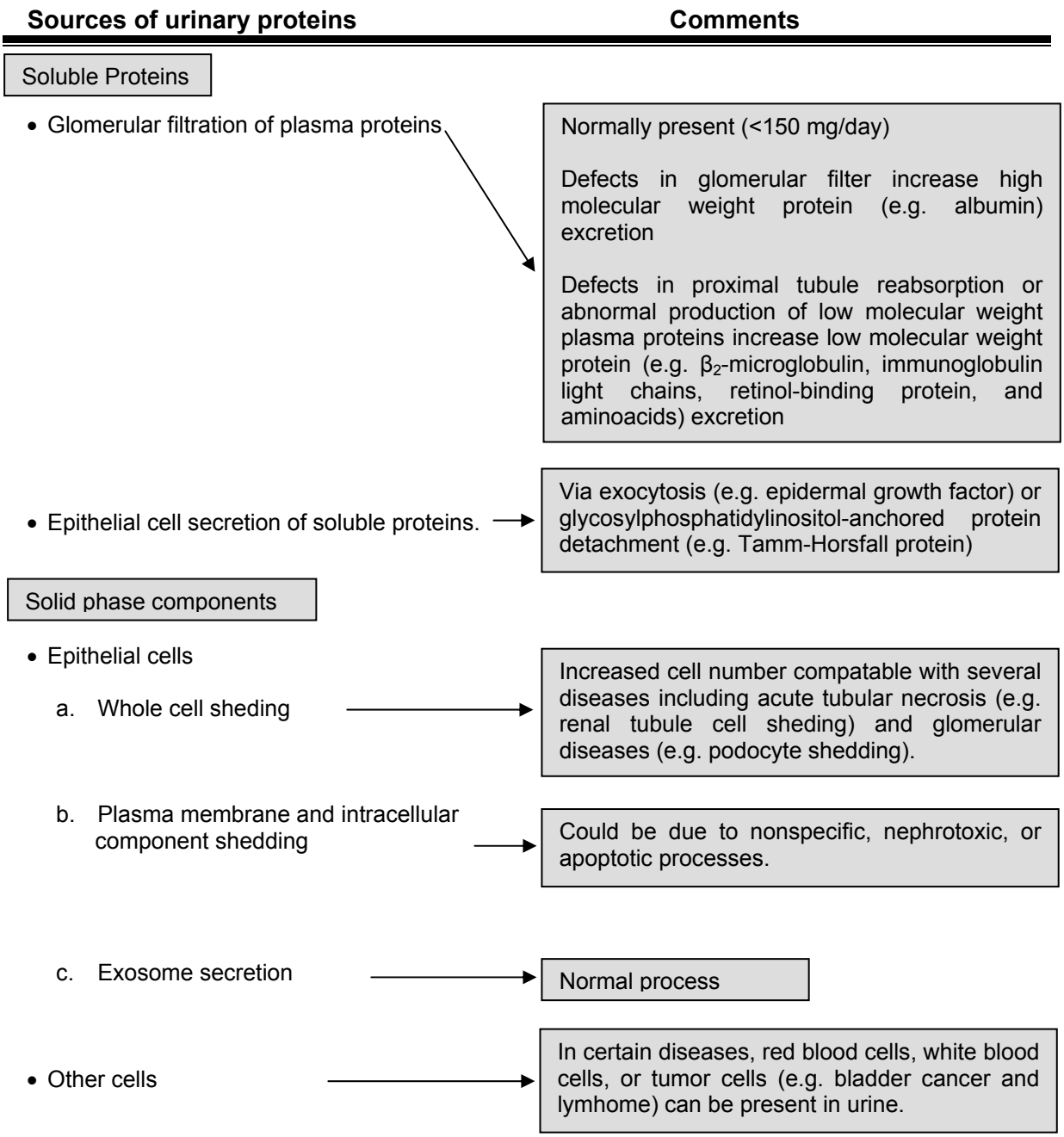


Table 1: Sources of urinary proteins. (Pisitkun et al., 2004).

The soluble proteins in urine are derived largely from glomerular filtration. The glomerular filter effectively retards passage of high molecular weight proteins. However, even with very low sieving coefficients, proteins that are abundant in the blood plasma such as albumin and various globulins can pass the glomerular filter in substantial amounts to enter the lumen of the nephron. Beyond this, peptides and small proteins (10 kDa) are freely filtered by the glomerulus. Most of the proteins and peptides that pass the glomerular filter are scavenged and proteolyzed in the proximal tubule by highly specialized apical uptake processes that involves receptor-

like recognition of the polypeptide molecules (Christensen and Birn, 2001; Christensen, 2002). Thus, a change in the amount of a given soluble protein that reaches the final urine can result from a change in its concentration in the blood plasma, a change in the function of the glomerular filter, or an alteration in the proximal tubule scavenging system (Fig. 9).

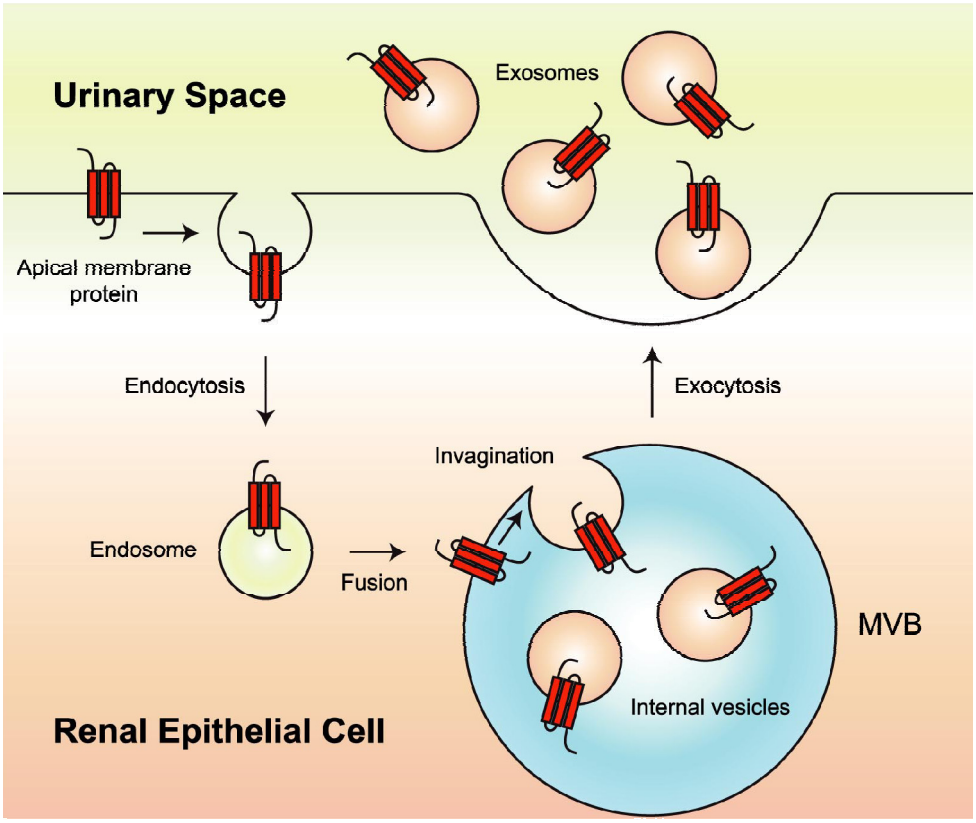


Figure 9: Mechanism of urinary exosome formation and excretion. Apical membrane proteins undergo endocytosis followed by targeting to the.multivesicular body (MVB). The membrane proteins are segregated initially in the MVB outer membrane and then are internalized by membrane invagination, encapsulating cytosolic proteins in the process. After accumulation of numerous internal vesicles, the outer membrane of the MVB fuses with the apical plasma membrane releasing its internal vesicles, called exosomes, into the urinary space. Exosomes contain both membrane and cytosolic proteins (Hoorn et al., 2005).

Based on these mechanisms, changes in excretion rate of specific urinary proteins can be indicative of systemic disease, glomerular disease, or diseases affecting the proximal tubule, respectively. Some of the soluble proteins in urine originate as membrane-bound proteins that are proteolytically cleaved from their membrane attachments.

1.4 Bladder cancer

Bladder cancer is one of the most common cancer type occuring worldwide. The rate for these tumors is highest in developed countries, where they rank as the sixth most frequent neoplasm. The disease currently ranks as the fourth most common cause of cancer death in men and the eighth in women. Estimated new cases are 63,210 and deaths from bladder cancer are 13,180 in the United States in 2005 (American Cancer Society, Atlanta, 2005). The incidence of bladder cancer increases with age and is particularly high after age of sixty and is uncommon before age fourty. The urinary bladder has a flat, smooth, shiny, watertight lining consisting of layers of cells tightly connected with each other. The lining of the bladder can be imagined to be similar to the lining in the oral cavity (mouth). Underneath this lining is the muscle tissue of the bladder. The muscle is responsible for pushing out the urine at the time of voiding. The capacity of bladder is around 350-400 cc in adults. The bladder lies in the pelvis anterior and inferior to the peritoneal cavity, and posterior to the pubic bones. In the female, it rests directly on the muscular pelvic floor, while in the male the prostate gland is attached directly to the base of the bladder and separates it from its muscular support.

Bladder cancer is an abnormal growth or tumor arising from the lining of the bladder. The cells grow abnormally fast causing a tumor to sprout up from the flat lining into a growth projecting into the interior of the bladder cavity. Bladder cancer occurs within the domain of human neoplasms by many different types of etiological factors such as some aromatics (i.e. xylamine, β-naphtylamine, benzydine, 4-nitro diphenyl), coffee, artificial sweeteners and smoking (Cohen et al., 1979; Morrison et al., 1982; Wynder and Stellman, 1977). Most of bladder cancers present as superficial disease and are amenable to local excision by endoscopic resection. The rate of recurrence ranges from 50% to 75% and the rate of progression in stage is from 15% to 25%. Cystoscopy is the gold standard for routine surveillance for bladder tumor recurrence, with biopsy of suspicious lesions and histopathologic evaluation required to confirm the diagnosis (Tsihlias and Grossman, 2000).

1.4.1 Staging of bladder cancer

The clinical staging of carcinoma of the bladder is determined by the depth of invasion of the bladder wall by the tumor (Fig. 10). Based on morphologic evaluation and natural history, urothelial neoplasms have been classified into two groups having

distinct behavior and prognosis; (*i*) low-grade tumors (always papillary and usually superficial) and (*ii*) high-grade tumors (either papillary or non-papillary and often invasive). Clinically, superficial bladder tumors (stages Ta, Tis, and T1) account for 75% to 85% of neoplasms, whereas the remaining 15% to 25% are invasive (T2, T3, T4) or metastatic (N+, M+) lesions at the time of initial presentation (Rabbani and Cordon-Cardo, 2000). The American Joint Committee on Cancer (AJCC) has designated staging by TNM classification to define bladder cancer (American Joint Committee on Cancer, 2002) (Tab. 2). AJCC stage grouping is shown in table 3.

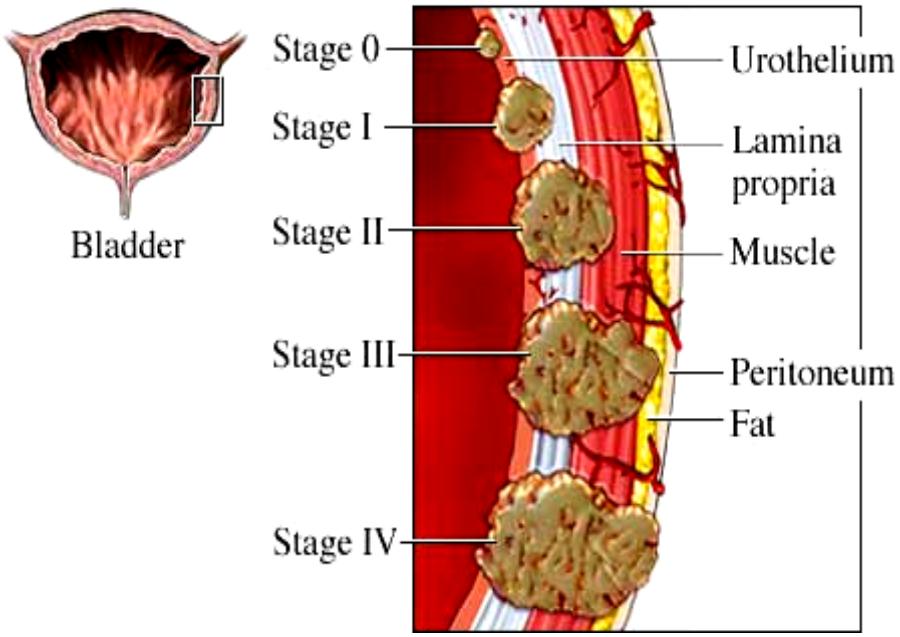


Figure 10: Representation of bladder cancer stages. Scheme for bladder tumor classification based-on the depth of tumor invasion into the bladder wall (From Hautmann and Huland, Urology, Springer-Publisher).

1.4.2 Cellular classification

More than 90% of bladder carcinomas are transitional cell carcinomas derived from the uroepithelium. About 6% to 8% are squamous cell carcinomas and 2% are adenocarcinomas (Mostofi et al., 1988). Adenocarcinomas may be either of urachal origin or of nonurachal origin; the latter type is generally thought to arise from metaplasia of chronically irritated transitional epithelium (Wilson et al., 1991). Pathologic grade, which is based on cellular atypia, nuclear abnormalities, and the number of mitotic figures, is of great prognostic importance. Resection of the tumor in his entirety, with inclusion of the lamina propria and muscularis propria, and biopsy of

all endoscopically suspicious areas (i.e., nontraumatized erytematous areas) are generally performed to ensure proper staging.

TNM Definitions	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma in situ (i.e., flat tumor)
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
pT2a	Tumor invades superficial muscle (inner half)
pT2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
PT3a	Microscopically
PT3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following ; prostate, uterus, vagina, pelvic wall, or abdominal wall
PT4a	Tumor invades the prostate, uterus, vagina
PT4b	Tumor invades the pelvic wall, abdominal wall
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
NO	No regional lymph node metastasis
N1	Metastasis in a single lymph node, =2 cm in greatest dimension
N2	Metastasis in a single lymph node, >2 cm but =5 cm in greatest dimension; or multiple lymph nodes, =5 cm in greatest dimension
N3	Metastasis in a lymph node, >5 cm in greatest dimension
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
MO	No distant metastasis
M1	Distant metastasis

Table 2: Staging of bladder cancer. Definition of bladder cancer which was designed by American Joint Committee on Cancer (AJCC) using TNM classification. (Lamm, 1994).

Directed biopsies may be performed to evaluate adjacent or distant mucosa if urine cytology is positive. Biopsy of the prostatic urethra to evaluate its involvement in the overall tumor diathesis (especially in the setting of CIS) is also important (Lee and Droller, 2000).

AJCC stage groupings	
Stage 0a	Ta, N0, M0
Stage 0is	Tis, N0, M0
Stage I	T1, N0, M0
Stage II	T2a, N0, M0; T2b,N0, M0
Stage III	T3a, N0, M0; T3b, N0, M0; T4a, N0, M0
Stage IV	T4b, N0, M0; Any T, N1,M0; Any T, N2,M0; Any T, N3,M0; Any T, any N,M1

Table 3: AJCC stage groupings. Classification of bladder cancer by American Joint Committee on Cancer (AJCC) (Lamm, 1994).

1.5 Clinical proteomics and biomarkers

Many diseases manifest themselves through severe changes in human physiology, which forms the basis for clinical chemistry and confers its value in diagnoses and subsequent therapeutic interventions (Bischoff and Luider, 2004). Clinical proteomics include the analysis of proteins expressed by the genome of an organism, with the typical aim being the evaluation of quantitative changes that occur as a function of disease, treatment, or environment (Somiari et al., 2003). Proteomics strategies are being used to identify disease-specific protein markers called biomarkers that could provide the basis for the development of new diagnosis methodologies, treatments, and early detection of diseases particularly of cancer (Hanash, 2000; Somiari et al., 2003). Already for a number of years, 2-DE followed by protein identification using mass spectrometry has been the primary technique for biomarker discovery in conventional proteomic analyses (Gorg et al., 2000; Hanash, 2000).

1.6 Urinary biomarkers

Because urine can be collected noninvasively in large amounts, it provides an attractive alternative to blood plasma as a potential source of disease biomarkers (Thongboonkerd et al., 2004). The use of urinary biomarkers to diagnose disease is a long-standing practice. Studies to identify biomarkers of disease in urine have been an underlying component of investigative medicine throughout the 20th century and early 21st century. These studies have been based on knowledge of the pathophysiology of disease to identify biomarkers that could be tested in clinical trials. Large scale proteomics profiling of normal human urine samples has revealed the presence of at least 1000 different protein gene products and many more peptide fragments of larger proteins (Castagna et al., 2005; Jurgens et al., 2005; Oh et al., 2004; Pisitkun et al., 2004; Smith et al., 2007; Sun et al., 2005). There is hope,

therefore, for discovery of urinary protein excretion profiles that can be used clinically for tasks such as early detection and classification of diseases particularly of cancer, choice of therapeutic agents, assessment of prognosis, and monitoring of a particular therapeutic regimen. Several potential diagnostic markers for bladder cancer have been identified, including nuclear matrix protein 22, bladder tumor antigen, and telomerase.

1.7 Tumor angiogenesis

One of the critical events required for cancer growth is the development of a new network of blood vessels. This is provided by angiogenesis as well as postnatal vasculogenesis (Folkman and D'Amore, 1996). Angiogenesis is defined as the sprouting of new vessels from pre-existing blood vessels (Folkman, 2003; Luttun and Carmeliet, 2004), while postnatal vasculogenesis describes the formation of new vessels by endothelial precursor cells circulating in peripheral blood or derived from the bone marrow (Asahara and Kawamoto, 2004; Gehling et al., 2000; Rafii et al., 2002) from the vessels wall as recently demonstrated (Zengin et al., 2006).

Angiogenesis as well as postnatal vasculogenesis are regulated by angiogenic activators and inhibitors (Hanahan and Folkman, 1996). The structural formation and maturation of blood vessels during vasculogenesis and angiogenesis is a very complex process that runs in successive steps including proliferation and tube formation of endothelial cells, construction of the basement membrane, integration of peri-endothelial cells into the vascular wall, and embedding of blood vessels into the peri-vascular tissue (Ergun et al., 2006; Folkman et al., 1989; Folkman and D'Amore, 1996). Numerous angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoietins (Ang1, Ang2), and their receptors, which belong to the receptor tyrosine kinase family, are involved in several steps of this process (Maisonpierre et al., 1997).

VEGF is the key regulator of physiological and pathological angiogenesis and acts as a survival factor for endothelial cells (EC), both in vitro and in vivo (Ferrara, 2005). The biological effects of VEGF are mediated by two receptor tyrosine kinases (RTKs), vascular endothelial growth factor receptor-1 (VEGFR-1) and VEGFR-2 (Ferrara et al., 2003). Particularly, the interaction of VEGF with its receptor VEGFR-2

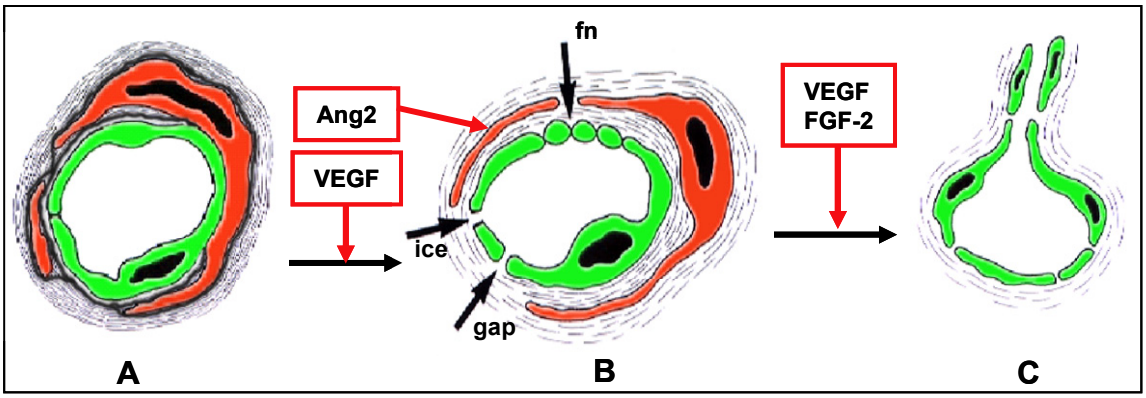


Figure 11: Vascular destabilization and initiation of angiogenesis. A normal stabilized capillary with a dense BM (gray) enclosing both endothelial cells (green) and pericyte (red) (A) will be destabilized by action of pro-angiogenic factors as exemplarily shown by VEGF and Ang2 leading to endothelial fenestration (fn), opening of inter-endothelial contacts (iec), development of transendothelial gaps (gap), degradation of BM and finally detachment of pericytes from the endothelial layer (B). These morphogenetic events are accompanied by an abnormal vascular leakiness. The further duration of pro-angiogenic action would finally lead to sprouting of new nascent and instable blood vessels (C), a process defined as angiogenesis (Adopted from Ergün et al., Cancer Encyclopedia, 2007, in press).

(known also as KDR in human or Flk-1 in mouse) intiates the angiogenic activity by causing the structural destabilization of the vascular wall with subsequently increased vascular leakeness (Ergun et al., 2006; Thurston et al., 2000). Also angiopoietin-2 (Ang2), a partial antagonist of the angiopoietin-1 (Ang1) and both Ang1 and Ang2 act via the same tyrosin kinase receptor Tie-2, is essentially involved in the destabiliazion of the vascular wall as shown in figure 11.

The abnormal vascular leakeness results in the extravasation of enzymes degrading the vascular basement membrane. Among these enzymes is the uPA, the urokinase type plasminogen activator, a key enzyme involved in the remodelling extracellular matrix supporting the migration of endothelial cells and thus the angiogenic activity of these cells (Agirbasli, 2005; Rakic et al., 2003). The action of this enzyme is modulated by the PAI-1 (plasminogen activator inhibitor-1) which has been shown to have a dual role in the interaction with uPA or the receptor of uPA, uPAR (Binder et al., 2007). The concerted action of the mentioned factors above howeever results in the complete disintegration of endothelial cells of pre-existing blood vessels with subsequent migration and proliferation of endothelial cells, and finally in the outgrowth of new vessels from pre-existing blood vessels.

1.8 One of urinary proteins: Orosomucoid (ORM)

Orosomucoid (ORM), also known as alpha1-acid glycoprotein (AGP), was firstly described in 1950 by Schmid (Schmid, 1950; Schmid, 1953). It belongs to a group of

acute phase proteins (APPs) and plays a role in the modulation of the immune response to stress, along with many other functions (Schmid K, 1975; Bennet M, 1980). The relatively high concentration of ORM in the serum of healthy humans is known to rise two- to fivefold in response to different conditions such as acute infection, inflammatory and lymphoproliferative disorders and cancer (Schmid, 1975). The structure of ORM is well characterized and is composed of a polypeptide chain containing about 45% carbohydrate including a large amount of fucosylic and sialic acid. Thus, its proposed immunomodulatory activities have been attributed to its glycosylation pattern, since the strongly fucosylated and sialylated ORM glycoforms have the ability to bind E-selectin and to inhibit complement activation (De Graaf et al., 1993).

Type 1 APPs, including ORM, complement component 3, serum amyloid A, C-reactive protein, haptoglobin and hemopexin, are regulated by IL-1, IL-6 and glucocorticoids and type 2 APP, including the three chains of fibrinogen and several proteases inhibitors, are regulated by IL-6- type cytokines and glucocorticoids (Baumann et al., 1989). ORM is synthesized in liver and various extrahepatic cell-types e.g. granulocytes (Fournier T, 2000). IL-1, IL-6 and glucocorticoids are the major modulators of ORM gene expression in liver cells (Alam et al., 1993; Baumann et al., 1989; Baumann and Gauldie, 1990). In most instances, a strong synergistic action is achieved by the combination of the three factors (Alam et al., 1993; Baumann et al., 1987). An induced expression of sialyl Lewis X (sLeX) on ORM during acute inflammation has been reported, leading to the speculation that it might influence the E- or P-selectin-mediated influx of sLeX-expressing leukocytes into inflamed areas. It has been suggested that an increased level of sLeX-expressing ORM could have a feedback inhibitory effect on the extravasation of leukocytes, by competition for the E-selectin adhesion molecules (Lasky, 1992). ORM is thought to modify the permeability of the vascular endothelium, possibly by interacting with the endothelial glycocalyx (Curry and Michel, 1980). Thus far it has been shown that ORM binds to the vascular endothelial cell surface (Fig. 12) and then causes transcytosis across the cell without passing the intercellular junction (Predescu et al., 1998).

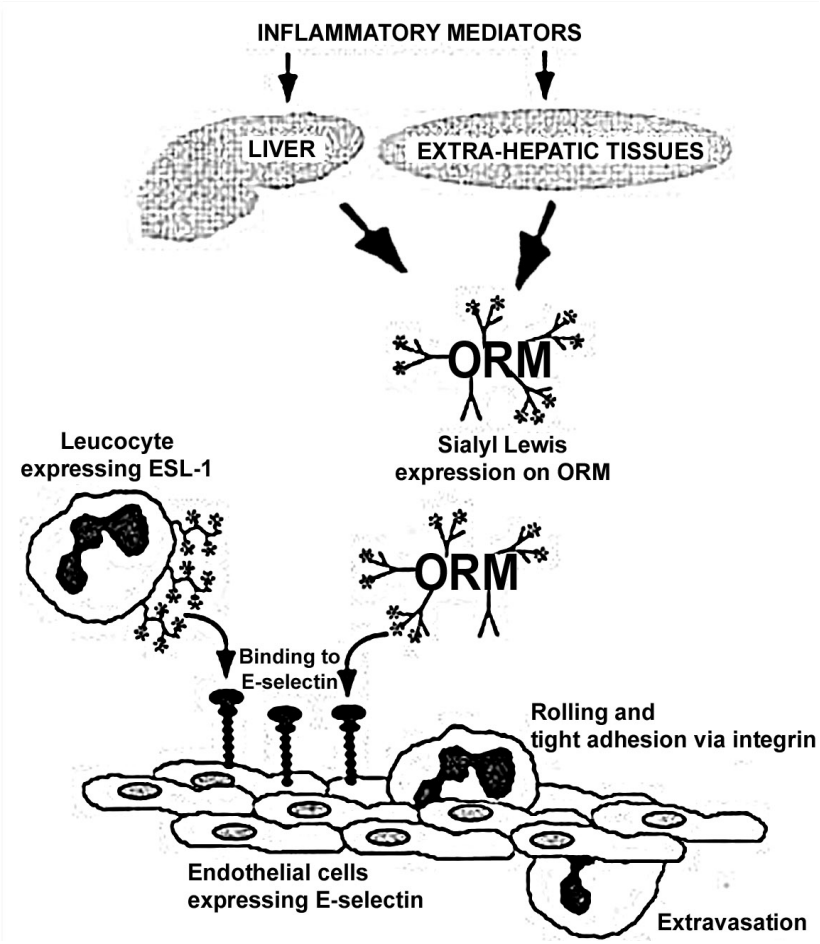


Figure 12: Hypothesis for the inhibition of leukocyte extravasation by ORM. Events leading to transmigration of leukocytes to a site of inflammation are summarised. ORM expressing sialyl Lewisx interacts with E-selectin expressed at the surface of endothelial cells and compete with leukocytes expressing ESL-1, the ligand of E-selectin. Consequently, rolling, adhesion and extravasation of leukocytes may then be inhibited. (From E.C. Havenaar, Thesis, Vrije Universiteit, Amsterdam, Netherlands.).

1.9 The plasminogen activation system

The plasminogen activation system regulates the formation of the serine protease plasmin and subsequent fibrinolysis. The process of fibrinolysis starts with conversion of an inactive proenzyme, plasminogen, a 92-kDa single-chain glycoprotein consisting of 791 amino acids into the active enzyme, plasmin (Forsgren et al., 1987). Plasmin is, in turn, able to degrade fibronectin, laminin, vitronectin, proteoglycans, as well as fibrin and activate latent collagenases. Angiogenic growth factors induce the expression of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) on the surface of endothelial cells (Flaumenhaft et al., 1992; Pepper et al., 1991). Both tPA and uPA are serine-proteases that can generate plasmin by proteolytic cleavage of its zymogen plasminogen. Plasminogen, like fibrinogen and other plasma components of the

provisional matrix, is synthesized in the liver and deposited in response to hyperpermeability. The formation of plasmin is essential for invasion and migration of endothelial cells into the tissue to be vascularized. The plasminogen activation system is not limited to endothelial cells. While tPA is almost exclusively expressed by endothelial cells (Mandriota and Pepper, 1997), uPA also facilitates migration of other cells like epithelial cells, fibroblasts and tumor cells (Del et al., 1990; MacDonald et al., 1998). A variety of cell types can bind components of the fibrinolytic system, including plasminogen (Miles and Plow, 1985), plasmin (Correc et al., 1990), uPA (Vassalli et al., 1985) and tPA (Hajjar et al., 1987). There are two main plasminogen activator inhibitors, PAI-1 and PAI-2. Plasminogen activator inhibitor type 1 (PAI-1) is the main inhibitor of plasminogen activators (Carmeliet et al., 1993; Fay et al., 1992). The PAI in blood represents 60% of plasminogen-activation inhibitor activity in plasma (Fig. 13). PAI-1 inhibits both tPA and uPA, whereas PAI-2 inhibits only uPA.

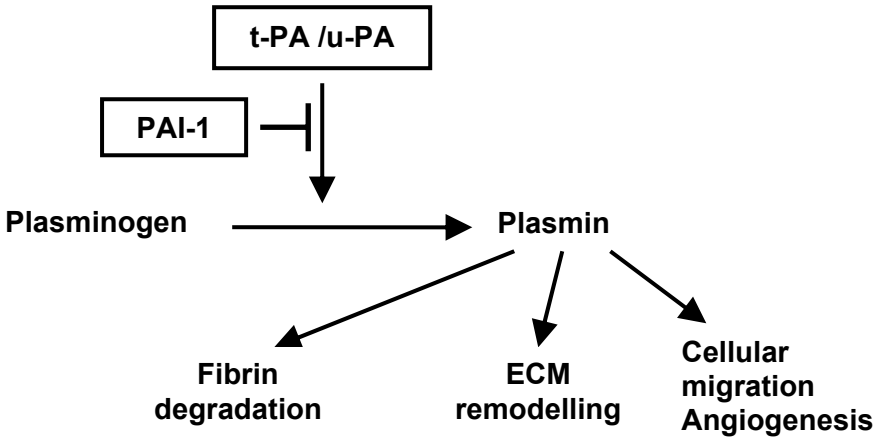


Figure 13: Plasminogen activation system. The plasminogen-activator inhibitor 1 (PAI-1) molecules is the main plasminogen-activator inhibitor in blood and it is important for fibrin degradation, remodelling of ECM and angiogenesis (Agirbasli, 2005).

Unlike the simple digestive proteases, in which only a signaling peptide and a short activation domain are attached to the catalytic region, the fibrinolytic proteases bear large noncatalytic regions (Patthy, 1985).

These regions contain functionally autonomous modules, such as "kringle", "growth factorlike" or "EGFlike" and "finger" domains, which also occur outside the serine protease family. A graphic representation of the domain arrangement in plasminogen, uPA and tPA is shown in Fig. 14.

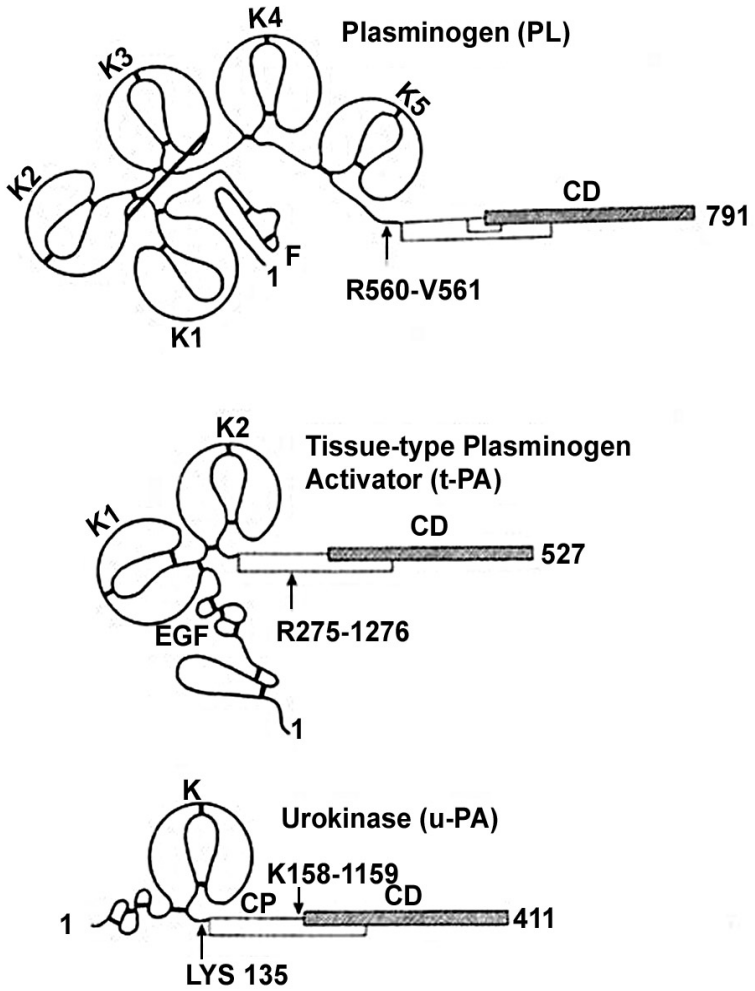


Figure 14: Graphical representation of the protein domains of plasminogen, tissuetype plasminogen activator (tPA) and urokinase (uPA) proenzymes. The kringle (K), Finger (F), EGFlike (EGF) and catalytic (CD) domains are marked. The activation sites are indicated by an arrow.

The serine proteinase plasmin consists of two disulfide bond-linked polypeptide chains. The N-terminal A chain contains five so called kringle domains. The C-terminal B chain contains a typical serine proteinase domain, which is responsible for catalytic activity (Fig. 15). Plasmin catalyzes the hydrolysis of peptide bonds on the C-terminal side of Lys and Arg residues. Conversion of plasminogen to plasmin can be catalyzed by either uPA or tPA.

The active form of PAI-1 is thought to have an exposed center loop that is available for interaction with proteinases (Lawrence et al., 1995; Lawrence, 1997). The conversion from active PAI-1 to its latent form involves incorporating this surface-exposed loop containing the reactive center into the central β-sheet of the protein (Lawrence et al., 1994; Mottonen et al., 1992). ORM, one of the major acute phase proteins, can interact with PAI-1 and stabilize its inhibitory activity toward

plasminogen activators (Boncela et al., 2001). The ORM-PAI-1 co-localization was found in thymosin β4 (Tβ4)-activated but not in quiescent HUVECs.

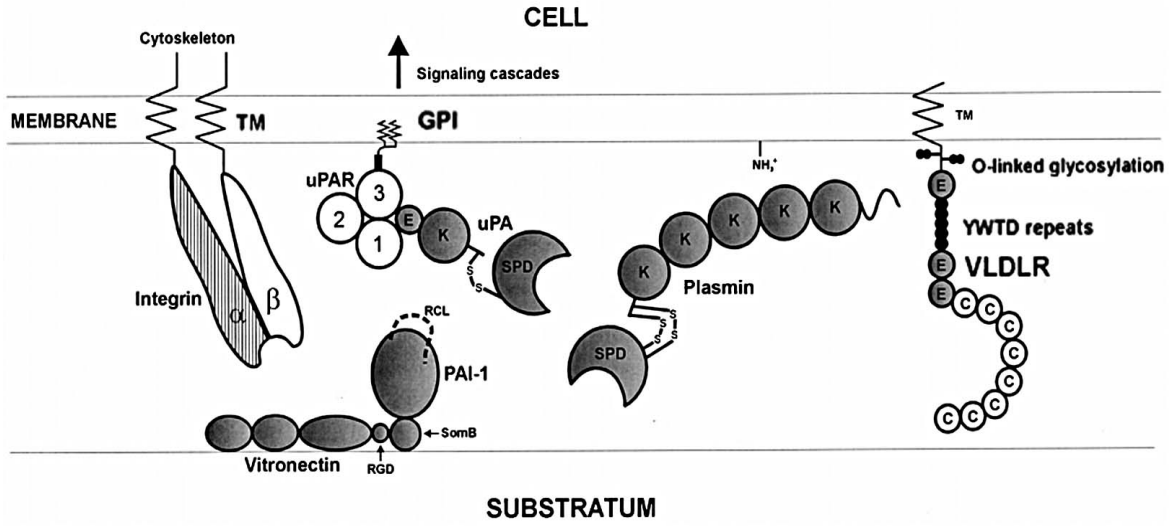


Figure 15: Schematic presentation of uPA and its interacting partners. uPA consists of a serine proteinase domain (SPD), a linker, a kringle (K), and a uPAR-binding epidermal growth factor-like domain (E). uPAR has three domains and is anchored to the membrane by a GPI anchor. Domains 1 and 3 make contact with uPA. Plasmin has a serine proteinase domain (SPD) and five kringles (K) and binds to pericellular proteins with C-terminal lysines. The RCL of PAI-1 is able to bind to the active site of uPA. Vitronectin, in the substratum, has an N-terminal PAI-1- and uPAR-binding somatomedin B domain (SomB) next to an integrin-binding RGD sequence. α and β integrin subunits have transmembrane helices (TM) and are anchored to the cytoskeleton. Integrins can interact with uPAR (Andreasen et al., 2000).

2 The aim of study

The aim of this study was: (*i*) to analyse the whole pattern of urine proteins present in urine samples of healthy persons in comparison to that of patients with bladder cancer, and to find out the potential relation between urine proteomics and bladder cancer stages. (*ii*) to explore the potential effect of ORM, as one of urinary proteins found as an increase amount in urine of patients with bladder cancer, in vascular and capillary morphogenesis. (*iii*) to study potential effect of the interaction between ORM and PAI-1 in angiogenesis and capillary morphogenesis.

3 Material and Methods

3.1 Materials

3.1.1 Chemical and Consumables

Acetonitril	Sigma (Taufkirchen)
Acrylamide	Serva (Heilderberg)
Agarose	PeqLab (Erlangen)
Ampicillin	Sigma (Taufkirchen)
APS	Merck (Darmstadt)
Bovine Trypsin	Sigma-Aldrich (Steinheim)
Bench Mark Prestained Protein Ladder	Invitrogen (Groningen, Holland)
CHAPS	Sigma (Taufkirchen)
Collagen, PureCol	Inamed (Nutacon,Finnland)
Coomassie Brillant Blue G-250	BioRad (München)
DMEM	Gibco BRL (Karlsruhe)
DMSO	Merck (Darmstadt)
DTT	BioRad (München)
Ethidiumbromide	Serva (Heidelberg)
FCS	Gibco BRL (Karlsruhe)
Histomount	ThermoShandon, (Pittsburg, USA)
Iodacetamide	Sigma (Taufkirchen)
LB Agar	Sigma (Taufkirchen)
L-Glutamin	Gibco BRL (Karlsruhe)
Nonidet (NP-40)	Calbiochem (Darmstadt)
MEM	Gibco BRL (Karlsruhe)
Normal Swine Serum (NSS)	Dako, (Hamburg)
Normal Rabbit Serum (NRS)	Dako, (Hamburg)
Nitrocellulose-Membrane	Schleicher & Schuell BioScience (Dassel)
Oligonucleotides (Primer,siRNA)	MWG Biotech (Ebersberg)
PBS Dulbecco	Seromed (Berlin)
Penicillin/Streptomycin	Gibco BRL (Karlsruhe)
Protease Inhibitor Cocktail	Sigma (Taufkirchen)
Röntgenfilms	KodakX-AR5 Kodak (Stuttgart)
Silvernitrate Solution	Sigma (Taufkirchen)

SDS-PAGE marker wide range	Sigma (Taufkirchen)
T4 DNA Ligase	New England Biolabs (Schwalbach/Taunus)
TBP	BioRad (München)
TEMED	Sigma (Taufkirchen)
Thiourea	Sigma (Taufkirchen)
Triton X-100	Sigma (Taufkirchen)
Trypsin/EDTA (1x)	Gibco BRL (Karlsruhe)
Urea	Sigma (Taufkirchen)
Mowiol	Calbiochem (Darmstadt)
Cell culture flasks, plates, and tubes	Falcon (Heidelberg)
Cryo-Save1 (conservation medium)	C.C.pro (Neustadt)

Unless otherwise stated chemicals and consumables were purchased from following companies: Amersham Biosciences (Freiburg), BioRad (München), Biozym (Hameln), Invitrogen (Groningen, Holland), Roche (Mannheim), Merck (Darmstadt), New England Biolab (Schwalbach/Taunus), Promega (Heidelberg), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) and Sigma-Aldrich (Taufkirchen).

3.1.2 Kits

Avidin-Biotin Complex (ABC) kit	Vector (USA)
BCA Protein Assay Kit	Pierce (USA)
RNeasy Mini Kit	Qiagen (Hilden)
ECL Plus Western Blotting Detection Kit	Amersham Biosciences (Freiburg)
Silencer siRNA Construction Kit	Ambion (Darmstadt)
HMVEL-L Nucleofector Kit	Amaxa-Biosystem (USA)
TOPO TA Cloning kit	Invitrogen (Groningen, Holland)
Nucleospin® Plasmid Kit	Macherey-Nagel (Düren)
EndoFree Plasmid Maxi Preparation Kit	Qiagen (Hilden)
Gel Extraction Kit	Qiagen (Hilden)
GFX PCR DNA and Gel Band Purification Kit	Amersham Bioscience (Freiburg)
Microcon YM-10 Centrifugal Filter Unit	Millipore/Amicon (Bedford, USA)
Centricon YM-10 Centrifugal Filter Unit	Millipore/Amicon (Bedford, USA)
ReadyPrep Sequential Extraction Kit	BioRad (München)
IPG Strips (11,17 cm) pH 3-10	BioRad (München)
Criterion Precast Gel	BioRad (München)

3.1.3 Stock Solutions and Buffers

Standard media and stock solutions were prepared according to standard procedures using deionised water. Solutions were sterilised by autoclaving (25 min/ 121ºC/ 2 bars). Heat sensitive components were prepared as stock solutions, filter-sterilised (0.2 µm) and added to the medium/buffer after cooling to 50ºC.

5x SDS-PAGE Running Buffer (TGS Buffer)

Tris-HCl	125 mM
Glycin	960 mM
SDS	0.5% (w/v)

2D-PAGE Rehydration Buffers:

Reagent 3 (ReadyPrep Sequential Extraction Kit)

Urea	5 M
Thiourea	2 M
CHAPS	2% (w/v)
SB 3-10	2% (w/v)
Tris	40 mM
Biolyte 3-10	2% (v/v)
TBP(fresh)	2 mM
DTT(fresh)	0.5% (w/v)
Bromphenolblue	trace

Reagent 2 (ReadyPrep Sequential Extraction Kit)

Urea	8 M
CHAPS	4% (w/v)
SB 3-10	2% (w/v)
Tris	40 mM
Biolyte3-10	0.2% (v/v)
TBP (fresh)	2 mM
DTT (fresh)	0.5% (w/v)
Bromphenolblue	trace

2D-PAGE Equilibration Buffer I

Urea	5 M
SDS	20% (w/v)
Tris-HCl (pH 8.8)	1.5 M
Glycerol	50% (w/v)
DTT(fresh)	0.5% (w/v)

2D-PAGE Equilibration Buffer II

Urea	5 M
SDS	20% (w/v)
Tris-HCl (pH 8.8)	1.5 M
Glycerol	50% (w/v)
Iodacetamid(fresh)	2.5% (w/v)

Fixer Solution I for gel staining

Methanol	50% (v/v)
Acetic acid	10% (v/v)

Fixer solution II for gel staining

Methanol	5% (v/v)
Acetic acid	7.5% (v/v)

Developer Solution	
Sodiumcarbonate	6% (v/v)
Formaldehyde	6 mM

Coomassie Staining Solution	
Coomassie BB G-250	0.1% (w/v)
$(NH_4)_2SO_4$	8% (w/v)
H_3PO_4	2% (v/v)

5x Laemlie Protein Loading Buffer	
Tris-HCl (pH 6.8)	50 mM
Glycerol	30% (v/v)
SDS	4% (w/v)
Bromphenolblue	0.5% (w/v)
DTT (fresh)	0.1 M

RIPA Lysis Buffer	
Tris-HCl (pH 7.4)	50 mM
NaCl	150 mM
NP-40	1% (v/v)
EDTA	1 mM
SDS (fresh)	0.1% (w/v)
Protease Inhibitor cocktail (fresh)	

Triton X-100- SDS Lysis Buffer	
Triton X-100	1% (v/v)
SDS	1% (w/v)

10x Blotting Buffer	
Tris-HCl	1 M
Glycin	1.93 M

10x TBS Buffer, pH 7.6	
Tris-HCl	200 mM
NaCl	1.37 M
SDS	0.5% (w/v)

TBS-T Buffer	
1x TBS (pH 7.6)	
Tween-20	0.1% (v/v)

SDS Polyacrylamide Gels

Stacking Gel (4%):	Tris; pH 8.8	1.5 M
	SDS	4%

Running Gel (12%):	Tris; pH 6.80	5 M
	SDS	4%

Stripping Buffer	
Tris-HCl (pH 6.7)	62.5 mM
β-Mercapthoethanol	100 mM
SDS	2% (w/v)

RNA/DNA-Loading Buffer	
Bromphenolblue	0.4% (w/v)
Xylencyanol	0.4% (w/v)
EDTA	1 mM
Glycerin	50% (v/v)

TFB1 (pH 5.8)	
RbCl	100 mM
$MnCl_2$	50 mM
Potassium acetate	30 mM
$CaCl_2$	10 mM
Glycerol	15% (v/v)

TFB2 (pH 6.8 by KOH)	
MOPS	10 mM
RbCl	10 mM
$CaCl_2$	15 mM
Glycerol	15% (v/v)

0.1 M PBS (pH 7.4)	
Na_2HPO_4	0.2 M
NaCl	0.2 M

0.1 M PB (pH 7.4)	
KH_2PO_4	0.018 M
$Na_2HPO_4.2H_2O$	0.082 M

Antibody dilution buffer (PBS/ BSA/ NaN_3)	
0.1 M PBS (pH 7.4)	
BSA	0.2% (w/v)
NaN_3	0.1% (w/v)

50x TAE Buffer (pH 7.5)	
Tris-Base	2 M
Glacial acetic acid	5.7% (v/v)
EDTA	50 mM

Glucoseoxidase solution (for 50 ml)

PB 0.1 M (pH 7.4)	45 ml
DAB 0.063 M	1 ml
NH_4Cl 3.35 M	100 µl
$NiSO_4$ 0.05 M	900 µl
Glucose (10%)	900 µl
Glucose-oxidase 179 U/ml	150 µl

Collagen Gel Mixture (for 10 ml)

Sterile water	3.95 ml
10x MEM Medium	1 ml
L-Glutamin 200 mM	100 µl
Sodiumpyruvate 100 mM	100 µl
Collagen	4 ml
Sodium bicarbonate (7.5%)	500 µl
Sodium hydroxyle 0.1 M	40 µl

3.1.4 Equipment and Applications

Nucleofector: Amaxa., BioSystems

Cameras: ProgRes^{TM}C10^{plus}, Jenoptik

Microscopes: Phase contrast microscope (Zeiss, Jena); Axiovert 25 (Zeiss, Jena); SM-Lux (Leitz)

Centrifuges: 5415D Centrifuge (Eppendorf)

Incubator: Hera Cell 240 (Thermo)

PCR-Thermocycler: T3000 Thermocyler (Biometra)

Photometer: UV-Spectrometer, SmartSpec 3000 (BioRad)

MALDI-TOF spectrometer: Axima CFR (Shimadzu Biotech)

Gel electrophoresis and electroblotting equipment: Criterion Dodeca Cell, Protean IEF Cell, Disposable Rehydration/Equilibration Tray (BioRad), Mini protean and Trans-Blot-System (BioRad).

3.1.5 Antibodies

Antibody	Source	Working dilution	Supplier
Anti-Alpha1 acid glycoprotein (AGP)	Rabbit	1:200	Dako, Hamburg
Anti-Zinc Alpha Glycoprotein(ZAG)	Goat	1:200	Santa Cruz, USA
Vimentin	Mouse	1:1000	Dako, Hamburg

Table 4: Primary antibodies used for western blot and Immunohistochemistry

Antibody	Working dilution	Supplier
Anti-mouse IgG	1:8000	Sigma, Taufkirchen
Anti-rabbit IgG	1:30,000	Sigma, Taufkirchen
Anti-goat IgG	1:30,000	Sigma, Taufkirchen

Table 5: Secondary HRP antibodies used for western blot

3.1.6 Cell lines and medium for cultivation of cell lines

Unless purchased sterile, all media, solutions and supplements used in cell culture work were sterilised with a 0.2 µm filter, strored at 4°C and prewarmed to 37°C prior to use.

Cell Lines	Characteristics	Source
HDMECs	Human Dermal Microvascular Endothelial Cells (HDMECs) derived from dermis	PromoCell, Heildelberg
RT4	Human Bladder Cancer Cells	Obtained from Department of Urology, University Hospital Hamburg-Eppendorf

Table 6: Used cell lines

Cell Lines	Medium
HDMECs	Endothelial Cell Growth Medium MV (PromoCell): After adding the SupplementMix, the concentrations of growth factors are; 0.4% ECGS/H; 5% Fetal Calf Serum; 0.1 ng/mL Epidermal Growth Factor; 1µg/mL Hydrocortison; 1ng/mL basic Fibroblast Factor
RT4	McCoy's Medium (Gibco,USA): 10% Fetal Calf Serum; 1% Glutamine; 1% penicillin/ streptomycin

Table 7: Cultivation medium for cell lines

3.1.7 Bacterial strains

For cloning applications, the E. coli strains DH5α and XL1-Blue were used. Ligation reactions were transformed into XL1-Blue strain; plasmids requiring digestion with methylation sensitive restriction enzymes were transformed into DH5α strain. PCR products were cloned using the TOPO TA cloning kit (Invitrogen) with the supplied Top10F' bacteria.

Strains	Genotype	Source
DH5α	F' ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk^-,mk^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Invitrogen (Groningen, Holland)
XL1-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac* {F', *pro*AB, $lacI^qZ\Delta M15$, $Tn10(Tet^R)$}	Stratagene(Heildelberg)
Top10F'	F^- {$lacI^q$ Tn*10* (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen (Groningen, Holland)

Table 8: Bacterial Strains. Names, genotypes and suppliers of the bacterial strains used.

3.1.8 Primers

Oligonucleotides used for PCR and contruction of siRNA were purchased from MWG Biotech.

Primer Name	Line	Sequence (5→3)	Genbank Number	T_m (°C)	PCR cycle numbers
ORM	Forward	ACGTGCCTCCTGGTCTCAGTA	NM_00067	62.1	35
	Reverse	TTAGCTGTTCCAAACACAGAAG		56.5	
GAPDH	Forward	TGATGACATCAAGAAGGTGG	NM_002064	55.3	35
	Reverse	TTTCTTACTCCTTGGAGGCC		57,3	

Table 9: Oligonucleotides used for in PCR

Primer Name	Line	Sequence (5→3)	T_m (°C)
siRNA ORM TS231	sense	AAGACTTATTGTACTCCTCGTCCTGTCTC	65.3
	antisense	AAACGAGGAGTACAATAAGTCCCTGTCTC	65.3
siRNA ORM TS266	sense	AATGAAGTAAAAGAAGGTTGCCCTGTCTC	63.9
	antisense	AAGCAACCTTCTTTTACTTCACCTGTCTC	63.9
siRNA Luciferase	sense	AATCGAAGTATTCCGCGTACGCCTGTCTC	55,3
	antisense	AACGTACGCGGAATACTTCGACCTGTCTC	55,3

Table 10: Oligonucleotides used for in siRNA construction

3.1.9 Urine samples

Spontaneous urine samples of 45 patients with bladder cancer of different stages (pTa, pT1, pT2-3, GI, GII, GIII), patients on follow-up and healthy volunteers were collected and after centrifugation at 5000 rpm for 5 min stored at –20°C (for a longer time than 2 months at –80°C) until their use.

Bladder Cancer Patients (n=45)		
pTa (n=12)	**PT1(n=8)**	**PT2-3(n=8)**
Age, 60-80	Age, 60-80	Age, 60-80
Female, n=7	Female, n=2	Female, n=1
Male, n=5	Male, n=6	Male, n=7
GI, n=4	GI, n=1	GIII, n=8
GII, n=8	GII, n= 3	
	GIII, n=4	
	Patients on follow-up (n=10)	**Healthy Volunteers (n=7)**
Age	60-80	30-50
Female	n=4	n=3
Male	n=6	n=7

Table 11: Urine samples used in 2-DE analyses and western blotting

3.1.10 Tissue samples

Tissue samples were provided from the Department of Pathology of the University Hospital Hamburg-Eppendorf. Normal tissue samples from human bladder were obtained by biopsy or by cystoprostatectomy because of prostate cancer, and tumor tissues of bladder cancer were obtained from patients who had undergone surgical therapy. The determination of tumor stage was made by the Department of Pathology according to the WHO (World Health Organization) classification. The tissue blocks were fixed in formaldehyde and embedded in paraffin and subsequently sectioned for use in immunohistochemistry.

3.2 Methods

3.2.1 Protein analyses

3.2.1.1 Determination of total protein

The concentration of proteins was determined by BCA protein assay kit which is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of

total protein. The complex giving colour exhibits a strong absorbance at 562 nm. BSA (Bovine Serum Albumin) was also serially diluted and used as standard. 2 ml of the BCA working reagent mix was added to each sample and to standard tubes. After a short vortex the tubes were incubated at 37°C for 30 min. For each dilution, measurements were performed in duplicate and the average was taken for the calculation of the protein concentration.

3.2.1.2 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Two dimensional gelelectrophoresis separates protein mixtures due to differences in their isoelectric point (pI), in the first dimension and subsequently by their molecular weight (MW) in the second dimension represented in figure 16.

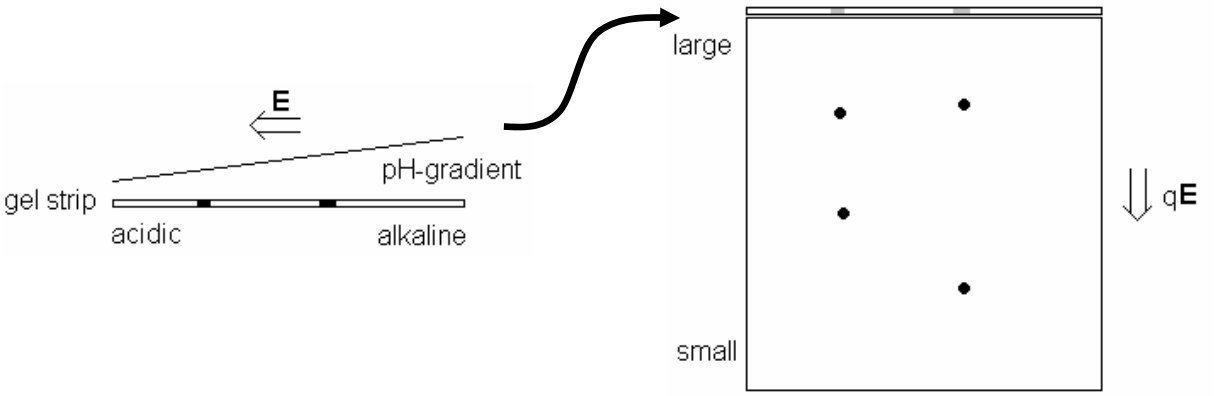


Figure 16: Schematical representation of two dimensional separation. Proteins are separated in two dimensions horizontally by iso-electric point (pI) and vertically by molecular weight (MW).

3.2.1.3 Preparation of urine samples for 2-DE

After protein determination, urine volume containing 200 µg total protein was applied onto a Centrifugal Filter Column (Amicon) to remove molecules smaller than 10 kDa such as urea, electrolytes, salts and to increase the protein concentration of the urine samples. The centrifugal column containing urine volume was centrifugated at speed linearly increased from 3000 rpm to 6000 rpm at 4°C until complete urine volumes were filtered through the filter membrane. Remained protein molecules were washed with 50 µl distilled water and centrifugated at 6000 rpm for 30 min. Filtered urine samples were diluted in rehyration buffer 2 (3.1.3). After fresh adding of TBP, DTT and trace of bromophenolblue, protein lysates were centrifugated at 15,000 rpm for 10 min at 4°C to remove insoluble particles. Supernatant containing urinary proteins was used for sample loading step of 2-DE procedure.

IPG Strip Length / pH Range	Loaded protein amount
11 cm, pH 3-10, nonlinear (NL)	200 µg (in 200 µl volume)
17 cm, pH 3-10, Linear (L)	300 µg (in 300 µl volume)

Table 12: IPG strips used for 2-DE analyses

Additionally, TCA (tricholoroaceticacid) precipitation was performed on the urine samples to precipitate all proteins present in urine. Shortly, an equal volume of 20% TCA were added to the urine samples and incubated on ice for 30 min. After centrifugation at 13,000 rpm for 10 min at 4°C, the supernatant was removed and protein pellets were diluted in rehydration buffer2-3 (3.1.3). But, this method was not effective for 2-DE analysis; it led to vertical streaks due to remained TCA particles (data was not shown). To precipitate a specific protein present in urine, immunoprecipitation was performed on urine samples. However, the 2-DE results of immunoprecipitated samples were not informative due to remained IgG (Immunglobulin G) coming from secondary antibody (data was not shown). Therefore ultracentrifugation was used for all urine samples prior their use in 2-DE process.

3.2.1.4 Rehydration

The PROTEAN IEF cell with integrated power supply was used for rehydration and IEF protocols. Diluted protein samples were loaded onto IPG strips in IEF focusing tray for rehydration and first dimensional separation. IPG strip gels were placed side down in the channel of a focusing or rehydration tray that contains the sample in rehydration solution. For urine samples mostly 11 cm nonlinear IPG strips with 3-10 pH range were used and for a large scale of gel the strips with 17 cm length were used (Tab. 12). Strips were covered with 2 ml of mineral oil to prevent evaporation which causes the urea to precipitate as it becomes more concentrated. IPG strips with sample in a focusing tray were active rehydrated by running of IEF cell under low voltage as 50 V overnight.

3.2.1.5 First dimensional separation: Isoelectric focusing (IEF)

After the strips have rehydrated the ends of each strip was lifted and wet electrode wicks were inserted between the strip and the electrodes. Strips were covered with mineral oil before starting the focusing run to prevent evaporation and carbon dioxide

absorpsion during focusing. Isoelectric focusing was performed at voltage linearly increased from 250 to 5000 V during the first 5 h, followed by 8000 V for a total of 70000 Vh/h (Tab. 13).

Temperature 20˚C
Current max. 0.05 mA per strip
Sample volume 200 µl for 11 cm strip, 300 µl for 17 cm strip

Step	Voltage	Time / Volt-hour
1	150 V	30 min. rapid
2	300 V	60 min. slow
3	1000 V	60 min. slow
4	1500 V	60 min. slow
5	2000 V	60 min. slow
6	3000 V	60 min. slow
7	5000 V	60 min. slow
8	8000 V	4 h slow
9	8000 V	70,000 Vh linear
10	500 V	3 h linear

Table 13: Applied voltage steps for IEF

3.2.1.6 Equilibration

This process reduces disulfide bonds and alkylates the resultant sulfhydryl groups of the cysteine residues. Rehydration/equilibration trays sized for each size strip were used for equilibration. Each strip was moved to equilibration tray and the channels were filled with the equilibration buffers. Strips were first incubated in DTT equilibration buffer-I (3.1.3) that reduces sulphydryl groups for 15 min at RT, than the channels were refilled with iodacetamide equilibration buffer-II (3.1.3) which alkylates sulfhdryl groups and incubated for 15 min at RT. For the strips with 11 cm length, 3 ml of each equilibration buffer and for the strips with 17 cm length 5 ml of each equilibration buffer were used. After equilibration, IPG strips were removed and embed into the prepared second-dimension gel.

3.2.1.7 Second dimensional separation

The second dimension electrophoresis of isoelectric focused proteins was carried out on pre-prepared polyacrylamide gels obtained from BioRad (Criterion Precast Gel 10% Tris-HCl, 1.0 mm). The equilibrated IPG gel strips were placed on top of the polyacrylamide gel and overlaid with 2 ml 0.5% melted agarose prepared in SDS-

PAGE running buffer. A small amount of Bromophenolblue was added to the agarose overlay to track the ion front during the run. The SDS gel running was performed with a voltage setting of 120 V for 45 min and 150 V for 20 min. BioRad Dodeca Cell which accommodate 12 gels per run was mostly used for proteomics analyses. To estimate the molecular mass of each spot, marker proteins (SigmaMarker^{TM}, wide molecular weight) were placed on a filter strip left to the strip on the gel. Polyacrylamide gels were stained with silver and/or Coomassie Brillant Blue (BioRad) according to the procedures described previously (Merril et al., 1981).

3.2.1.8 Protein detection

To visualize proteins after separation in 2-DE gels it is necessary to use an adequate staining method. The enormous variation in characteristics and abundance of the individual proteins put high demands on the staining technique. The most important requirements are high sensitivity, high linear dynamic range, high reproducibility and compatibility with mass spectrometry. Major developments of protein staining methods have occurred during the last few years. Since silver staining is the most sensitive non-radioactive detection technique and the costs for reagents are relatively low when compared to fluorescence techniques, it is still widely used. There are presently more than 100 different modifications of the original silver staining protocol that was introduced by (Merril et al., 1981). In this study silver staining and coomassie blue staining methods were used to visualize the protein spots.

3.2.1.9 Silver staining

After second dimensional separation the gels were taken out and incubated first with fixer solution-I (3.1.3) containing 50% methanol and 10% acetic acid for 30 min following second incubation with fixer solution-II (3.1.3) containing 5% methanol and 7.5% acetic acid for 10 min at RT. After fixation, the gels were washed twice with distilled water for 5 min at RT on a shaker. Gels were then incubated with 0.002% sodiumthiosulphate for 1 min and washed twice with distilled water for 1 min. For staining, fresh prepared 0.2% silver nitrate solution was used. The gels were incubated with silver nitrate solution in dark for 30 min at RT. After a washing step as 2x1 min, the gels were incubated with fresh developer solution (3.1.3) containing 6% sodium carbonate and 6 mM formaldehyde for 5-20 min until the protein spots

became visualized. A few solid citric acid particles were added to stop the development of the gels. After washing, gels were scanned for the image analysis.

3.2.1.10 Coomassie staining

For coomassie staining, the gels were incubated with fixer solution-I (3.1.3) for 30 min at RT on a shaker. After washing twice with distilled water for 5 min, gels were stained by incubation using coomassie solution (3.1.3) for overnight. Stained gels were washed several times with distilled water to remove remained coomassie crystals and excess dye from gel.

3.2.1.11 Image and data analysis

The resulting 2-DE images were analysed using PDQuest software programme (BioRad). Gel analysis includes spot detection, spot quantitation, gel comparison, and statistical analysis. Before the software automatically detected the protein spots of a 2-DE gel, the raw image data were corrected and background was subtracted.

3.2.1.12 Mass spectrometry and bioinformatics

Some of the visible protein spots on the stained gels have been identified by mass spectrometric analysis in Heinrich-Pette-Institute of Hamburg University. Coomassie Brilliant Blue-stained protein spots (Coomassie G250) were excised, cut in 1 mm^3 pieces, washed twice with distilled water and destained with 50% v/v acetonitrile (MeCN) in 25 mM ammonium bicarbonate for 2x15 min, shrunk by dehydration in acetonitrile for 15 min, and dried in a vacuum centrifuge for 10 min. The gel pieces were reswollen in 10-20 µl of 25 mM ammonium bicarbonate, containing 200-400 ng of bovine trypsin (proteomics sequencing grade; Sigma-Aldrich) at 4°C. After 60 min, 10-20 µl of 25 mM ammonium bicarbonate were added if necessary to keep the gel pieces wet during tryptic cleavage (37ºC, overnight). The peptides were extracted sequentially with water, 0.1% TFA, 10% MeCN/0.1% TFA, 20% MeCN/ 0.1% TFA and 40% MeCN/0, 1% TFA for 30 min each. The separated liquids were combined and dried under vacuum. The peptides were redissolved in 4 µl matrix solution containing 10 mg dihydroxybenzoic acid, 5 mg diammonium hydrogen citrate, 0,1% TFA, and 0,05% phosphoric acid in 50% MeCN. For MS-analysis 1 µl were spotted onto the target plate and air-dried. MALDI-MS measurements were performed on an

Axima CFR MALDI-TOF spectrometer (Shimadzu Biotech) in positive mode with reflectron, delayed extraction was optimized for 2500 mu. Dependent on spectra quality 200-400 laser shots were accumulated. Control of the spectrometer and spectra processing was done with the Axima CFR software V2.2.1. Mass spectra were calibrated using known autolytic fragments of trypsin, Angiotensine III and acetylated Insulin B chain as internal standards. The peptide masses were measured as monoisotopic masses.

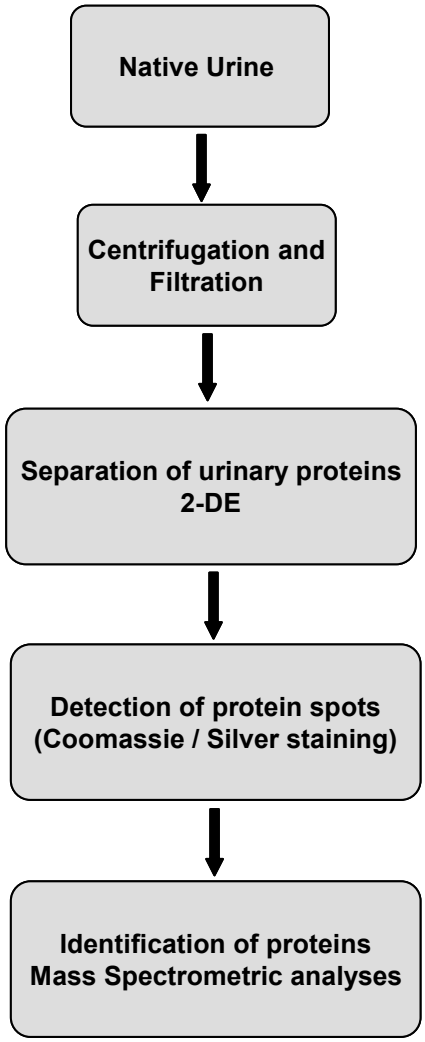


Figure 17: Experimental flow diagram. This diagram shows the experimental steps of analyses of urine samples. Native urine containing a certain amount total protein was centrifugated and filtrated. After first and second dimensional separation using 2-DE technique, the 2-DE gels were stained using silver and/or coomassie blue staining protocol to visualize protein spots. The spots of interest were cut out from gel and identified by mass spectrometric analyses.

Proteins were identified by searching the NCBI database using the program MASCOT (Matrix Science; http://www.matrixscience.com/serach_form_select.html). The parameters for the search were as follows: the modifications acetamide on

cysteine residues and methionine oxidation were considered as a partial modification and three was used as the maximum missed tryptic cleavage sites.

3.2.1.13 SDS-polyacrylamide gelelectrophoresis

One dimensional gel electrophoresis (Laemmli, 1970) under denaturing conditions (in presence of SDS) was used for separation of the proteins on the base of their molecular size. The polyacrylamide gel was prepared as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus from BioRad. After sample proteins are solubilised by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel line, and the individual proteins are separated electrophoretically. The mobility of the proteins is inversely proportional to the logarithm of their molecular mass. SDS is employed to effect denaturation of the proteins, to dissociate protein complexes and to impart upon the polypeptide chains net negative charge densities proportional to the length of the molecule. A reducing agent as DTT was used to reduce any existing disulphide bond. After one dimensional separation the proteins was detected using staining methods which is able to detect all proteins and by immunoblotting for detection of a specific protein.

3.2.1.14 Western blotting

Electroblotting of proteins from polyacrylamide gels onto retentive membranes was performed for immunoblotting following detection of accumulated proteins using a specific antibody. Proteins were separated by SDS-PAGE and electrophoretically transferred from the polyacrylamide gel to a nitrocellulose membrane at 350 mA constant current for 1 hour.

Proteins were separated on 12% SDS-Acrylamide gel which was prepared according to the „Mini Protean Manual Instruction' from BioRad. For preparation of gels 30% acrylamide solution obtained from Serva was used. 25 µl of different protein samples containing 50 µg total protein were denaturated by adding of sample loading buffer (3.1.3) including DTT and following heating at 95ºC for 5 min. Protein samples were loaded onto the gel and running was performed at 120 volt for 90 min. Prestained protein marker from invitrogen was used to estimate molecular size of separated proteins. For transferring of proteins from SDS gel to nitrocellulose membrane, equipment as "Mini Trans-Blot-System" obtained from BioRad was used according to

the manufacturer's recommendations. For blotting nitrocellulose membranes (Schleicher & Schuell, BioScience) were cut in size as 7x9 cm and wetted by transfer buffer shortly before placing. The fiber pads and filter papers were pre-wetted with 1x Transfer buffer. Gel containing proteins and nitrocellulose membranes were allowed to contact by a sandwich model placing in order as a fiber pad, a filter paper, gel, nitrocellulose membrane, a filter paper, a fiber pad (from cathode plate to anode plate). The cassettes were placed in the chamber and a cold pack was put beside and filled with pre-chilled 1xTransfer buffer (3.1.3). Electrophoretically transfer was performed 1 h at 350 mA current on a stir plate. After the transfer onto nitrocellulose, the blots were taken out and washed with distilled water. To prevent unspecific bindings, blots were incubated with blocking buffer containing 5% non-fat milk solution in 1xTBS-T buffer (3.1.3) overnight at 4ºC. Next day the blots were incubated with the primary antibody in 1xTBS-T solution for 1 h at RT. After 2x10 min washing in 1xTBS-T buffer the membranes were incubated for 1 h at RT with the horseradish peroxidise-coupled secondary antibody in blocking buffer. Finally, the membranes were washed 2x10 min and 2x20 min with 1xTBS-T buffer. The antigen-antibody complex was detected by enhanced chemiluminescence using ECL reagents (Amersham-Pharmacia) and visualized by auroradiography. The X-ray films were digitalized for subsequent densitometric quantification of the bands using the morphometric program Optimas^{TM} (Seattle, USA).

3.2.2 Molecularbiological methods

3.2.2.1 Cultivation and storage of *E. coli*

Bacteria were cultivated in Luria-Bertani (LB)-broth or on LB-agar plates at 37°C. For selection of transformed bacteria, broth and plates were supplied with 50 mg/l ampicillin.

Freshly plated bacteria were viable for approximately one month when stored at 4°C. For long-term storage, glycerol stocks were prepared by mixing 500 µl of an overnight liquid culture with 500 µl of 15% sterile glycerol. The stocks were stored at –80°C.

3.2.2.2 Preparation of competent cells

DH5-α bacterial were obtained from Invitrogen and competent *E. coli* cell stocks were prepared using protocol described previously (Sambrook, 1989) 200 µl of DH5-α cells were thawed on ice and were mixed with 10 ml LB-medium without antibiotic. After overnight incubation at 37°C with shaking at 225-250 rpm, 200 µl of mixture were plated on an agar plate without antibiotic and incubated overnight at 37°C. A single colony was picked and 10 ml LB medium was inoculated with. After overnight incubation at 37°C, 1 ml of overnight culture was added to 100 ml pre-warmed LB medium without antibiotic. The culture was incubated and shaked at 37°C until an OD_{600} OF 0.5 was reached (approximately 90-120 min). The culture was cooled on ice for 5 min and transferred to a sterile round-bottom centrifuge tube. The cells were collected by centrifugation at low speed (5 min, 1500 rpm, 4°C). After supernatant was removed, the cells were resuspended in cold TFB1 buffer (3.1.3) (30 ml for a 100 ml culture) and suspension was incubated on ice for 90 min., followed centrifugation for 5 min at 1500 rpm at 4°C. The cells were resuspended in 4 ml ice-cold TFB2 buffer (3.1.3). Aliquots of 100 µl in sterile microcentrifuge tubes were prepared and frozen in liquid nitrogen. The competent cells were stored at -80°C.

3.2.2.3 Bacterial transformation

Bacterial transformation which is needed for cloning application was performed using *E. coli* strains DH5α and XL1-Blue (Tab. 8). 50 µl of competent cells were thawed on ice. 1-5 ng purified DNA or 1-5 µl of ligation reaction was added to one aliquot of cells and mixed by pipetting. The tubes containing cells and DNA were incubated on ice for 30 min, and then were heat-pulsed in a 42°C water bath for 45 seconds, followed incubation on ice for 2 min. After adding 500 µl pre-warmed LB medium to each sample, the tubes were incubated at 37°C for 1 hour with shaking at 225-250 rpm. After incubation 50-500 µl of transformation mixture were spread on LB agar plates containing 50 mg/l ampicillin. The plates were incubated overnight at 37°C. Single colonies were picked and incubated with LB medium containing ampicillin at 37°C overnight with shaking. The plasmid DNA was isolated using either Plasmid Maxi Kit (Qiagen) or for further transfection application, EndoFree Plasmid Maxi Kit (Qiagen). Transformed bacteria were stored as 1:1 mixture with sterile glycerol stock at –80°C.

3.2.2.4 Purification of DNA from solution and gel bands

For isolation and purification of DNA from PCR product and/or agarose gel bands, GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) was used. This kit uses a chaotropic agent that denaturates proteins, dissolves agarose, and promotes the binding of double-stranded DNA (100 base-pairs to 48 kilobase-pairs) to a glass fiber matrix. Purification of DNA was performed using the protocol provided by GFX purification kit. First DNA was denatured and for gel band purification, agarose was dissolved. DNA samples were passed through the GFX column provided with kit to capture the DNA onto the glass fiber matrix. Then Matrix-bound DNA was washed with an ethanolic buffer to remove salts and other contaminants. Purified DNA was eluted from GFX column in a low ionic strength buffer (TE pH 8.0, 10 mM Tris-HCl pH 8.0, or water).

3.2.2.5 Minipreparation of plasmid-DNA

For small-scale preparation of plasmid DNA, Nucleospin® Plasmid Kit (Macherey-Nagel) was used. Isolation of plasmid DNA was performed according to protocol from Nucleospin® Plasmid kit. The pelleted bacteria were resuspended and plasmid DNA was liberated from the *E.coli* host cells by SDS/alkaline lysis. SDS precipitate and cell debris were pelleted by a centrifugation step, the supernatant was loaded onto a Nucleospin® Plasmid column. Pure plasmid DNA was finally eluted under low ionic strength conditions with slightly alkaline buffer (5 mM Tris-Cl, pH 8.5) which provided with kit. Concentration of DNA was measured by UV-spectrophotometer.

3.2.2.6 Maxipreparation of plasmid-DNA

For isolation of plasmid DNA in high culture volume, Plasmid Maxi Kit (Qiagen) was used. The purification protocol of kit is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to Qiagen Anion-Exchange Resin under appropriate low-salt and pH conditions. 100 ml of LB-Medium containing Ampicillin was inoculated by a picked single colony from a selective agar plate and then cultured by over night incubation at 37°C on a rotation shaker. Plasmid DNA was isolated using the protocol from Plasmid Maxi Kit and diluted in 200 µl TE buffer or in buffer containing 10 mM Tris-Cl, pH 8.5. DNA concentration was determined by UV spectrophotometry and quantitative analysis on an agarose gel. Plasmid DNA which

was further transfected into the cells was isolated using EndoFree Plasmid Maxi Kit (Qiagen) due to endotoxin effects efficiency of gene transfer.

3.2.2.7 Restriction digest of DNA

Restriction enzymes and appropriate buffers were obtained from NEB. Restriction digest and double restriction digest of DNA were performed according to the manufacturers' protocol. The general protocol for restriction digestion of DNA is shown in table 14.

Components	Concentration / Volume
DNA	1-3 µg
Restriction Enzyme	3 U/ µg DNA
10x Buffer	2 µl
Distilled water	ad 20 µl

Table 14: Composition of restriction digests reaction

After incubation of the reaction mixture at 37°C for 1 h per 5U of restriction enzyme, the reaction was inactivated by incubation at 75°C for 20 min.

3.2.2.8 Agarose gel electrophoresis

PCR fragments and/or digested DNA were electrophoretically separated on 1-2% (w/v) analytical agarose gels prepared in TAE buffer containing 0.1 g/ml ethidium bromide.
1x TAE buffer (3.1.3) was used as running buffer. DNA samples were mixed with 6x loading buffer (3.1.3) and loaded onto the gel. 500 ng of DNA molecular marker (Invitrogen) were used for evaluation of sample size and integrity. The DNA was visualised on an UV transilluminator at 302 nm and documented by a gel documentation instrument (Intas). Interested DNA bands were cut out from agarose gel using a sterile scalpel. DNA was isolated from cut gel using GFX PCR DNA kit or in some cases Gel Extraction Kit (Qiagen). After isolation and purification, DNA samples were electrophoretically separated on agarose gel for controlling. After that, DNA concentration was measured by UV-spectrophotometry.

3.2.2.9 Sequencing of DNA

All sequencing of plasmids was performed by MWG Biotech. The sequence data were compared using sequence comparing programme, BLAST (Basic Local

Alignment Search Tool) in EMBL (European Molecular Biology Laboratory)-Databank (http://www.ncbi.nlm.nih/gov/Blast/).

3.2.2.10 Determination of DNA concentration

For determination of DNA concentration SmartSpec3000 UV-Spectrometer (BioRad) was used. DNA samples were diluted as 1:100 with distilled water, which used also as blank and 100 µl of diluted DNA samples were placed into micro-quartz cuvetes. Absorbance was measured for double strand DNA using UV-light by 260 nm wavelength.

3.2.2.11 Construction of pcDNA3.1(-)/ORM expression vector

For overexpresssion of ORM in HDMECs via nucleofection technology, the expression vector pcDNA3.1(-)/ORM was constructed. For this, the full-length cDNA encoding ORM was cloned into the *Xho*I and *Hind*III restriction sites of the expression vector pcDNA3.1/Hygro(-) (Clontech). Firstly the cDNA encoding for ORM gene was amplified by PCR from cDNA of human granulocytes using gene specific primers. Subsequently the PCR product was ligated into the 2.1-TOPO® vector.

3.2.2.12 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR), a procedure for rapid in vitro enzymatic amplification of a specific segment of DNA, was used for the amplification of cDNA encoding ORM. cDNA of granulocytes which obtained as a gift from the Department of Clinical Chemistry of University Hospital Hamburg-Eppendorf, was used directly as a template for PCR to amplificate ORM genes present in granulocytes.

Components	Concentration / Volume
DNA	1 µl
dNTP	10 mM
$MgCl_2$	50 mM
Primer	20 pmol each
Taq DNA polymerase	1 U
10xPCR Buffer	1x
Distilled water	ad 25 µl

Table 15: Composition of PCR reaction

Step	Temperature [°C]	Time
Initial denaturation	95	10 min
35 Cycles:		
Denaturation	95	45 sec
Primer annealing	60	45 sec
Elongation	72	1 min
Final elongation	72	10 min

Table 16: PCR conditions for ORM

The reactions were performed in 0.2 ml PCR tubes (Biozym), using a PCR thermocycler (MWG Biotech). Specific primers annealing to the vector backbone were used for PCR reaction (Tab. 15). GAPDH was used as control reaction. Thermocycler conditions were used as described in table 16. The PCR products were analysed on a 1% agarose gel (3.2.2.8) and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) (3.2.2.4).

3.2.2.13 Ligation of PCR product into pCR® 2.1-TOPO® Vector

The PCR product coding for ORM gene was purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). For direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector, TOPO TA cloning Kit (Invitrogen) was used. The pCR® 2.1-TOPO® vector provided with kit was used as linearized with single 3´-thymidine (T) overhanging 3´-deoxythymidine (T) residues which allows PCR inserts to ligate with the vector.

Insertion of PCR product into the TOPO vector was performed according to manufacturer's protocol provided with kit. PCR product and TOPO vector were mixed and incubated at RT for 5 min. TOPO cloning reaction was transformed into chemically competent cells and single colonies were selected to isolate DNA.

The selected single colonies were cultured in LB-medium overnight and DNA was isolated using protocol from Nucleospin® Plasmid kit. After DNA concentration measurement, DNA samples were digested at *HindIII* and *XhoI* restriction site and electrophoretically separated on agarose gel. One of positive clones was selected and used for subcloning of *TOPO/ORM* into the expression vector as next step.

3.2.2.14 Subcloning into pcDNA3.1(-)

The pcDNA3.1(-) is 5.4 kb vector designed for high-level stable and transient expression in mammalian cells. The pcDNA3.1(-) expression vector and *TOPO/ORM* plasmid vector were digested at *HindIII* and *XhoI* restriction site and after extraction of digested pcDNA3.1(-) and ORM insert from agarose gel ligation reaction were performed using the T4-Ligase. Ligation reaction was incubated overnight at 16 °C and directly transformed into *E. coli* strains DH5α or XL1. Single colonies on LB-agar plates were selected and analysed for the presence of insert by *HindIII* and *XhoI* restriction digestion. A transformant which has correct restriction pattern was selected and sequenced by MWG Biotech to confirm that ORM gene was cloned in the proper orientation.

3.2.2.15 siRNA construction

For ORM gene silencing via small interfering RNA (siRNA) the Silencer™ siRNA Construction Kit (Ambion) was used.

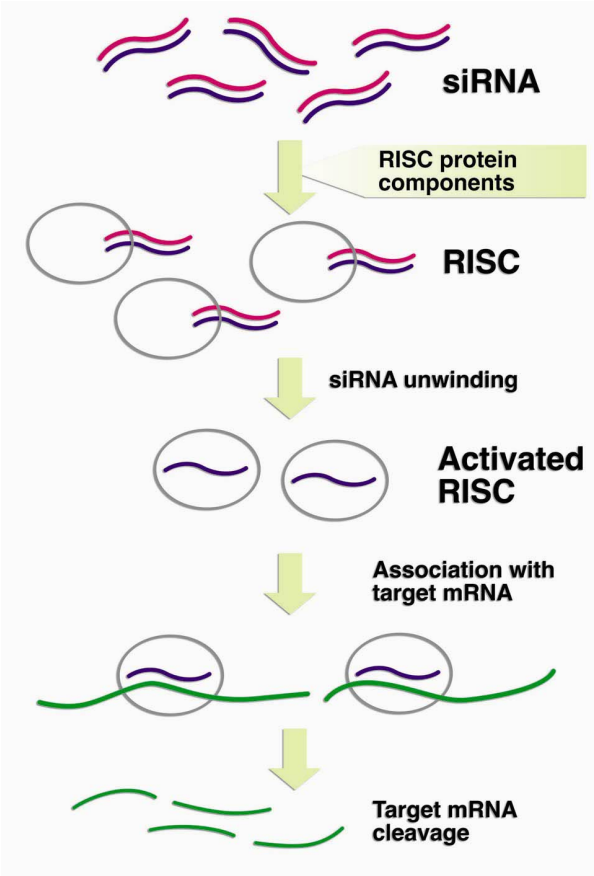


Figure 18: Mechanism of gene silencing via siRNA

The siRNAs are 21-23 nucleotides double-stranded RNAs, which induce post-transcriptional gene silencing. After introducing the siRNAs into the cells, they were assembled with protein components into a RNA-induced silencing complex (RISC). An ATP-generated unwinding of the siRNA activates the RISC, which in turn binds to the homologous transcripts by base pairing interaction and cleaves the mRNA. This sequence specific degradation of mRNA results in gene silencing (Fig. 18).
Following target regions for the siRNA sequences are selected from the cDNA of ORM according to the guidelines described by Elbashir et al. (Elbashir et al., 2001). Target sequence (cDNA) from the firefly luciferase gene was chosen as a control for ORM silencing studies.

3.2.3 Cellbiological methods

General cell culture work was performed under sterile conditions under a laminar flow hood using disposable plastic ware.

3.2.3.1 Culturing of cells

Cell lines (Tab. 6) were placed in polystyrene culture flasks 25 cm^2 (T25) and 75 cm^2 (T75) (Falcon) provided with 0.2 µm hydrophobic vent caps and maintained in a humidified 5% CO_2 atmosphere in an incubator (Hera Cell 240) at 37°C. All culture media (Tab. 7) were sterilized by filtration. Glass wares were sterilized by heating at 180°C for 8 h, and plastic wares were autoclaved at 120°C and 1.2 bar for 20 min. Adherent cells were cultured until a confluence of 70-90%. Medium was removed and after two times PBS washing, the cells were incubated with trypsin for one minute at 37°C and the reaction was stopped by addition of double used trypsin volume of culture medium. Cell suspension was harvested by centrifugation (1200 rpm /5 min/ RT). The supernatant was removed and cells were washed by resuspension in PBS with subsequent centrifugation. For subculturing cells were diluted approximately 1:5 fold with fresh medium.

3.2.3.2 Freezing and thawing of cells

500,000 viable human dermal microvascular endothelial cells (HDMECs) (Tab. 6), derived from dermis and cryopreserved in 1ml serum-free freezing medium, after thawing were obtained from PromoCell. HDMECs were cultured and passaged until

passage four, and then used for experiments. HDMECs were not used after passage eight and were also not frozen and stored. For all experiments thawed and viable HDMECs were shipped from PromoCell.
RT4 cells (Tab. 6) were harvested at a concentration of $0,5x10^6$ cells/ml, washed with PBS and resuspended in 1 ml ice cold cryo-conservation medium. The cryovials (Nunc) with 1 ml cell suspension each were placed in a -80°C freezer and transferred to liquid nitrogen for long-time storage.
Cryopreserved cells were thawed rapidly in a 37°C water bath, transferred to 20 ml prewarmed medium and centrifuged (1200 rpm/ 5 min). Supernatant was discarded and cells were seeded at high density in culture flasks (25 cm^2 and 75 cm^2, respectively).

3.2.3.3 Determination of cell number

For determination of cell number 10 µl of the cell suspension was mixed with 90 µl trypan blue solutions. After 3 minutes of staining, only dead cells turn blue, while living cells remain unstained. The latter were counted in a Neubauer chamber. The numbers of cells were calculated according to the equation below.

Number of cells/ml = number of cells over a large square x dilution factor x 10^4

3.2.3.4 Cell extraction

Cell lysates of HDMECs and RT4 cells were prepared by chemical lysis. The cells in culture were washed two times with cold-PBS and after third adding of cold-PBS the cells were scraped using a scraper on ice. Cell suspensions were collected into a falcon tube and centrifuged at 1200 rpm for 6 min. Supernatant was removed and cell pellets were washed three times more with ice-cold PBS. RT4 cell pellets were lysed in RIPA-Lysis buffer (3.1.3). HDMECs pellets were lysed in Triton X-100-SDS Lysis buffer (3.1.3). After adding lysis buffer onto the cell pellets, cell suspensions were mixed first by pipetting followed vortexing. Cell lysates were incubated on ice for 30 min and after a short vortex, incubated at RT for 10 min. After that, the cell lysates were centrifuged at 15,000 rpm for 15 min at 4ºC. Supernatant were collected and stored at -80ºC until further analyses. Protein concentration was determined using BCA protein assay kit (Pierce).

3.2.3.5 Transfection of HDMECs via Nucleofector

HDMECs transfection was performed using the Nucleofector™ technology (Amaxa Biosystems). Nucleofector™ technology offers a non-viral alternative to primary cell transfection, which bases on a unique combination of electrical parameters and cell-type specific solutions. HDMECs were transfected using HMVEC-L Amaxa Nucleofection Kit along with program S-05 on the Nucleofector device according to the optimized protocol of manufacturer (Amaxa). After reaching 70% confluency, the cells were passaged 3-4 days before nucleofection. The cells after passage ten were not used for nucleofection because it may lead to lower gene transfer efficiencies and trypsin treatment is more difficult and may damage the cells. For transfection, cultivated cells were harvested by trypsinization and count to determine cell density. $5x10^5$-$1x10^6$ cells per nucleofection were centrifuged at 1200 rpm for 10 min and resuspended in 100 µl Nucleofector solution and mixed with 2 µg DNA or siRNA. Cell suspension was transferred into an Amaxa certified cuvette and inserted into the cuvette holder. After running the adequate program in the Nucleofector, 500 µl of full medium was added to the cells and the sample could be transferred into a prepared culture flask.

3.2.3.6 Overexpression/ gene silencing of ORM in HDMECs

To overexpress ORM in HDMECs the cells were transfected with the expression vector pcDNA3.1(-) ORM, in which the 770 bp full-length cDNA of ORM was cloned into the *XhoI* and *HindIII* restriction sites of the expression vector pcDNA3.1/Hygro(-) (Clontech). Empty vector pcDNA3.1(-) was used as a negative control.

ORM gene was silenced using constructed siRNA-ORM (3.2.2.15). The siRNA-luciferase was used as negative control. 2 µg of plasmid DNA or siRNA were transfected into the HDMECs using nucleofector technology (3.2.3.5). Transfected HDMECs were cultured for 1-2 days and used for further studies. After 2 days supernatant of transfected cells were collected and stored at -20°C and for later western blot analyses. Supernatants which were later used for tube formation assay (3.2.4) were centrifuged at 1200 rpm for 6 min to remove dead cells. Supernatant was taken and sterilised using 0.2 µm filter. To see if overexpression and gene silencing are effective at the protein level WB analyses were performed for supernatant and extract of transfected cells. Two days after transfection, cells were

lysed (3.2.3.4) and used for western blot analyses (3.2.1.14). After protein concentration measurement, same amount of total protein were loaded onto SDS-PAGE to compare ORM level for each sample. The same blot was then striped by striping buffer (3.1.3) and incubated with anti vimentin antibody as control for same amount of loaded protein.

3.2.4 Endothelial tube formation assay

This assay was carried out using three-dimensional type I collagen gel (PureCol, Inamed) which was prepared in 48-well cluster tissue culture dishes as described previously (Ergun et al., 2000; Oliveira-Ferrer et al., 2004; Pepper et al., 1990).
48-well plates were coated with 190 µl of ice-cold collagen gel mixture (3.1.3) and incubated over night at 37°C. After polymerization of the gel, wild type ORM-overexpressing and ORM-silenced endothelial cells (HDMECs) were seeded onto solidified gels at a concentration of $4x10^4$ cells /well in 300 µl of growth medium containing 5% FCS. At confluence, the medium was replaced by basal medium containing 2-4% FCS without further supplements. After 24 hours VEGF-A was added to the wells and was renewed every 3 days after taking photographs with phase contrast microscopy (Zeiss, Jena). The collagen gels containing endothelial tubes of interest were fixed overnight with Bouin´s fixative solution following overnight incubation with 70% ethanol and two times ethanol incubation for 1 h. The gels were left out from 48-well using a scalpel and embedded in paraffin to use for immunohistochemical studies.

Stimulating Factors	Description	Working concentration	Supplier
ORM	Orosomucoid , human serum	1. 100 ng/ml 2. 300 ng/ml	Calbiochem
AntiPAI-1	Anti-human PAI-1 monoclonal antibody (from mouse)	15 µg/ml	Oxford Biomedical Research
VEGF-A	Recombinant Human VEGF-A	50 ng/ml	Immunotools

Table 17: The stimulating factors used for tube assays

To find out if PAI-1 or/and the ORM-PAI-1 complex has any function modulating effects on VEGF-A induced endothelial tubes, endothelial cells was seeded into collagen gel and incubated until confluent. Afterwards cells were left untreated or stimulated with; (*i*) VEGF-A (positive control), (*ii*) ORM, (*iii*) VEGF-A together with ORM, (*iv*) VEGF-A and anti-PAI-1 antibody, (*v*) VEGF-A, ORM and anti-PAI-1

antibody, (*vi*) anti-PAI-1 antibody alone (Tab. 17). Endothelial cells and VEGF-A induced endothelial tube formation was examined and photographed day by day using phase contrast microscope (Zeiss, Jena) equipped with a digital camera (ProgRes^{TM}C10^{plus}, Jenoptik).

3.2.5 Histological methods

3.2.5.1 Fixation and HE staining for tissue samples and cells

Paraffin-embedded bladder tissue sections obtained from patients who have clinically been diagnosed to have bladder cancer as well as normal bladder tissue, obtained by biopsy, sections were used for immunohistochemical analyses. The bladder tissue sections used in this study were obtained from department of Pathology of University Hospital Hamburg-Eppendorf. Before immunohistochemical staning the sections were stained with Hematoxylin-Eosin to confirm presence of cells on tissue sections. Slides containing paraffin sections were deparaffinised by incubation in order as three times xylene, isopropanol, 96% and 70% ethanol, and lately deionized water for 5 min. After that, the sections were stained with Hematoxylin after a short washing with water, destained with acid ethanol and washed again with water. For cytoplasmatic staining the sections were treated with Eosin and dehydrated by 96% ethanol and subsequently isopropanol incubation.

3.2.5.2 Immunohistochemistry

Each section was deparaffinised (rehydrated) in descending alcohol row and incubated in 1xPBS. Then the sections were subjected to immunohistochemistry. After treatment with normal rabbit or normal swine serum to block the unspecific background for 30 min at RT, the sections were incubated with the corresponding primary antibodies against ORM and/or ZAG diluted in PBS-BSA-NaN_3 buffer (3.1.3) for 24 h at 4°C. The sections were washed with 1xPBS for 3x10 min and exposed to the secondary antibodies for 1 h at RT. Then washed again and treated with the peroxidase-anti-peroxidase (PAP) for 30 min at RT. Subsequently, the sections were treated with ABC-complex (Vector) for 30 min at RT, washed again for 2x10 min with 1xPBS and transferred to the solution for the immunostaining development. For the control reaction, same samples were incubated only with secondary antibody.

Peroxidase activity was enhanced and developed by means of the modified nickel-glucose oxidase technique (Ergun et al., 1997). Glucoseoxidase solution was prepared freshly in dark according to the receipt described in section 3.1.3. After washing with 1xPB buffer (3.1.3) for 10 min, the sections were incubated with glucoseoxidase solution for 10-30 min at RT. Developing was followed by microscope. After the staining became clear, the reaction was stopped by removing the developing solution followed washing 3x5 min with PBS.
Sections were counterstained with Calcium Red for 20 sec at RT and washed 2x5 min with distilled water. The sections were dehydrated by incubation in order as deionized water, 70%, 96%, 100% ethanol and three times xylene for 5 min. After that, were embedded using histomount (Thermoshandon). In some cases Mowiol (Calbiochem) was used directly after Calcium red staining to embed the sections. Using the microscope system equipped with a digital camera the immunostaining were studied and photographed.

3.2.5.3 Immunocytochemistry

Wild type cells were seeded and cultured in chamber slides. After fixation with 4% paraformaldehyde for 15 min and washing 3 times with 1xPBS, the cells were incubated in normal serum to block the unspecific background. The cells were incubated with primary antibody for 24 h. After washing in 1xPBS the cells were incubated with biotinylated secondary antibody. Afterwards, they were washed in PBS and incubated with peroxidase (PAP). After washing the slides were incubated in avidin-biotin complex (ABC Kit, Vector). The immunostaining were developed using Nickel enhanced-glucoseoxidase technique (Davidoff et al., 1990) as described in immunohistochemistry section. If counterstaining is needed the sections were stained in Calcium Red for 1-2 min. Using a microscope system equipped with a digital camera the immunostaining were studied and photographed. Using the same method we have studied the localization of ORM and known angiogenic factors and their receptors on endothelial tubes induced in vitro after ORM overexpression versus ORM silencing in HDMECs. Endothelial tubes on collagen gel were fixed using Bouin´s fixative over night and embedded in paraffin. On 5-7 µm thick tissue sections immunhistochemistry were performed as mentioned above.

4 Results

4.1 Profiling of urinary proteins by 2D gel electrophoresis

4.1.1 Optimization of the sample preparation method for 2D gel electrophoresis on human urine

In order to determine the changes in urinary protein patterns by presence of bladder cancer, 2-DE analyses were performed on urine samples of fourtyfive patients with bladder cancer of different stages (pTa (n=12), pT1 (n=8), pT2-3 (n=8), GI, GII, GIII), patients on follow-up (n=10) and healthy volunteers (n=7). The details of 2-DE analyses were described in the section of material and methods. Protein spots were visualized using Coomassie Brillant Blue or silver staining. Obtained 2-DE images were analysed by PDQuest software programme (BioRad). Several protein spots in coomassie stained 2-DE gel of a patient with bladder cancer were identified using mass spectrometric analyses, which were not present in normal urine samples.

It is well known that urine contains trace amounts of protein originating from blood plasma, the kidneys and the urogenital tract, and amounts of body filtrates such as water, salts, electrolytes and nitrogenous waste products (Anderson and Anderson, 2002; Beetham and Cattell, 1993). Thus, the sample preparation of urine samples is very important for protein analysis and preliminary screening of biomarkers.

In order to optimize 2-DE for urine samples several techniques including protein precipitation with TCA or in some cases immunoprecipitation, ultracentrifugation and filtration were used for sample preparation. TCA precipitation led to the vertical streaks on 2-DE gel probably due to residual TCA in protein sample and difficulties in resolubilization of proteins (data was not shown). Immunoprecipitation was used for detection of a specific protein and to determine changes in those proteins which suppose to be related to bladder cancer. However, immunoprecipitated proteins caused horizontal streaks and dark background on 2-DE gels probably due to the presence of remained immunoglobulin G leading to over loading of protein amount. Ultrafiltration was performed on native urine samples using Centrifugal Filter Column (Amicon) to remove high abundance of interfering molecules containing salts, electrolytes and/or some nitrogenous waste products, which disturb the electrophoresis process especially in the first dimension of 2-DE. After that, different rehydration buffers were used to solubilise filtrated and concentrated urine proteins. The efficiency of each buffer was compared to each other. Ultrafiltration, precipitation methods and different rehydration buffers were applied on urine samples and

compared each other to find the best method for sample preparation of urine samples. The use of optimized sample preparation methods (see 3.2.1.3) revealed clear detectable protein spots and reduced background and streaks.

The 2-DE protein pattern of urine sample of a patient with superficial bladder cancer is exemplary shown in figure 19. IPG strip with 17 cm length and 3-10 pH range was used for this urine sample and the protein spots were visualized using silver staining protocol. Many spots representing different proteins and focused particularly in the pH-spectrum ranging from pH 5 to 8 were seen in the silver stained 2-DE gel.

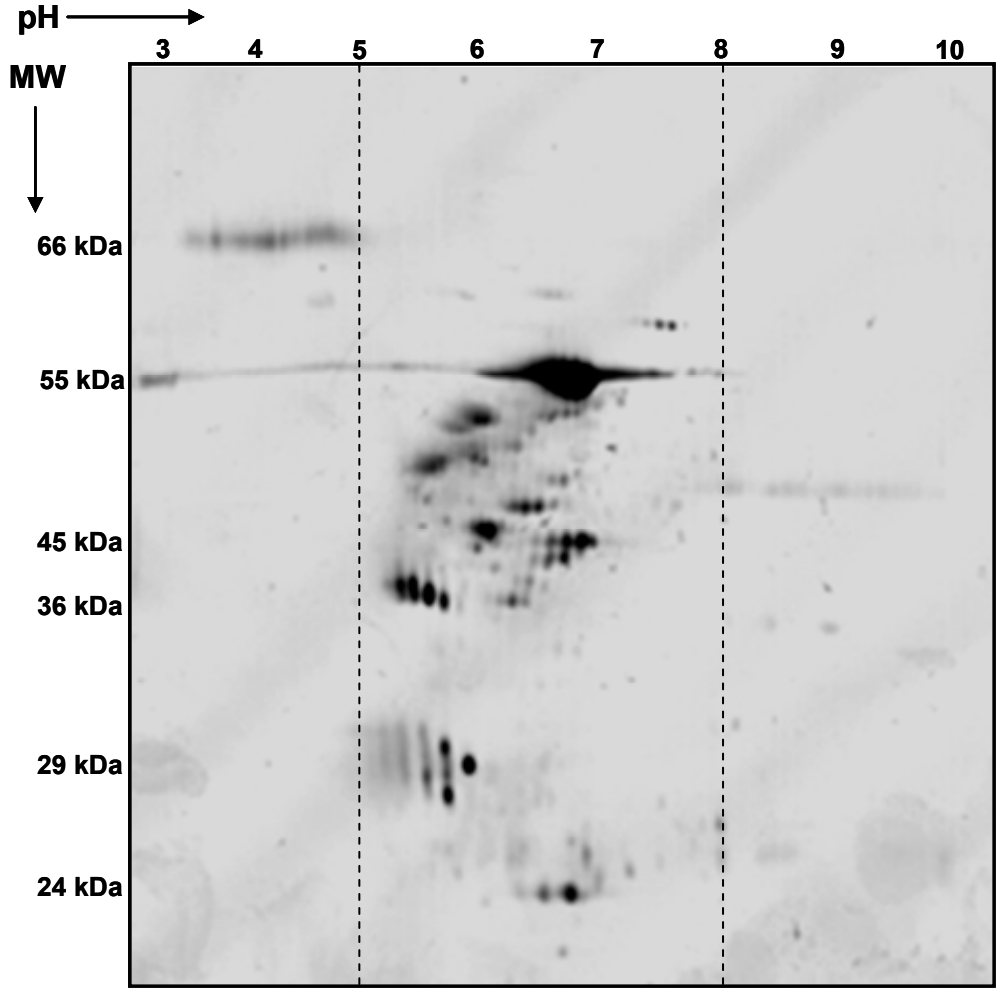


Figure 19: Protein pattern in urine sample of a patient with bladder cancer of the stage pTa. Urine sample containing 200 µg total proteins were analysed by 2-DE using 17 cm strip with pH range 3-10. Silver staining revealed numerous spots, particularly focused in the pH range 5-8.

4.1.2 Determination of urinary protein pattern in relation to bladder cancer by 2-DE

Urine samples were subdivided into three groups: urine samples of (*i*) healthy persons, (*ii*) patients with bladder cancer of different stages, and (*iii*) follow-up patients.

Firstly, it was aimed to find out possible changes in whole protein pattern of urine samples, which thought to be related to bladder cancer. To this aim the urinary protein patterns of healthy volunteers to that of patients with bladder cancer of different stages such as pTa, pT1, pT2 and pT3, as well as of patients on follow-up were compared to eachother. Several spots, which were present in urine samples of patients with bladder cancer and in those of follow-up, were not present in urine samples of healthy persons (Fig. 20-25). These protein groups were indicated with circles and arrows.

4.1.3 Protein pattern of normal urine versus urine of patients with pTa

Native urine samples obtained from healthy persons (n=7) and patients with bladder cancer of stage pTa (n=12) were analysed by 2-DE technique. Urine samples containing 200 µg total protein were loaded on IPG strips with 11 cm length and 3-10 (NL) pH range for the first dimension of 2-DE protocol. Protein spots were visualized using silver staining (3.2.1.9).

Comparison of urinary protein pattern of healthy volunteers (Fig. 20A) and patients with bladder cancer of stage pTa, GI (Fig. 20B) revealed big differences in pattern and intensity of protein spots. 2-DE images of urinary protein of different healthy volunteers revealed almost the same protein pattern as shown in figure 20A, 21A, 22A. The strongest spots were seen at the sizes of 55 and 66 kDa. Using the same protocols and conditions, 2-DE analyses on a urine sample of pTa patients resulted in protein patterns showing many additional protein spots (Fig. 20B). Compared to healthy urine, the number and the density of detectable protein spots were higher in urine samples of pTa patients. The proteins which were seen as faint spots in normal urine were also present in urine sample of patients but in significantly higher amount.

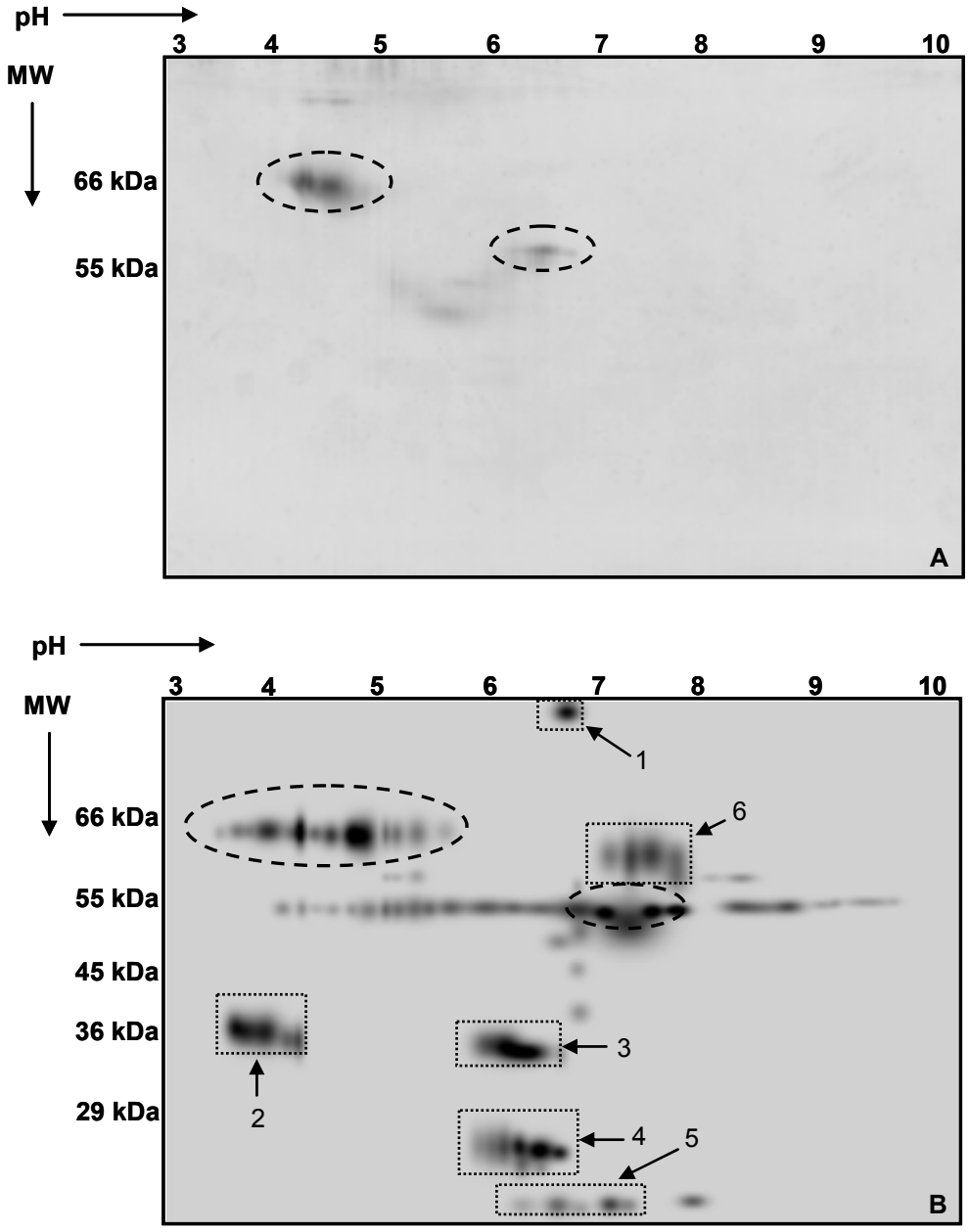


Figure 20: Comparison of protein pattern in normal urine (A) and in urine of patients with bladder cancer of the stage pTa,GI (B). Urine samples containing 200 µg protein were loaded on 11 cm IPG strips with pH range 3-10 non linear (NL). Protein spots were visualized by silver staining. 2-DE image of urine sample of a healthy volunteers (A) and urine of a patient with bladder cancer of the stage pTa, GI (B) are shown. The protein spots at 55 and 66 kDa indicated by circles are similar in both urine samples, but significantly stronger in urine samples of bladder cancer (B). In urine samples of bladder cancer patients there are numerous additional spots as numbered (1-6).

4.1.4 Protein pattern of normal urine versus urine of patients with pT1

Using IPG strips with 11 cm length and 3-10 NL pH range, native urine, containing 200 µg total protein, of patients with bladder cancer of pT1 stages (n=8) were analysed by 2-DE. The 2-DE gels were stained using silver staining protocol. Protein pattern of the urine sample obtained from a healthy person revealed two different

protein spots at 55 and 66 kDa (Fig. 21A) similar to healthy urine which was shown in figure 20A. The urinary protein pattern of a patient with bladder cancer of stage pT1,GIII revealed several additional protein spots, compared to normal urine (Fig. 21B).

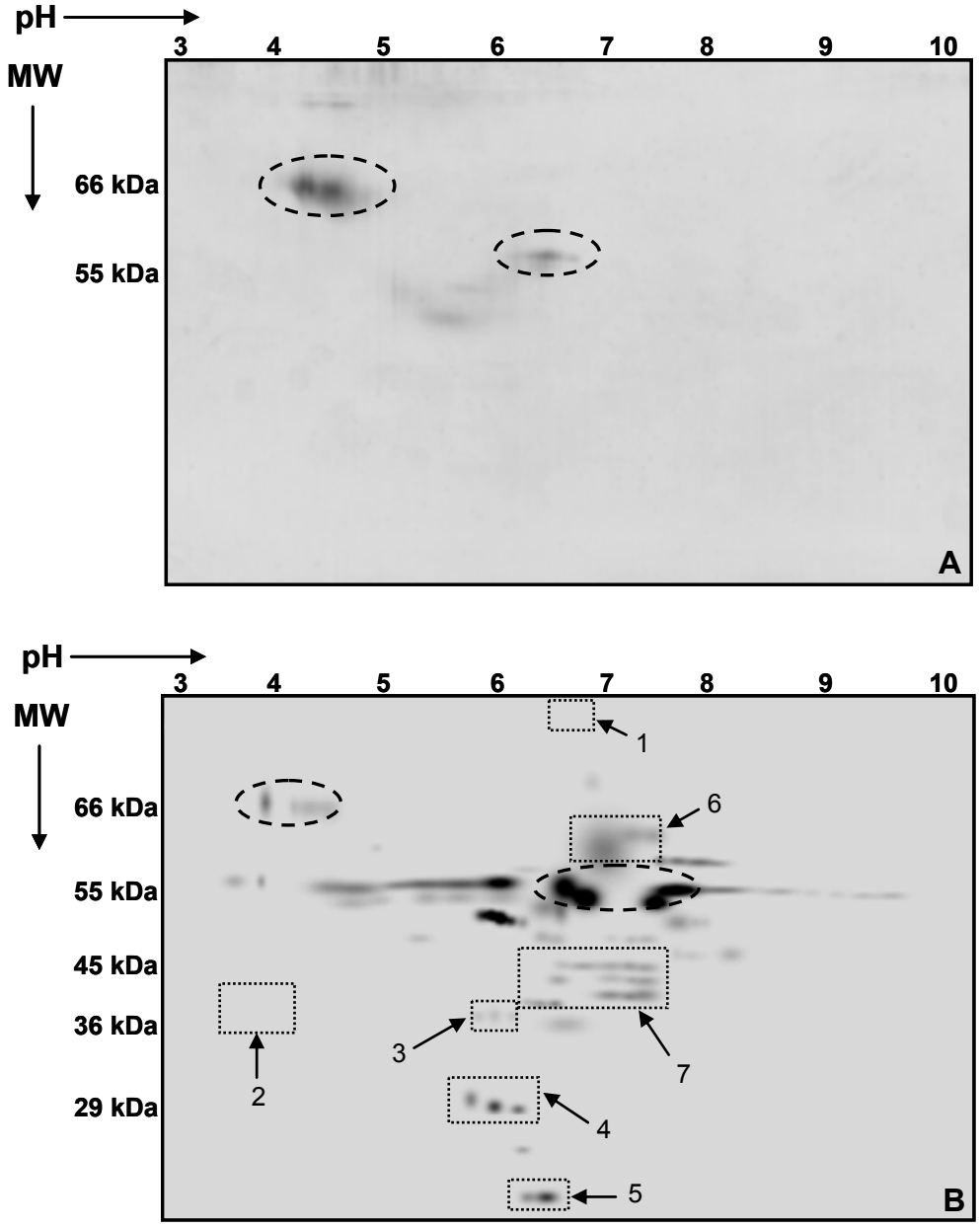


Figure 21: Comparison of protein pattern in normal urine (A) and in urine of patients with bladder cancer of the stage pT1, GIII (B). Urine samples containing 200 µg total proteins were loaded on 11 cm IPG strips with pH range 3-10 NL. Protein spots were visualized by silver staining. The protein spots indicated by cycles in healthy urine (A) and in urine of patient with bladder cancer of the stage pT1, GIII (B) are similar. There are additional spots which were numbered and indicated by doted sequares (1-7) in urine samples of patients. Among marked protein spots, especially the protein groups at 55 kDa are stronger in urine samples of patients (B).

The spots at 55 and 66 kDa, which were also present in normal urine, were seen in urine of pT1 patients too. But the protein group at 55 kDa was present in urine of pT1 patients in a significant higher amount than that in normal urine. Spot 1 and spot 2, detected in urine of pTa patients, were not present in urine of pT1 patients and also were not present in normal urine. Spot 3-6 were detected in urine samples of both patients, either pTa and pT1 bladder cancer but they were much stronger present in urine of pTa patients shown in figure 20B. In urinary pattern of pT1 patients, additional protein spots numbered as 7 were detected. Spot 7 representing several different proteins, at 35-45 kDa, was not present in the urine of samples pTa patients.

4.1.5 Protein pattern of normal urine versus urine of patients with pT2

Urine samples containing 200 µg total protein were obtained from patients with bladder cancer of invasive stages as pT2-3 (n=8) and were separated using 2-DE technique. For the first dimension IPG strips with 11 cm length and 3-10 NL pH range were used. The protein spots on 2-DE gels were visualized using silver staining protocol (3.2.1.9). Protein pattern of healthy urine (Fig. 22A) revealed the similar protein spots at 55 and 66 kDa as shown for urine samples of healthy volunteers in figure 20A and 21A. The 2-DE image obtained from analyses of urine sample of a patient of bladder cancer with stage pT2, GIII revealed protein spots at 55 and 66 kDa (Fig. 22B) which were also present in normal urine, as well as in urine of pTa and pT1 patients, but in comparison to the stages pTa and PT1, these protein spots were weaker in the urine samples of pT2 patients. The protein spot at 55 kDa was stronger in urine samples of patients with pT2 than the normal urine. Spot 1, which was not present in the urine samples of pT1 patients, was present in the urine samples of pT2 patients as well as in pTa patients. Spot 2 was not present in urinary protein pattern of pT2 patients, in similarity with that of pT1 patients. Spot 3 and 5, which were present in the urine samples of pTa and pT1 patients, were also detected in the urine samples of pT2 patients. Spot 4 and 6 were not detectable in urine of pT2 patients in contrast to the detection of these spots in urine samples of pTa and pT1 patients. Spot 7, which was seen only in the urine samples of pT1 patients (Fig. 21B), was not present in the urine samples of pT2 patient. Additional spots numbered as 8, 9 and 10 were present only in the urine samples of pT2 patients.

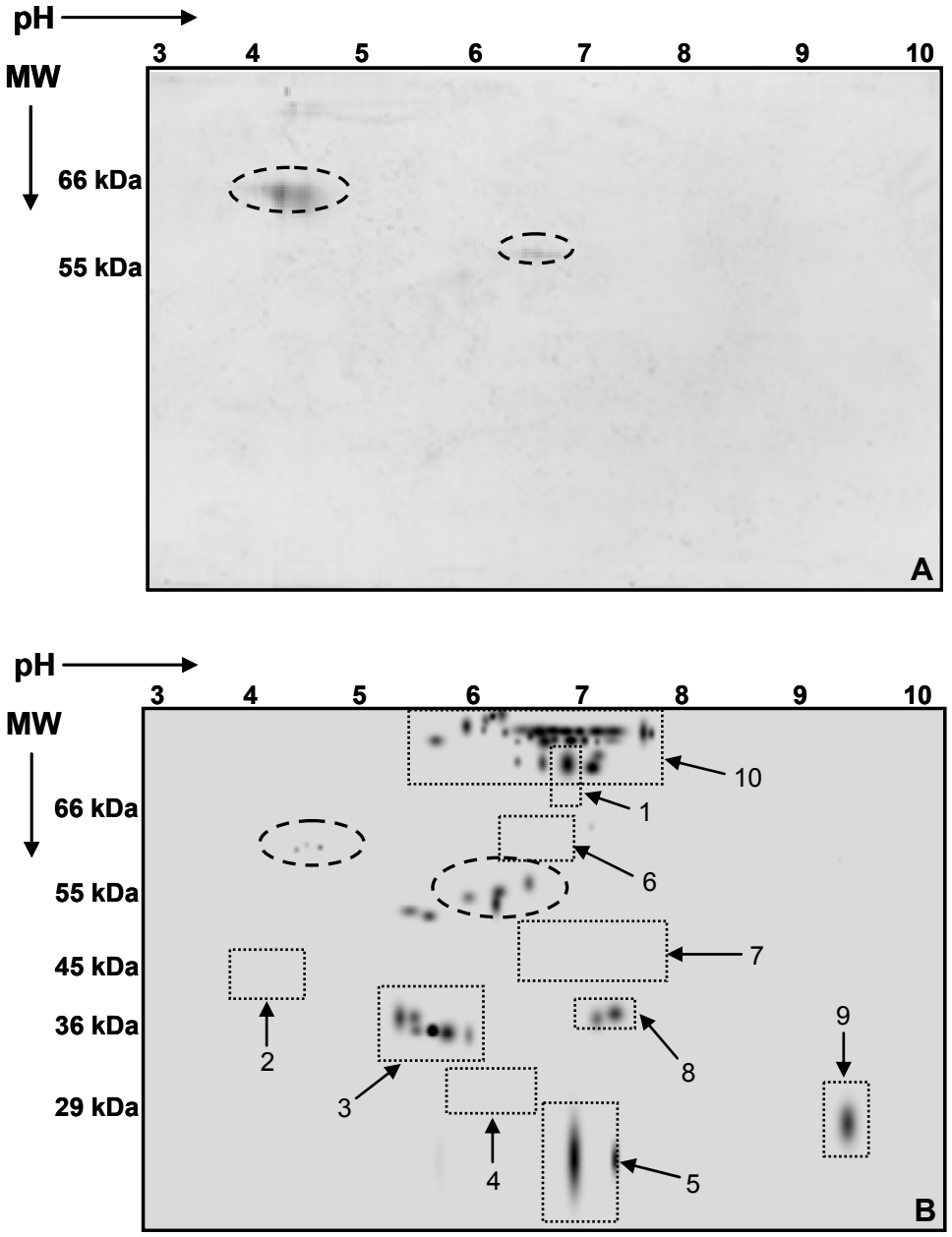


Figure 22: Comparison of protein pattern in normal urine (A) and in urine of patients with bladder cancer of the stage pT2, GIII (B). 200 µg urinary proteins were loaded on 11 cm IPG strips with pH range 3-10 NL. Protein spots were visualized by silver staining. The protein spots at 55 and 66 kDa indicated by circles are similar in both urine samples, healthy urine (A) and urine of a patients with bladder cancer of the stage pT2,GIII (B). In urine samples of patients with bladder cancer of the stage pT2,GIII additional protein spots and numbered as 1, 3, 5, 8, 9,10 were detected.

4.1.6 Protein pattern of urine samples of follow-up versus urine samples of bladder cancer pateints

In the next step, protein pattern of the urine samples of patients on follow-up (n=10) and patients with bladder cancer of different stage (pTa, pT1, pT2-3) were compared. For 2-DE analyses of urine samples obtained from patients on follow-up, IPG strips

with 11 cm length and 3-10 NL pH range were used. Native urine containing 200 µg total proteins were loaded and the gels were visualized using silver staining protocol.

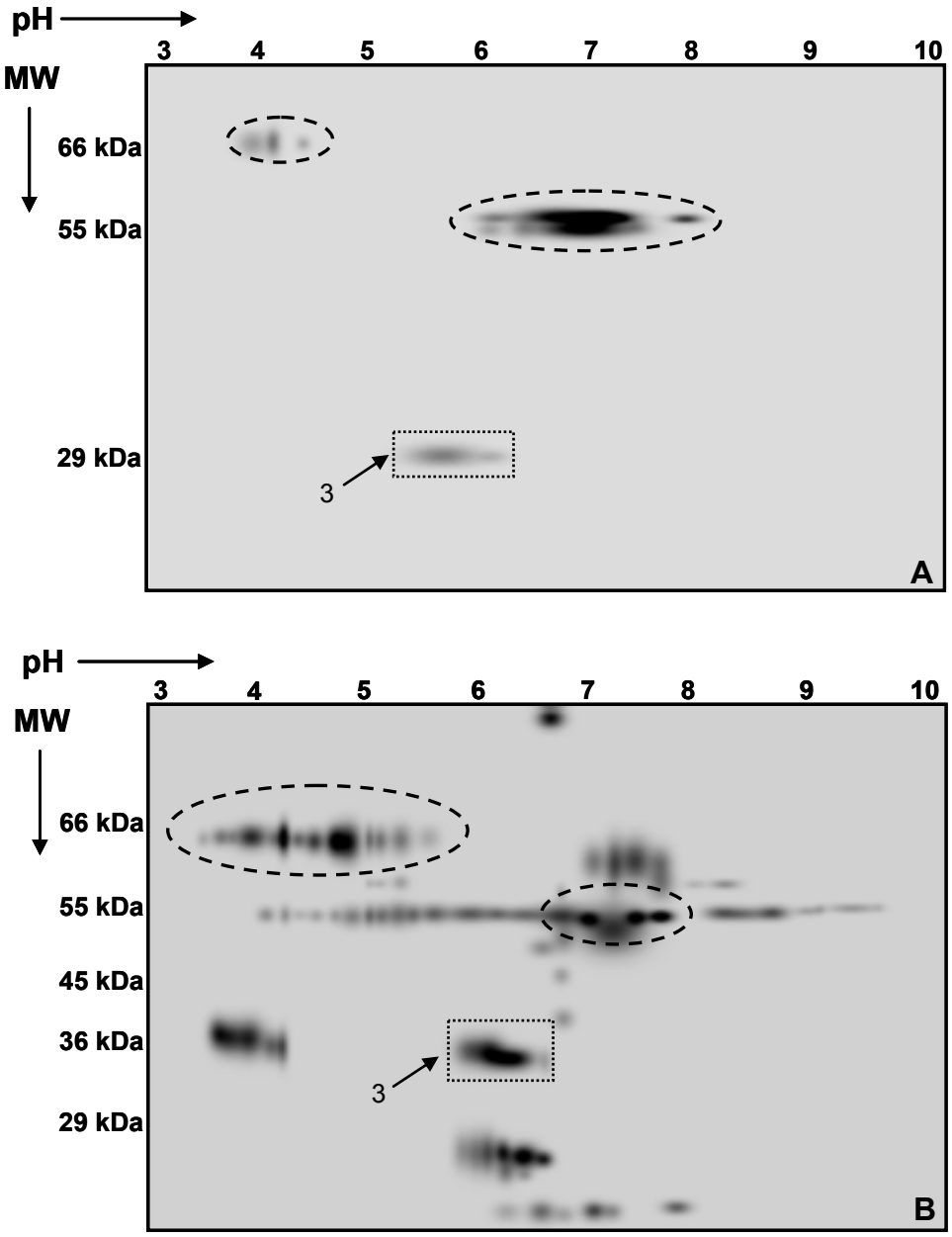


Figure 23: Comparison of protein pattern in urine of patients on follow-up (A) and patients with bladder cancer of the stage pTa, GI (B). 200 µg proteins, involved in urine samples, were loaded on 11 cm IPG strips with pH range 3-10 NL. Protein spots were visualized by silver staining. The protein spots, at 55 and 66 kDa, which are marked with circles, are seen in urine samples of patients on follow-up (A) and patients with bladder cancer of the stage pTa, GI (B). Additionally, the protein spot numbered as 3, which was detected in urine sample of patients with bladder cancer (shown in figure 21B, 22B, 23B) is also present in urine sample of patients on follow-up.

2-DE spots groups at 55 and 66 kDa marked with doted circles were detected in urine of follow-up patients with similarity to all urine samples used including urine samples of healthy persons and patients with bladder cancer. However, the density

of these proteins was stronger in urine samples of patients with bladder cancer of different stages and patients on follow-up than those in urine of healthy persons.

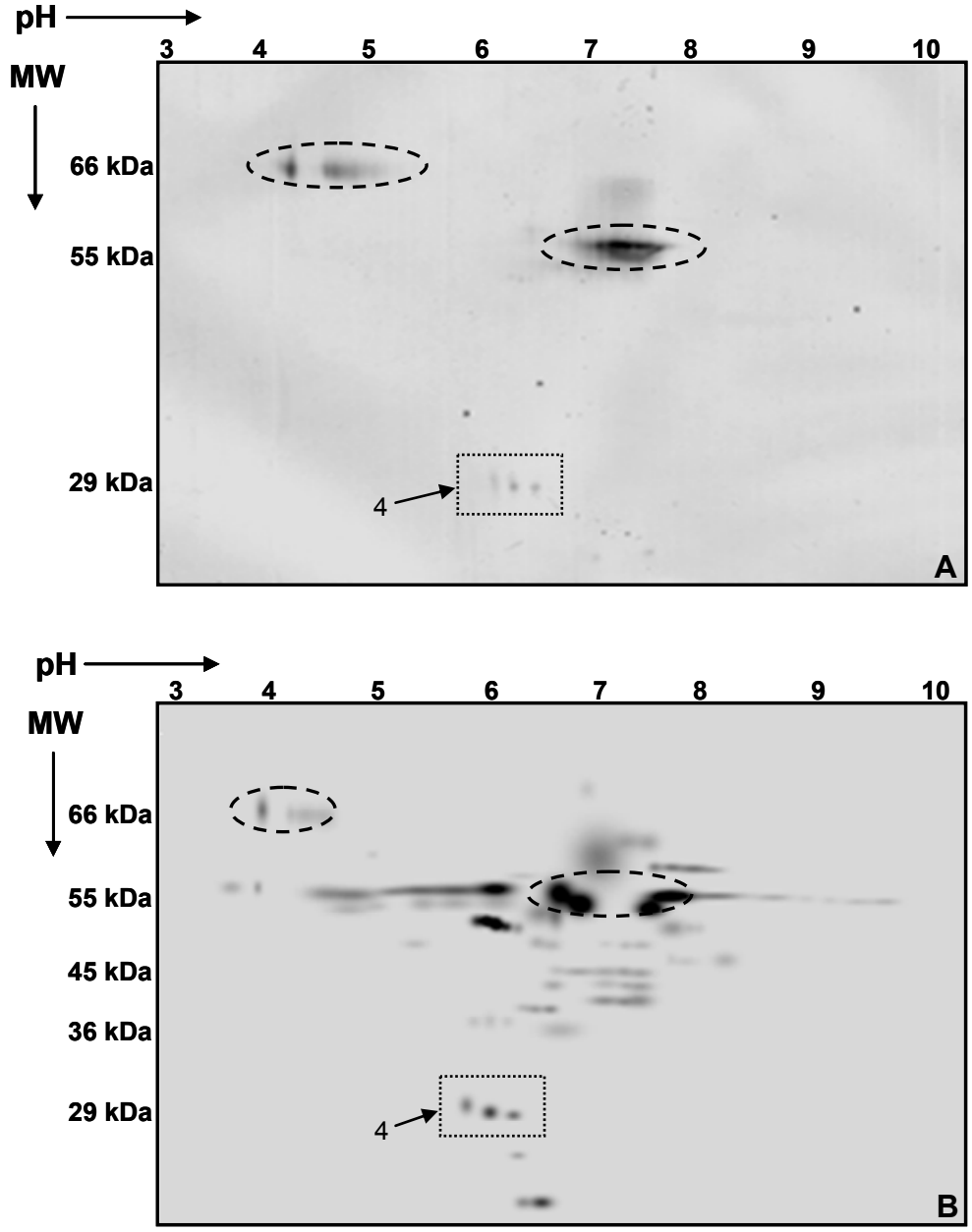


Figure 24: Comparison of protein pattern in urine of patients on follow-up (A) and patients with bladder cancer of the stage pT1, GIII (B). Urine samples containing 200 µg proteins were used for 2-DE analyses. 11 cm IPG strips with pH range 3-10 NL were used. For detection of proteins, silver staining was used. The spots groups, at 55 and 66 kDa, indicated by circles are similar in urinary protein pattern of patients on follow-up (A) and patients with bladder cancer of the stage pT1,GIII (B). The protein spots numbered as 4, which was seen in urine sample of pTa and pT1 patients (Fig. 21B, 22B), is also present in urine samples of patients on follow-up.

In comparison of urinary pattern of patients on follow-up (Fig. 23A, 24A, 25A) and patients with bladder cancer of different stage such as pTa,GI (Fig. 23B), pT1,GIII (Fig. 24B), pT2,GIII (Fig. 25B) revealed similar but also different spots in urinary

protein patterns. In 2-DE gels of urine samples of patients on follow-up, there are few detectable and faint stained protein spots in addition to protein spot groups marked by doted circles.

4.1.7 Protein pattern of urine samples of patients on follow-up versus that of patients of tumor stage pTa

As mentioned above the protein spots at 55 and 66 kDa were present in urine sample of follow-up patients as marked by doted circles (Fig. 23A). The densities of these protein groups seen in urine samples of patients on follow-up were as strong as in that of patients having bladder cancer. The additional protein spot numbered as 3 was detected in urine samples of patients on follow-up, which was not present in normal urine but prominently present in urine samples of patients with bladder cancer of stage pTa (Fig. 23B). Many additional spots representing different proteins, as shown and numbered for the same image in figure 20B, were detected in urine samples of patients with pTa, in comparison to urine samples of patients on follow-up.

4.1.8 Protein pattern of urine samples of patients on follow-up versus that of patients of tumor stage pT1

The protein spots at 55 and 66 kDa were present also in urine samples of another patient on follow-up as shown in figure 24A. These spots were marked as doted circles. The protein spot at about 30 kDa marked as number 4 was detected in urine sample of pateints on follow-up and also patients with bladder cancer of pT1 (Fig. 24A,B). The same protein spot was also present in urine sample of pTa patients and was indicated with the same number in figure 20B. The density of spot 4 was higher in urine sample of the patients with bladder cancer stage pT1 (Fig. 24B) then the follow-up patients. But the additional protein spots, which were numbered for the same image in figure 21B, were seen only in the urine samples of patients with bladder cancer.

4.1.9 Protein pattern of urine samples of patients on follow-up versus that of patients of bladder cancer stage pT2

The protein spot at 66 kDa as marked by doted circle was not present in urine sample of this follow-up patient as shown in figure 25A. But the spot at 55 kDa was present in urine sample of a patient on follow-up (Fig. 25A) in a higher intensity as in

the urine sample of a pT2 patient (Fig. 25B). The protein spots numbered as 3, 4, and 5 were present in urine samples of both patients on follow-up and those with bladder cancer stage pT2/GIII.

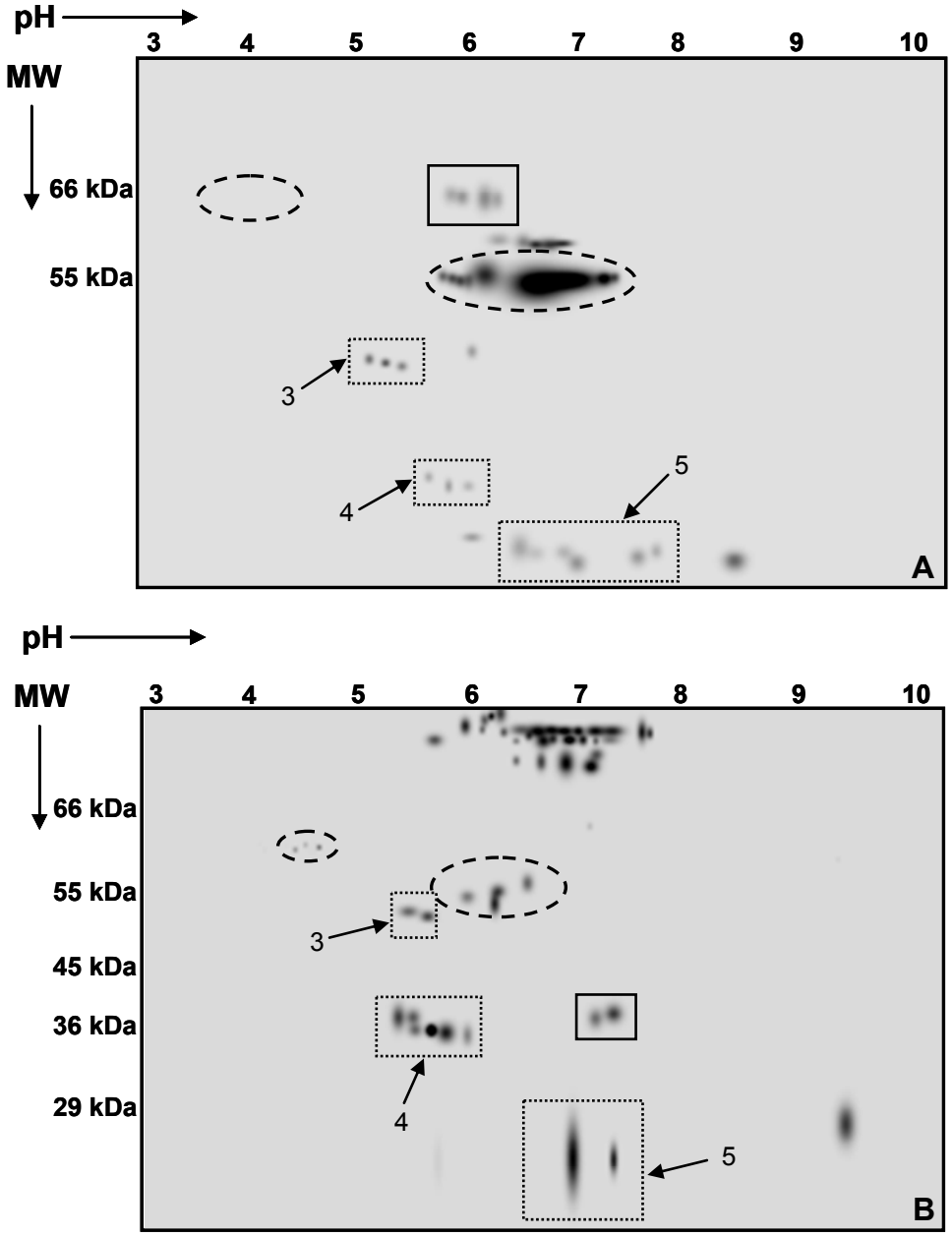


Figure 25: Comparison of protein pattern in urine of patients on follow-up (A) and patients with bladder cancer of the stage pT2, GIII (B). Urine samples containing 200 µg protein were loaded on 11 cm strips with pH range 3-10 NL. Silver staining was used for detection of protein spots. The protein spots seen at 55 kDa which marked by circles are similar in both sample, urine of follow-up patients (A) and and patients with bladder cancer of the stage pT2, GIII (B). The protein spot at 66 kDa, which was seen in other 2-DE images is not visible in urine sample of this follow-up patient (A). The protein spot marked by a fat square in figure A is not present in urine samples of pT2 patients (B). Another protein spot in figure B marked by fat square is not present in urine samples of follow-up patients. The protein spots numbered as 3, 4, and 5 are present in both urine samples.

The spot marked as fat square in 2-DE of urine sample of a patient on follow-up (Fig. 25A) was not present in urine sample of patient with bladder cancer stage pT2 (Fig. 25B). On the other hand, another protein spot ,marked by fat square, present in urine sample of a patient with tumor stage pT2 (Fig. 25B) was not seen in urine sample of a patient on follow-up (Fig. 25A). In summary, two different groups of spots at 55 and 66 kDa were present in all urine samples (Fig. 20-25), but they were particularly prominent in urine samples of patients with bladder cancer and patients on follow-up. Numerous additional proteins were detected in urine samples of patients with bladder cancer in comparison to other groups of urine samples, including healthy persons and follow-up patients, which were analysed in this study.

4.1.10 Identification of protein spots in urine samples of patients with bladder cancer

Proteins from urine samples of patients with bladder cancer stage PTa/GI were separated on a 2-DE gel and stained with both silver and CBB (Fig. 26).

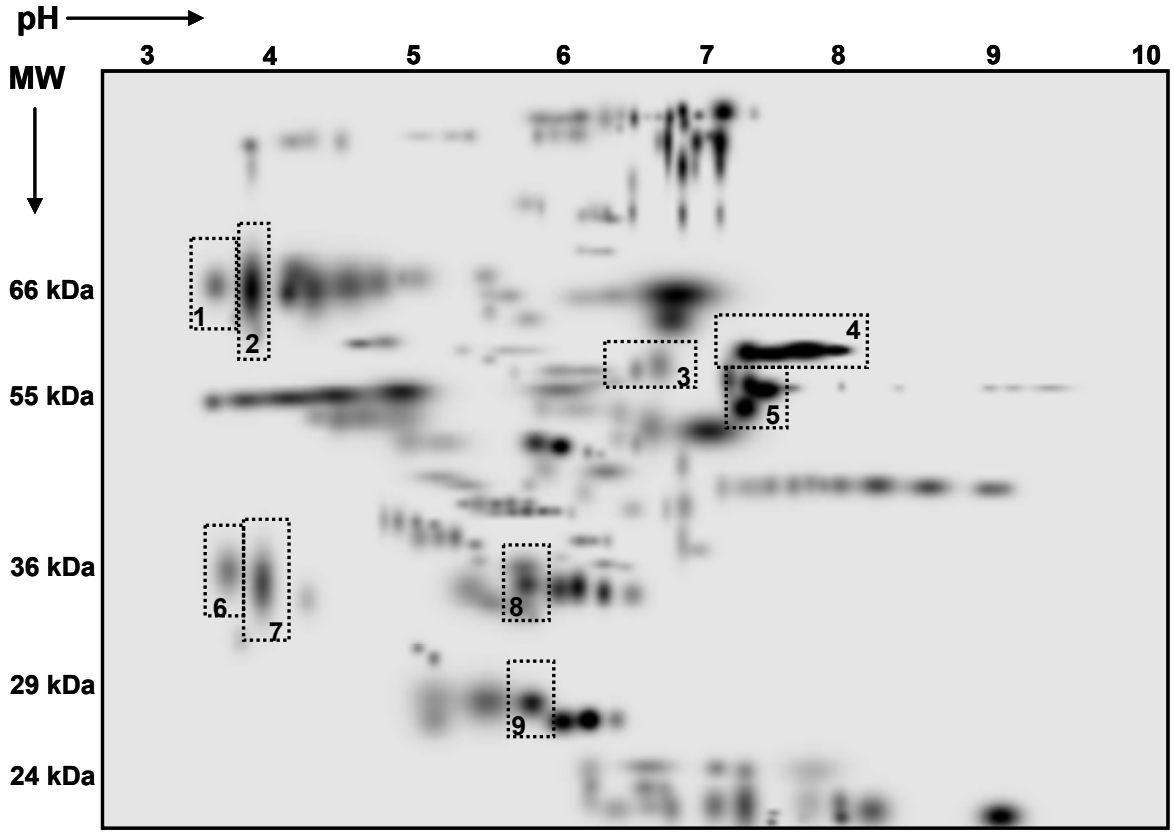


Figure 26: Protein identification from 2-DE image of urine of a patient with bladder cancer of the stage pTa/GI. The urinary pattern of a patient with bladder cancer of the stage pTa/GI was chosen for protein identification. Nine protein spots indicated by quadrates in Coomassie stained 2-DE gel were cut out and subsequently underwent mass spectrometric analyses.

Several protein spots observed in urine samples of patients with bladder cancer of the stage PTa/GI were cut out from the coomassie stained gel and underwent mass spectrometric analyses. After these studies, several proteins called as uromodulin, DNA-binding glycoprotein, human serum albumin (HSA), orosomucoid1 (ORM), human zinc-alpha-2-glycoprotein and complex-forming glycoprotein (HC) have been identified (Tab. 18).

Spot Number	Match to	Score	NCBI database reference	Sequence Coverage (%)
1	Uromodulin; Tamm-Horsfall glcoprotein; uromucoid (Homo sapiens)	38	gi | 4507833	11
2	Nuclear DNA-binding protein (Homo sapiens)	36	gi | 37550855	-
3	Ribosomal Protein S19 (Homo sapiens)	39	gi | 3164200	-
4	Human Serum Albumin (Homo sapiens)	164	gi | 4389275	63
5	Human Serum Albumin (Homo sapiens)	229	gi | 4389275	74
6,7	Orosomucoid 1 precursor (Homo sapiens)	152	gi | 20070760	38
8	Human Zinc-Alpha-2-Glycoprotein (Homo sapiens)	212	gi | 4699583	72
9	Complex-forming glycoprotein HC (Homo sapiens)	94	gi | 223373	41

Table 18: Proteins idendified by MS analyses. For spot 2 and 3 no sequence coverage was determined. These protein spots were predicted to be Nuclear DNA-binding protein (spot2) and Ribosomal protein S19 (spot3).

One of these proteins, tamm-horsfall glycoprotein (Fig. 26, spot 1), also referred to as uromodulin, is one of abundant protein in normal urine. In 2-D images the spot belonging to uromodulin (mentioned as the spot at 66 kDa in figure 20-25) was prominently present in the urine sample of patients with bladder cancer but almost equally present in the urine of healthy volunteers and patients on follow-up. DNA-binding protein (Fig. 26, spot 2) included in spot group at 66 kDa as shown in figure 20-25 was particularly prominent in urine samples of patients with bladder cancer but almost equally present in urine samples of patients on follow-up and healthy volunteers. The spots 4 and 5 (Fig. 26) were identified as human serum albumin, which were present in all urine samples analysed in this study. These spots were in similarity with spots marked by doted circles for the protein at 55 kDa as shown in figures 20-25. The spots representing human serum albumin were particularly prominent in urine samples of patients with bladder cancer and patients on follow-up. The proteins indicated as spot 6 and 7 in figure 26 were identified as orosomucoid 1

and they were present only in urine samples of patients with bladder cancer (shown in figure 20B as spot 2), but were absent in urine samples of healthy persons and patients on follow- up. The spot marked with the number 8 in figure 26 was identified as human zinc-alpha-glycoprotein and was prominently present in the urine samples of patients with bladder cancer (Fig. 20-25, included in spot 3) and in those of patients on follow-up. But it was absent in urine samples of healthy persons. The spot 9 in figure 26 was identified as complex-forming glycoprotein (alpha-1-microglobulin, also called HC). This protein was found in urine samples of patients with bladder cancer, particularly strong in the stage of pTa, and visible as a faint spot (Fig. 20-25, included in spot 4) in urine samples of patients on follow-up (Fig. 23-25). In contrast, it was not detectable in urine samples of healthy persons. The spot 3 in figure 26, which was identified as ribosomal protein S19, was not present in normal urine, but no considerable changes were seen for this protein in urine samples of patients with bladder cancer and patients on follow-up. It is included in the spot group marked by doted circle at 55 kDa (Fig. 20-25). The changes in density of identified proteins in urine samples were summarized in table 19.

Spot number	Idendified Proteins	Healthy	Follow-up	pTa	pT1	pT2-3
1	Uromodulin	-/+	-/+	↑↑	↑↑	↑↑
2	DNA-binding protein	-/+	-/+	↑↑	↑↑	↑↑
3	Ribosomal Protein S19	-/+	↑	↑	↑	↑
4,5	Human Serum Albumin	-/+	↑↑	↑↑	↑↑	↑↑
6,7	Orosomucoid 1	---	---	↑	↑↑	↑↑
8	Human Zinc-Alpha-2-Glycoprotein	---	---	↑	↑↑	↑↑
9	Complex-froming gylcoprotein	---	-/+	↑↑	↑	-/+

Table 19: Semiquantitative determination of protein levels of identified proteins in urine samples by 2-DE.

4.1.11 Human serum albumin in normal urine versus in urine samples of patients with bladder cancer and follow-up

The 2-DE results showed that most abundant proteins in urine samples, which were analysed in this study, were detected at 55 and 66 kDa. One of these protein spots, at 55 kDa, was identified as human serum albumin using mass spectrometry (Tab. 18). To view changes in the amount of human serum albumin in normal urine

samples and urine samples of patients with bladder cancer the urinary protein patterns of all groups of urine samples were compared to each other.

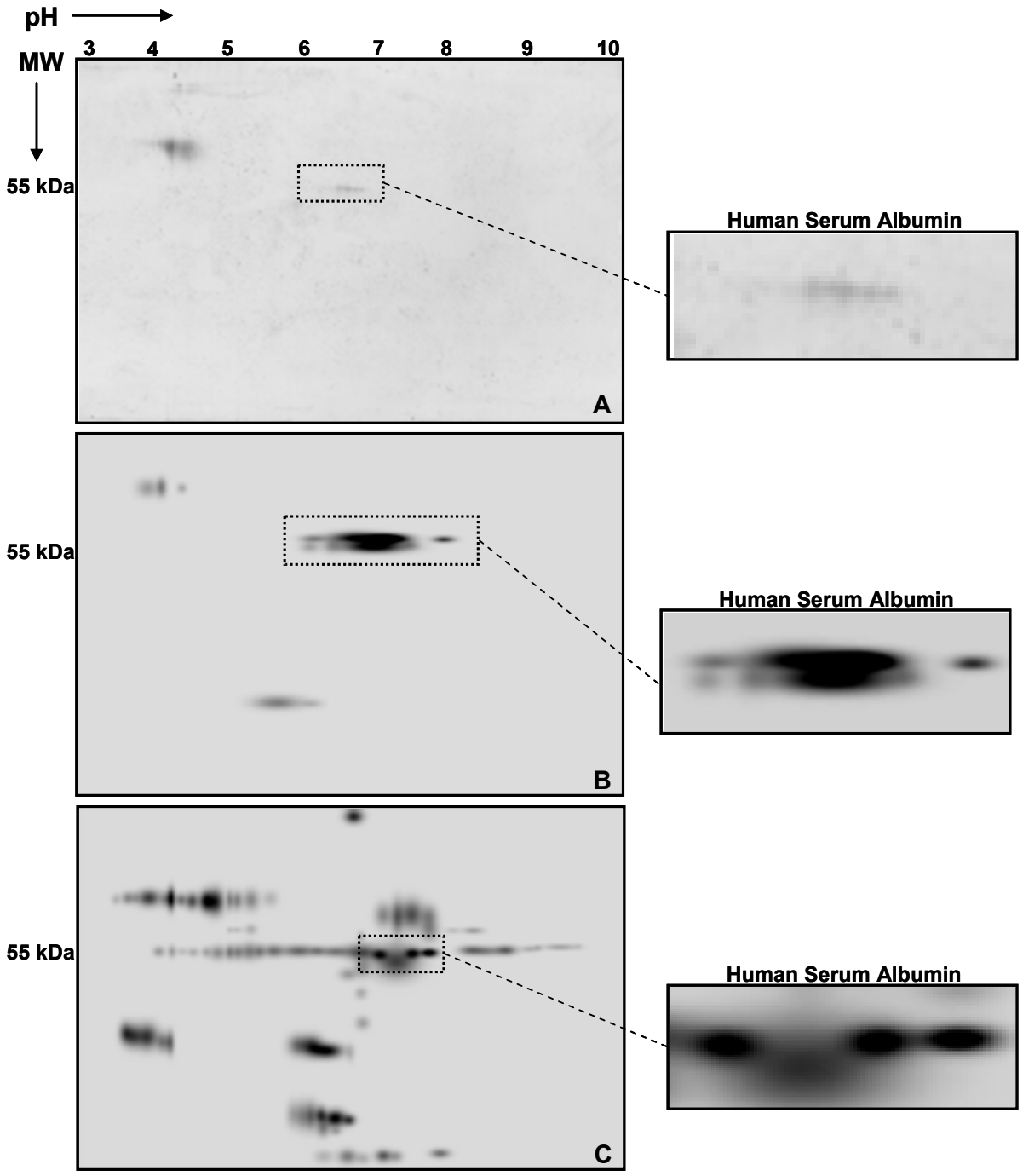


Figure 27: Comparison of protein spots indicating human serum albumin (HSA) in all urine sample groups. The protein spots at 55 kDa, which are present in all urine samples, were identified as Human Serum Albumin. The protein pattern of healthy urine (A), urine of patients on follow-up (B), and patients with bladder cancer with different stages (urinary pattern of a patient with pTa, GI was shown as exemplary) (C) were compared to each other for the spots showing HSA. The protein spots of HSA are stronger in urine samples of patients with bladder cancer and patients on follow-up in comparison to healthy persons.

Human serum albumin was present in all urinary protein patterns (Fig. 27 A-C), but the strongest protein spots which belong to human serum albumin were detected in urine of patients with bladder cancer (Fig. 27C) and patients on follow-up (Fig. 27B) when compared to those in healthy urine (Fig. 27A).

4.1.12 Mass spectrums and matched peptides for identified proteins

Proteins were identified by searching the NCBI database as shown for all identified proteins in urine samples of patients with bladder cancer (Fig. 26). Spectrum of mass spectrometric analyses and matched sequences were given for spot 1 which was identified as uromodulin (Fig. 28); spot 2 and 3 which were identified as similar to DNA-binding glycoprotein and ribosomal protein (Fig. 29A-B); spot 4 and 5 which were identified as human serum albumin (Fig. 30A,B); spot 6 and 7 which were identified as orosomucoid1 (Fig. 31); spot 8 which was identified as zinc-alpha-2-glycoprotein, and spot 9 identified as Complex-forming glycoprotein (Fig. 32A,B).

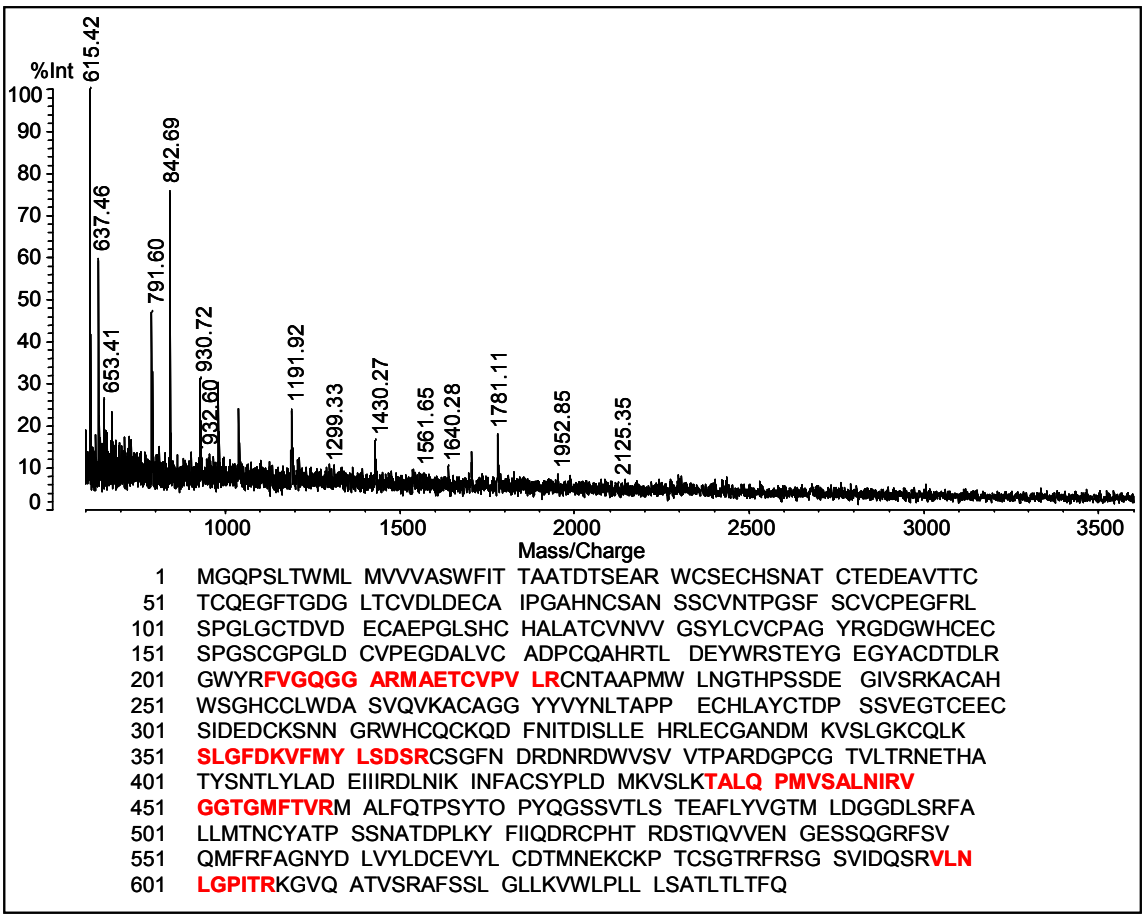


Figure 28: MS identification of Uromodulin (spot 1). Matched peptides are in red.

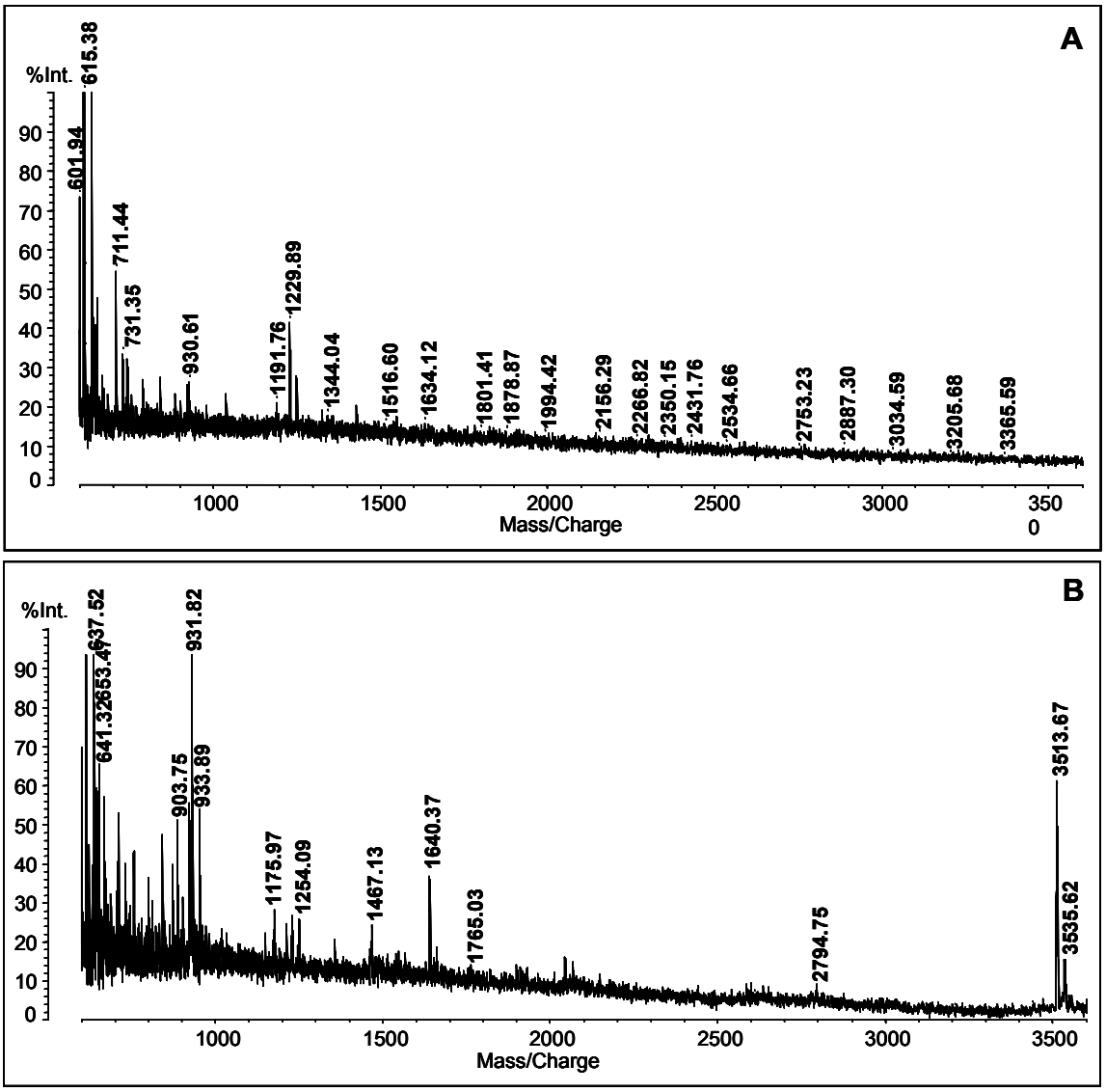


Figure 29: MS identification of DNA binding glycoprotein and ribosomal protein. Spot 2 was idendified as DNA binding glycoprotein (A) and spot 3 was identified as Ribosomal protein (B). Matched peptides were not given for this two proteins.

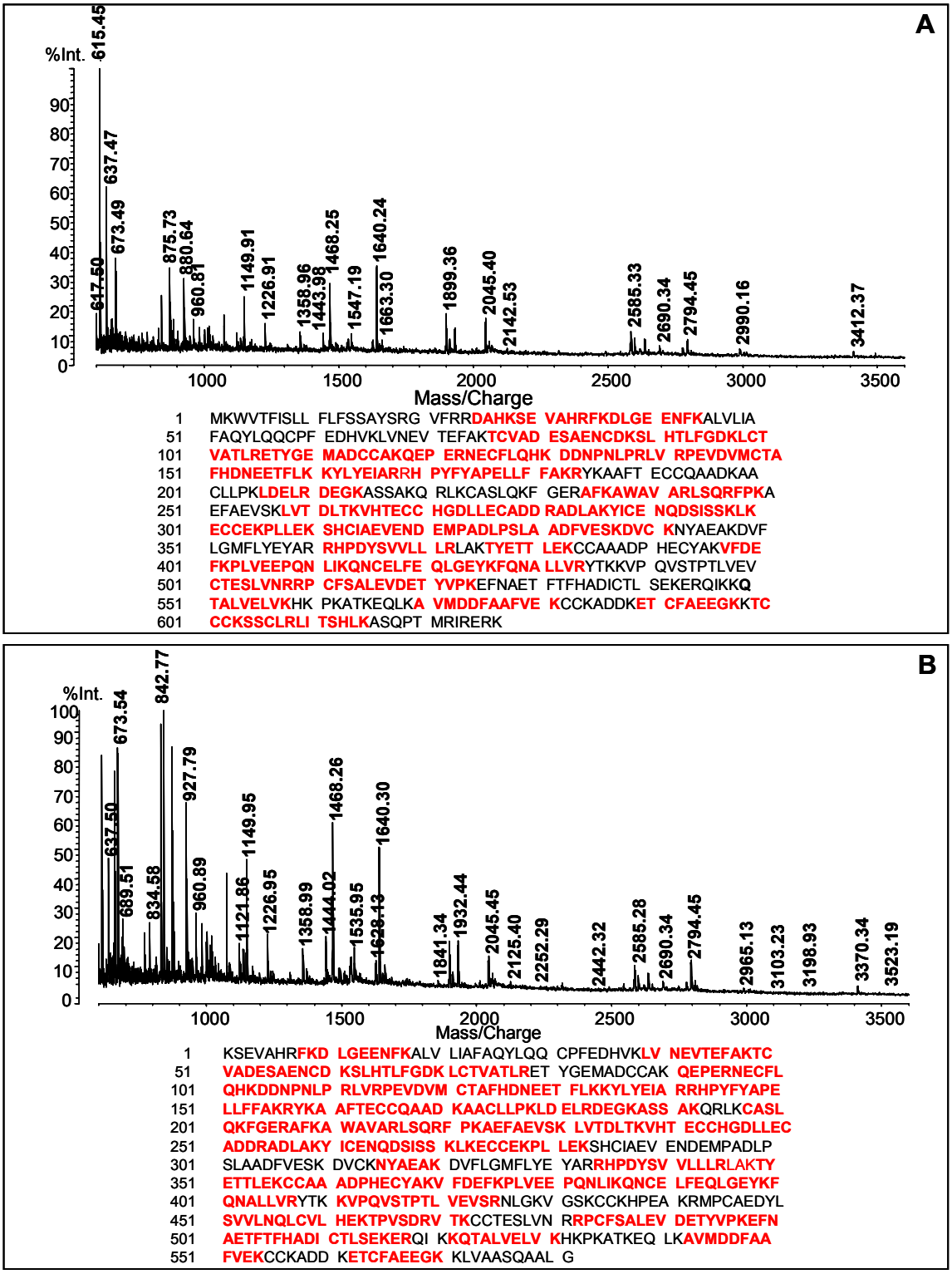


Figure 30: MS identification of Human Serum Albumin (HSA). Spot 4 (A) and spot 5 (B) were identified as human serum albumin. Matched peptides are in red.

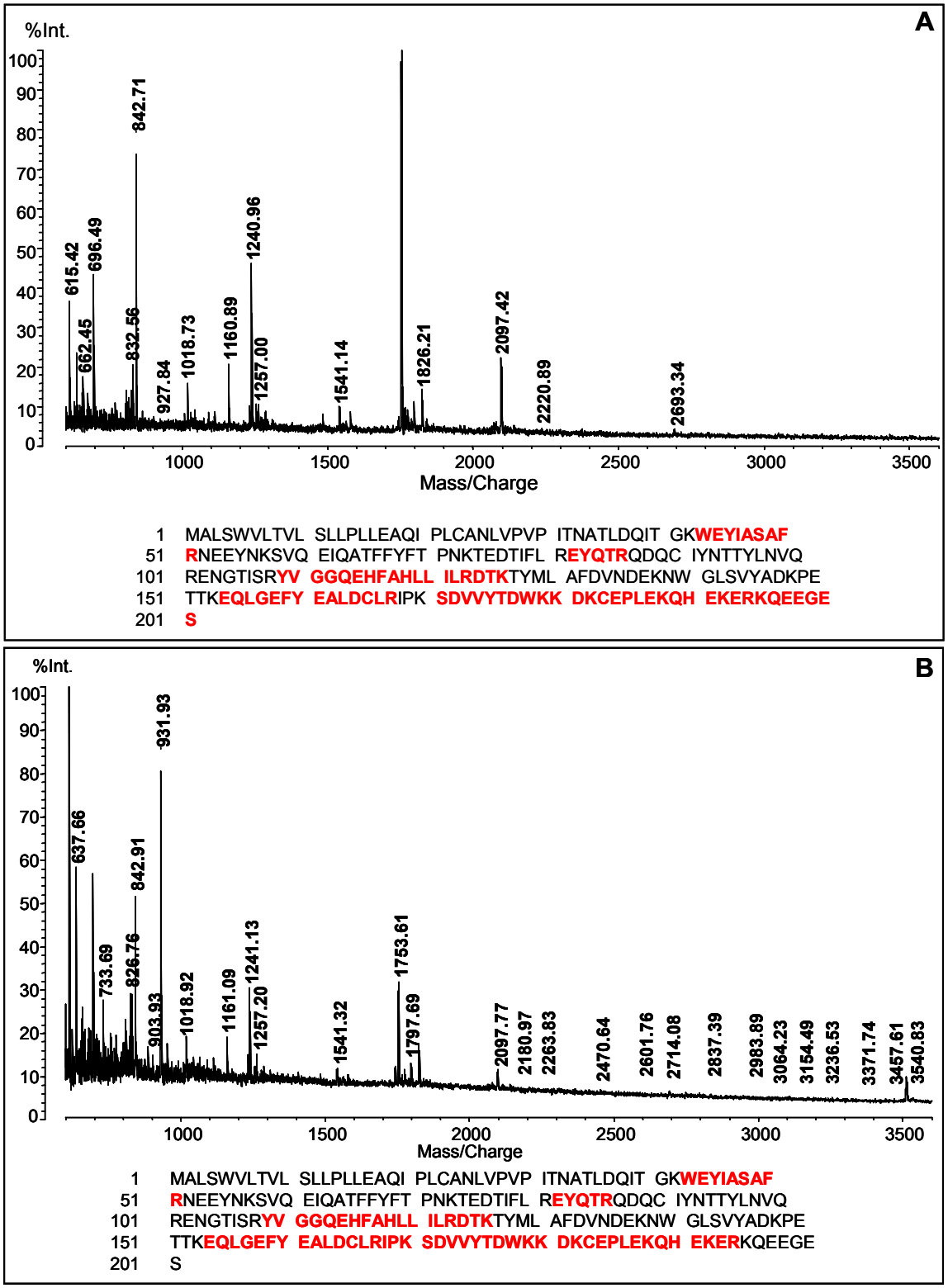


Figure 31: MS identification of orosomucoid 1. Spot 6 (A) and spot 7 (B) were identified as orosomucoid. Matched peptides are in red.

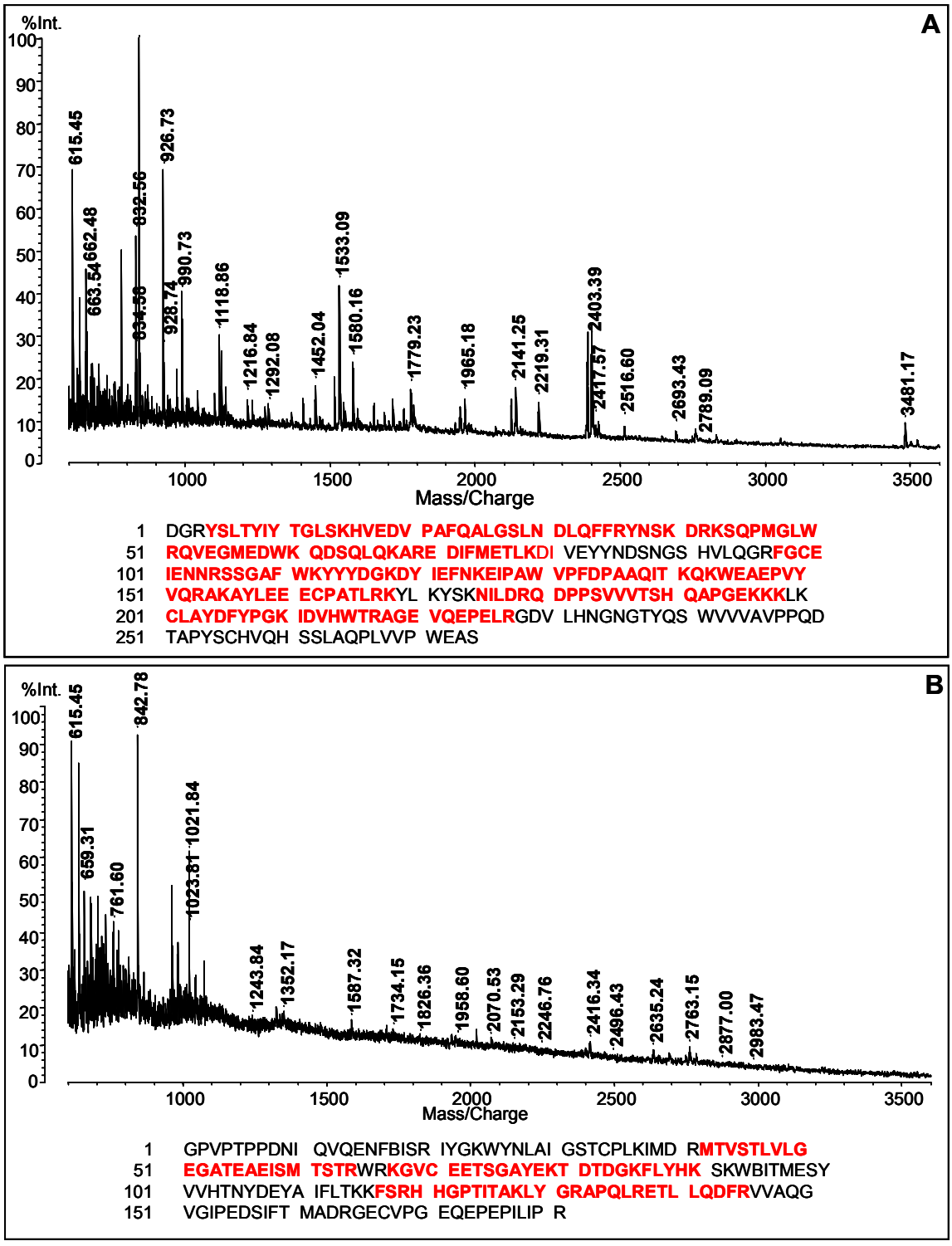


Figure 32: MS identification of Zinc-alpha-2-glycoprotein (ZAG) and Complex forming glycoprotein. (spot 8, 9). Spot 8 (A) was identified as Human zinc-alpha-2-glycoprotein and spot 8 (B), as Complex forming glycoprotein (HC). Matched peptides are in red.

4.2 Idendification of two urinary proteins using Western blot analyses

4.2.1 Detection of orosomucoid (ORM) in urine samples of healthy persons, patients with bladder cancer and patients on follow-up

In urine samples (n=45) of all bladder tumor stages classified as pTa, pT1, pT2, pT3 ORM was detected at the size of 41 kDa by Western blot analyses using the polyclonal anti-ORM antibody (Fig. 33A). ORM was also found in the urine samples of patients on follow-up and healthy volunteers, but prominently in the urine samples of patients with bladder cancer, particularly in the invasive tumor stages as pT2-3. Compared to healthy volunteers ORM was increased by three times in 85 % of urine samples of bladder cancer patients on follow-up, by seven times in 83 % of patients with pTa, 16 times in 85 % of patients with pT1, and twenty times in 71 % of patients with pT2-3.

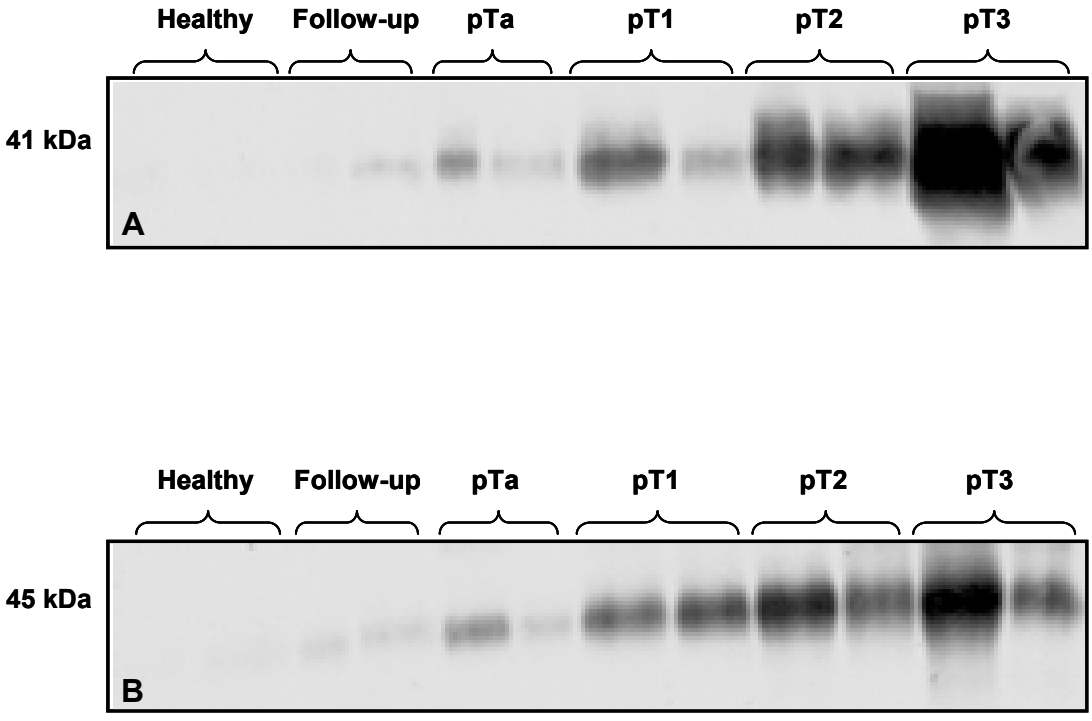


Figure 33: Detection of ORM and ZAG in urine samples. Western blot analyses using polyclonal anti-ORM antibody revealed ORM (A) at the referred size of 41 kDa in urine samples of patients of follow-up and in highest amount in urine samples of patients with invasive bladder cancer stages pT2-3. In similar pattern of ORM, western blot analyses using a polyclonal anti-ZAG antibody revealed ZAG at the expected size of 45 kDa in urine samples (B) of patients on follow-up and patients with bladder cancer.

4.2.2 Detection of zinc-alpha-2-glycoprotein (ZAG) in urine samples of healthy persons, patients with bladder cancer and patients on follow-up

Zinc-alpha$_2$-glycoprotein (ZAG) was detected at the size of 45 kDa in urine samples (n=45) of all bladder tumor stages classified as pTa, pT1, pT2, pT3 by western blot analyses using the monoclonal anti-ZAG antibody (Fig. 33B). ZAG was also found in urine samples of patients on follow-up and healthy volunteers but prominently in urine sample of patients with bladder cancer, particularly in invasive tumor stages such as pT2-3. Compared to urine samples of healthy volunteers, ZAG was increased by four times in 85 % of urine sample of bladder cancer patients on follow-up, by sixteen times in 75 % of those patients with pTa, by five times in 85 % of those of patients with pT1, and twenty times in 71 % of those of patients with pT2-3.

4.3 Expression pattern of ORM and ZAG in bladder cancer versus normal bladder tissue

To localize ORM and ZAG proteins in human urinary bladder tissue comparing tumor and normal tissue areas immunohistochemical analyses were performed on paraffin sections of bladder tissue.

Immunohistochemistry for ORM revealed no considerable staining in normal areas of bladder tissue (Fig. 34A) whereas endothelial cells of large and small blood vessels within tumor tissue (Fig. 34B) or in close proximity of tumor tissue (Fig. 35A) exhibited ORM. In addition to the blood vessels, also some single cells, probably inflammatory cells (Fig. 35B), within or around the tumor tissue were strongly positive for ORM. Also a part of tumor cells exhibited ORM, particularly those of invasive tumor stages as pT2 and pT3 (Fig. 35A). ZAG immunostaining was found at the luminal surface of normal urothelium (Fig. 36A) which disappeared in superficial bladder tumors as pTa (Fig. 36A). Concurrently, one or two cell rows at the basal side of pTa expressed ZAG (Fig. 36A). Interestingly, the ZAG immunostaining was most prominent in tumor cells located at the invasive front (Fig. 36B) where tumor cells invade the lamina propria of bladder wall marking the transition of tumor from pTa to pT1 stage.

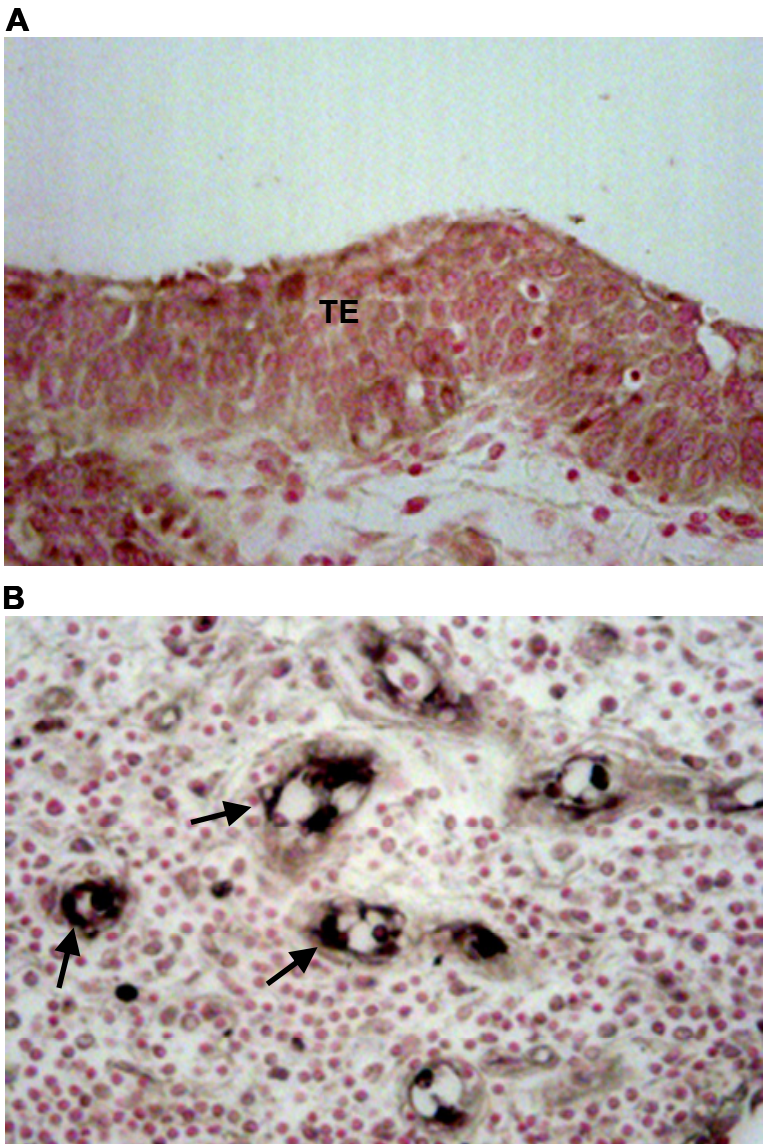


Figure 34: Localization of ORM in normal and bladder tumor tissue. In normal bladder tissue there is no considerable staining for ORM, neither in transitional epithelium (TE) nor in blood vessels (A). In contrast, blood vessels (arrows) of tumor tissue are strongly positive for ORM (B).

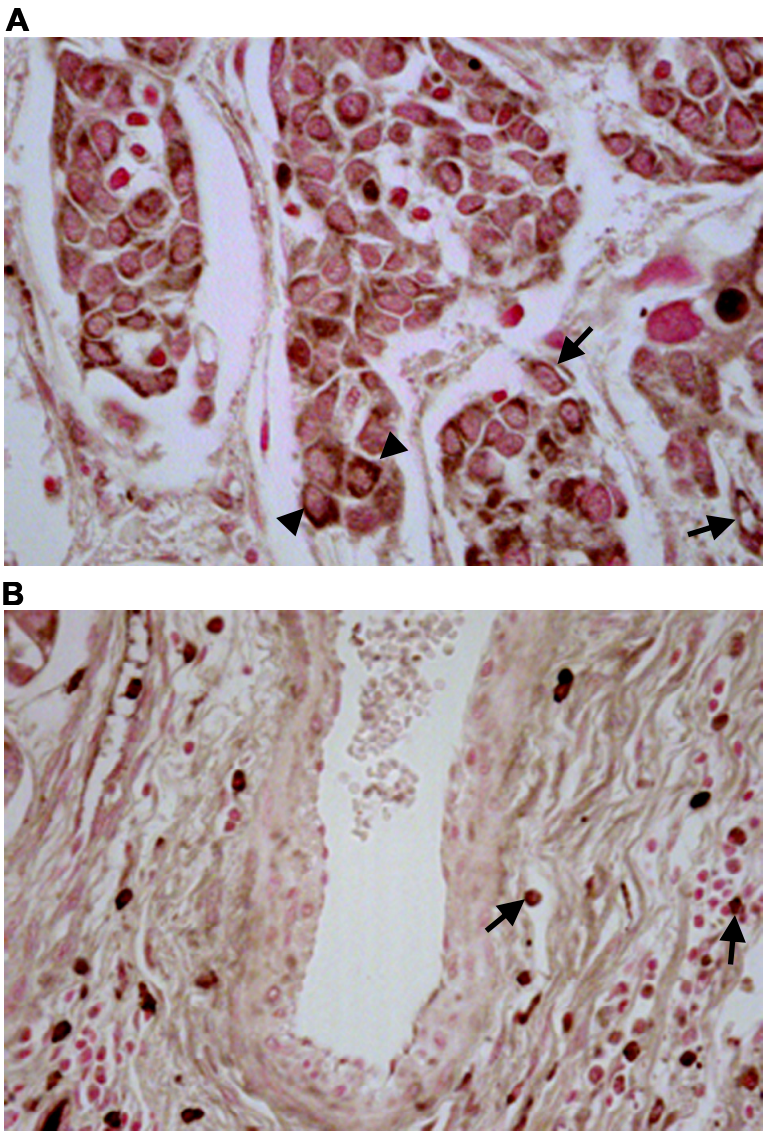


Figure 35: Localization of ORM in bladder tumor cells. Vascular endothelial cells (arrows) and positive for ORM in close proximity of bladder tumor tissue, but also some tumor cells (arrow heads) exhibit ORM immunostaining (A). Inflammatory cells (arrows) within the tumor tissue also show positive ORM staining (B).

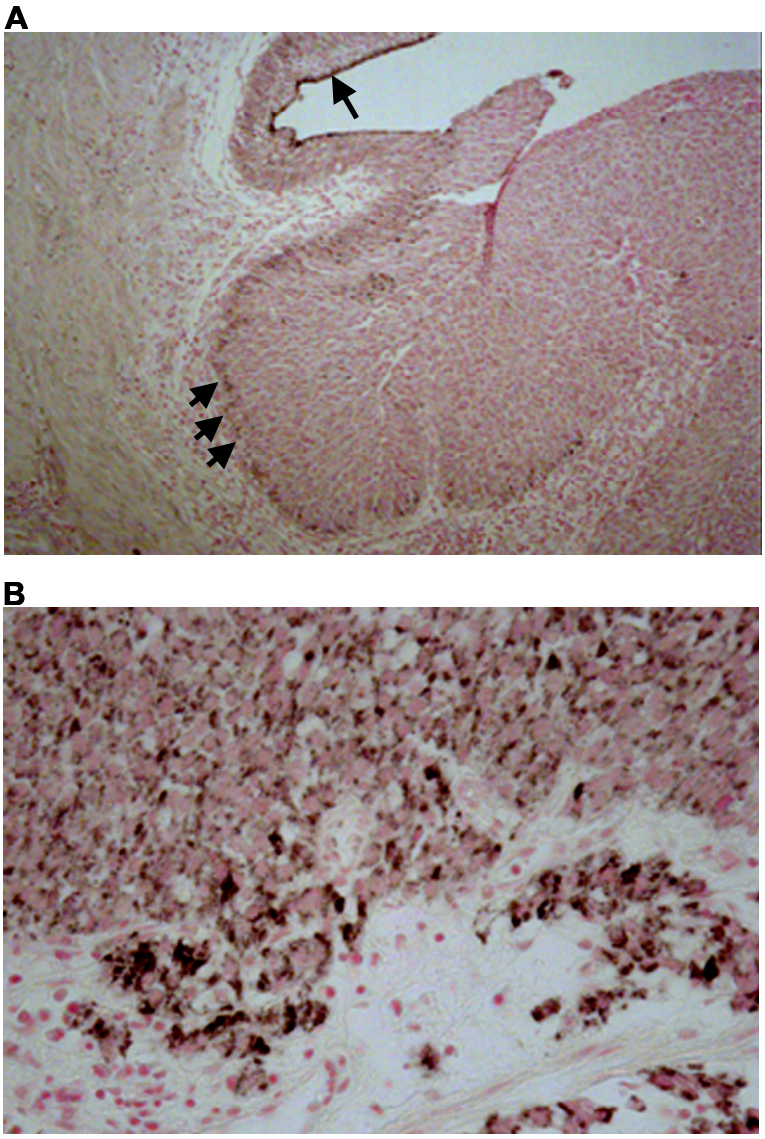


Figure 36: Localization of ZAG in bladder tumor tissue. ZAG immunostaining is localized at the luminal surface of normal transitional epithelium (arrows) which switches to the basal side of bladder epithelium (arrow heads) when superficial bladder tumor such as pTa is seen (A). The strongest staining of ZAG is found in tumor cells located at the invasive front (B).

4.4 Localization of ORM on endothelial cells (HDMECs) by immunocytochemistry

To determine whether the ORM staining in vascular endothelial cells due to endogenous production or only due to the endothelial binding of ORM produced by other cells immunostaining for ORM on cultivated human primary microvascular endothelial cells (HDMECs) were performed. These studies confirmed the results from immunohistochemistry on bladder cancer tissue and revealed a strong staining for ORM in HDMECs (Fig. 37A) while the cells were mostly negative for ZAG (Fig. 37B). ORM immunostaining was also present in cultivated bladder cancer cell line

RT4 but weaker than in HDMECs (Fig. 38A). In contrast, ZAG was not detectable in RT4 cells (Fig. 38B). The presence of ORM in endothelial cells was confirmed by western blot analyses (Fig. 39A) whereas ZAG was neither detectable in protein extract of RT4 nor in that of HDMECs.

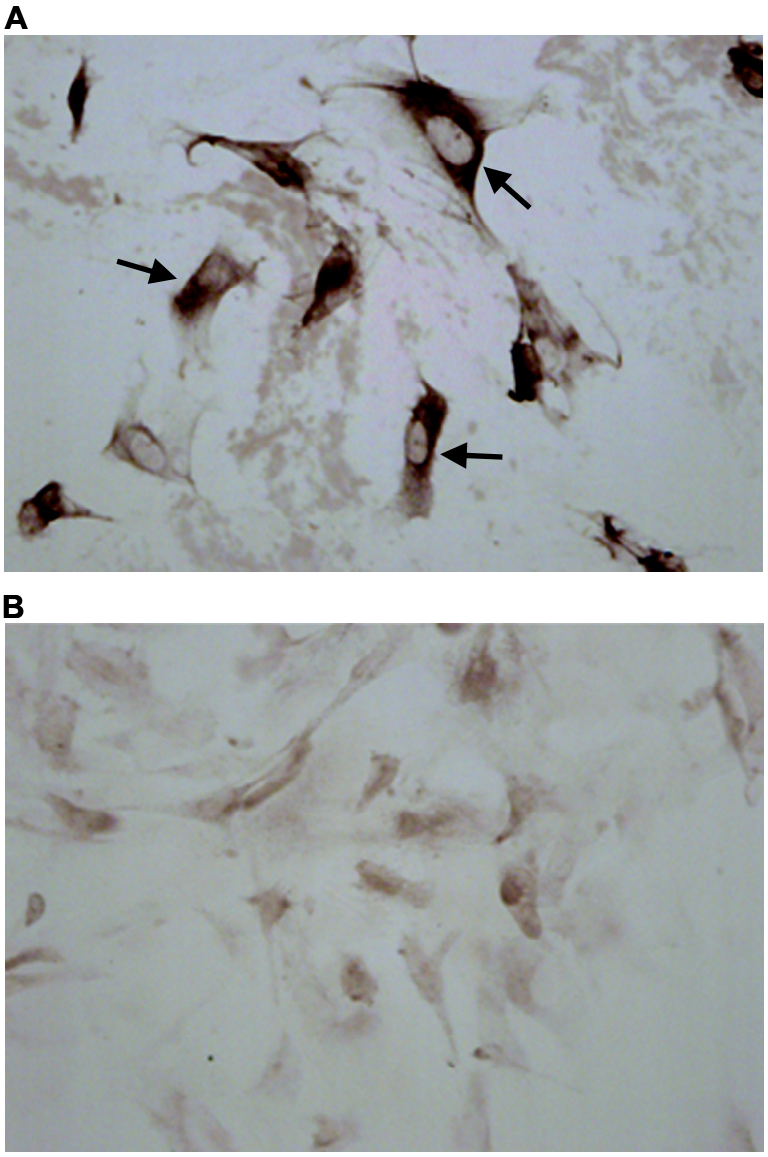


Figure 37: Immunocytochemical staining for localization of ORM and ZAG in HDMECs. Cultivated human dermal microvascular endothelial cells (HDMECs) are strongly positive for ORM (arrows, A), but are mostly negative for ZAG (B).

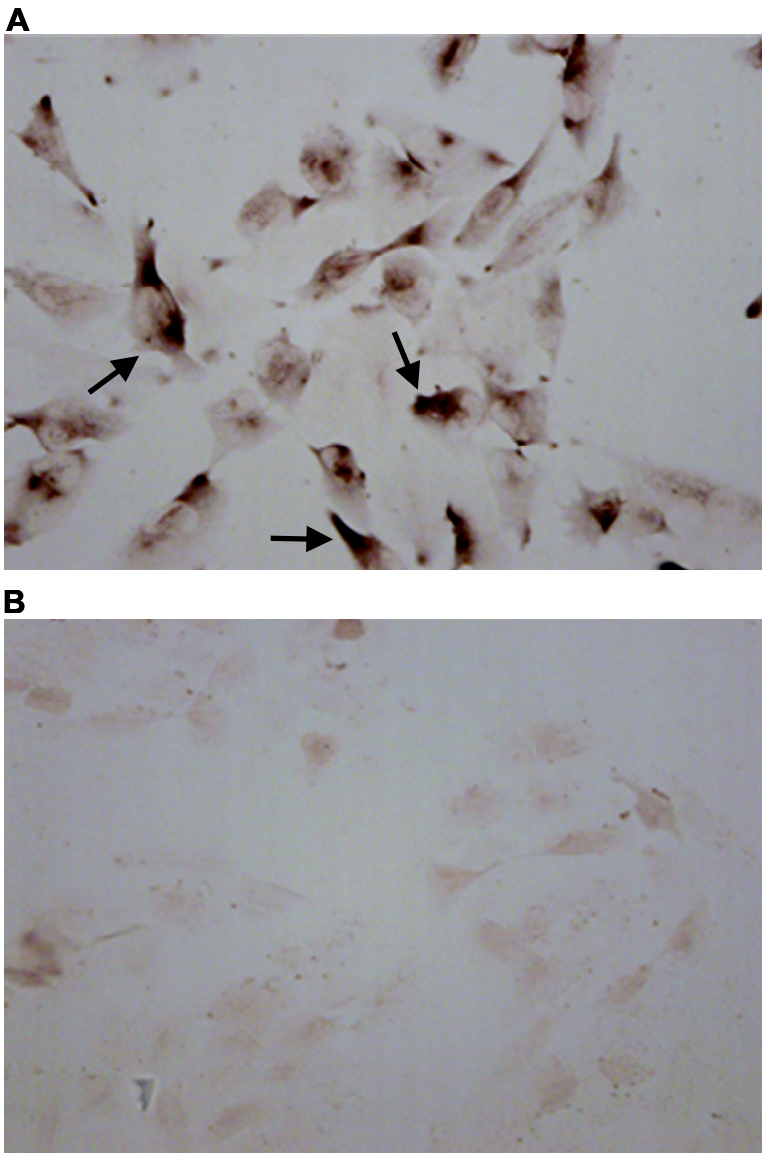


Figure 38: Immnucytochemical staining for localization of ORM and ZAG in bladder cancer cell line. ORM staining is also present in bladder cancer cell line RT4, but its expression is weaker than in HDMECs (arrows, A). ZAG is not detectable in RT4 cells (B).

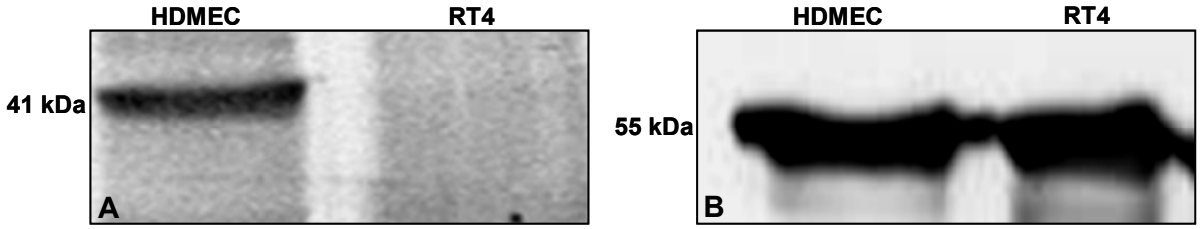


Figure 39: Detection of ORM in HDMECs and RT4 cell line. Western blot analyses using anti ORM antibody confirm the immunohistochemical data and show ORM at the expected size of 41 kDa in HDMECs (A). ORM was not seen in bladder cancer cell line RT4. The control of protein loading was performed by immunoblotting using anti-vimentin antibody (B).

4.5 ORM overexpression versus ORM gene silencing in HDMECs

To address the role of ORM in angiogenesis, ORM overexpression versus ORM gene silencing were performed in HDMECs. The effeciency was confirmed by western blot analyses which were performed on supernatant and protein extracts of transfected HDMECs (Fig. 40). ORM protein was detectable at 41 kDa in lysate of ORM overexpressing endothelial cells (Fig. 40A) and their supernatant (Fig. 40C) using polyclonal anti-ORM antibody. Empty vector transfected endothelial cells (Fig. 40A, Lane1) produce ORM as described in previously published data in the literature (Sorensson et al., 1999). A significantly higher amount of ORM was detected in the protein extract of ORM-overexpressing HDMECs in comparison to empty vector transfected HDMECs (Fig. 40A, Lane2). But ORM expression was almost completely reduced in HDMECs transfected simultaneously with ORM expressing vector plus ORM-siRNA (Fig. 40A, Lane3). The specificity of ORM siRNA was confirmed using siRNA for luciferase, which did not cause any significant reduction of ORM in contrast to ORM siRNA (Fig. 40A, Lane4). On the other hand, as shown in figure 40C, the majority of ORM after its overexpression in HDMECs was detected in supernatant of ORM expressing HDMECs (Fig. 40C, Lane2). ORM was detectable as a faint band in supernatant of empty vector transfected cells (Fig. 40C, Lane1).

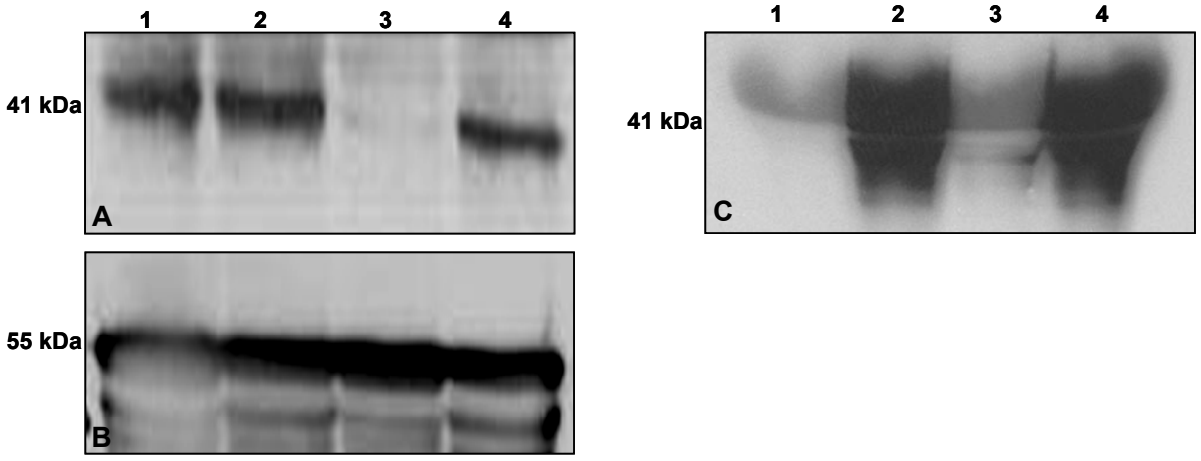


Figure 40: Overexpression and gene silencing via siRNA for ORM in human dermal endothelial cells (HDMECs). The ORM amount in cell extract of ORM-overexpressing HDMECs (A, Lane2) is only slightly higher as in the control. The use of ORM-siRNA reduces the ORM amount significantly below the endogenous level (A, Lane1). No change is seen by application of luciferase siRNA used as negative control (A, Lane4). Control of protein loading for cell lysates was performed by immunoblotting using anti-vimentin antibody (B). Immunoblotting using anti-ORM antibody reveals high amount of ORM at the size of 41 kDa in the supernatnats (C) of ORM-overexpressing HDMECs (C, Lane2), which is sucsessfully reduced by additional transfection with ORM-siRNA (C, Lane3) while luciferase siRNA used as control did not change the ORM amount (C, Lane4).

The transfection of HDMECs with ORM siRNA reduced ORM protein amount (Fig. 40C, Lane3) while luciferase siRNA which used as a negative control did not change the ORM amount (Fig. 40C, Lane 4).

4.6 Mechanistic studies via in-vitro angiogenesis assays

4.6.1 *In-vitro* endothelial tube formation using ORM-overexpressing versus ORM–silenced HDMECs

To find out the potential effects of ORM in angiogenesis and capillary morhogenesis, *in-vitro* endothelial tube formation assay was performed.

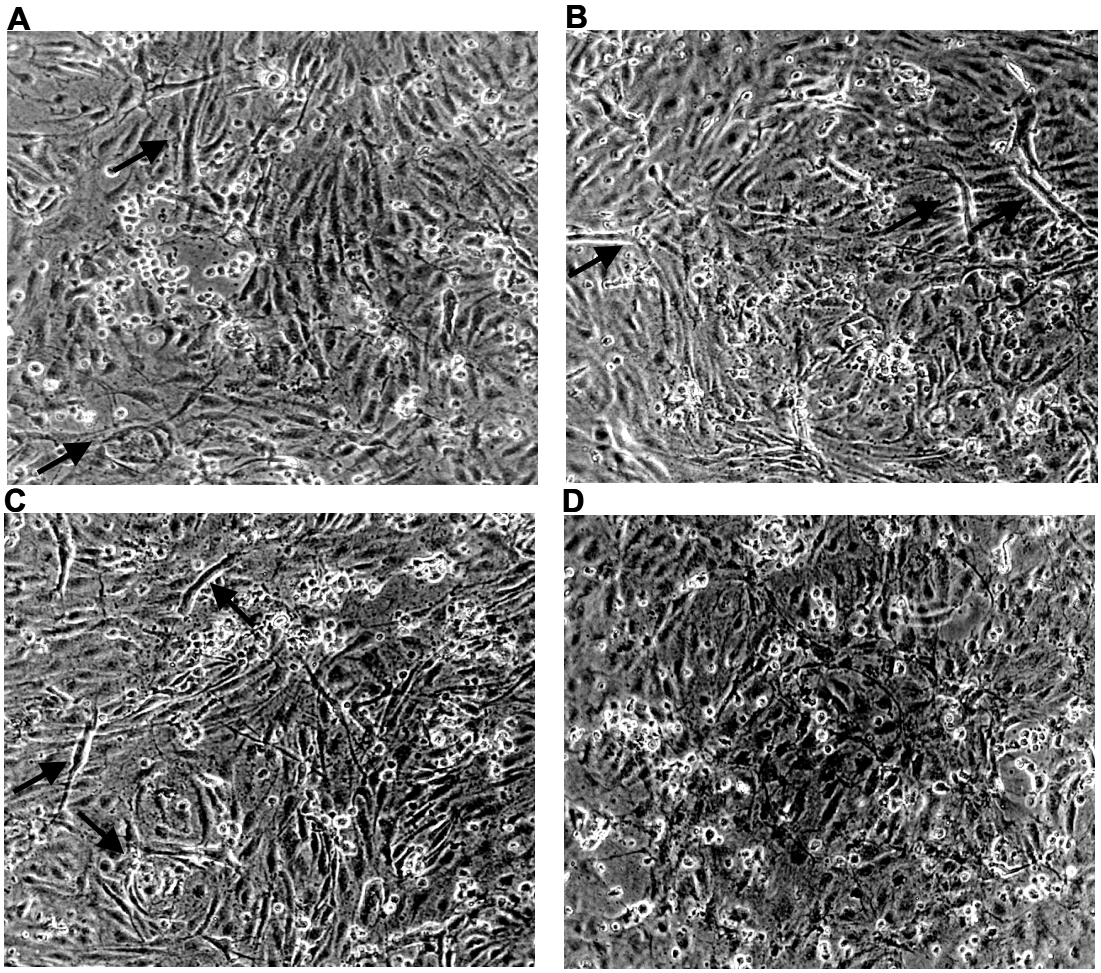


Figure 41: Endothelial tube formation assay. VEGF-induced endothelial tubes (arrows; A) are enhanced by combined application of VEGF-A and the supernatant of ORM-overexpressing HDMECs (arrows; B). The treatment with VEGF-A and supernatant of HDMECs transfected with ORM expressing vector plus ORM-siRNA reduced the tube formation (C) in comparison to VEGF-A alone (A) and VEGF-A plus supernatant of ORM overexpressing HDMECs (B). Untreated endothelial cells were used as a negative control (D).

The supernatant of ORM-overexpressing and ORM-silenced HDMECs were used in endothelial tube formation assay. HDMECs were transfected with empty vector, ORM expressing vector, and ORM siRNA using nucleofector technology as described in

material and methods (3.2.3.5). VEGF-A was used as a positive control which induced endothelial tube formation (Fig. 41A) as expected. The number and the network of VEGF-A induced endothelial tubes were increased by combined application of VEGF-A and the supernatant of ORM-overexpressing HDMECs (Fig. 41B). Combined application of VEGF-A, supernatant of HDMECs transfected with ORM expressing vector and ORM siRNA simultaneously did not affect VEGF-induced endothelial tubes (Fig. 41C). Untreated endothelial cells were used as a negative control and did almost not induce tubes (Fig. 41D).

4.6.2 The interaction between ORM and PAI-1 in endothelial tube formation

Since it was reported that a complex composed of PAI-1 and ORM on the endothelial cells surface influences the behaviour of endothelial cells, it was aimed to study potential effect of the interaction between ORM and PAI-1 in capillary morphogenesis. To this aim, untreated endothelial cells were cultured and at passage 4 the cells were seeded onto collagen gel and cultured until confluence. Afterwards, endothelial cells were stimulated with: (*i*) ORM, (*ii*) VEGF-A, (*iii*) anti-PAI-1 antibody alone and with different combination of these factors. Two different concentration of ORM as 100 and 300ng/ml were used for stimulation of HDMECs. Endothelial cells exposed only to basal medium were used as negative control and did not form tubes (Fig. 42A).VEGF-A was used as a positive control and induced endothelial tube formation as also mentioned in tube assay studies above (Fig. 42B). ORM, at 100 ng/ml concentration, alone did almost not induce tube formation as also observed in cases of untreated cells (Fig. 42C). Treatment with anti-PAI-1 antibody alone, which was used to block PAI-1 protein, induced the formation of a few endothelial tubes in contrast to ORM alone (Fig. 42D). To test the effects of the combined application of ORM and anti-PAI-1 the cells were stimulated with these factors alone or in combination with VEGF-A. The application of ORM at 100 (Fig. 43A) and 300 ng/ml (Fig. 43B) plus VEGF-A simultaneously increased the VEGF-A induced endothelial tubes depending on the applied concentration of ORM. Combined application of VEGF-A and anti-PAI-1 increased the network and the number of VEGF-induced tubes too (Fig. 43C). Treatment of endothelial cells with ORM at 300 ng/ml and anti-PAI-1 induced a few tubes (Fig. 43D). The combined application of all three factors as ORM, anti-PAI-1, and VEGF simultaneously exhibited the strongest effect on endothelial tube formation. The application of anti-

PAI-1 and ORM at 300 ng/ml (Fig. 44B,C) in combination with VEGF-A increased more endothelial tubes than the combination of VEGF-A and 100 ng/ml of ORM with anti-PAI-1 (Fig. 44A).

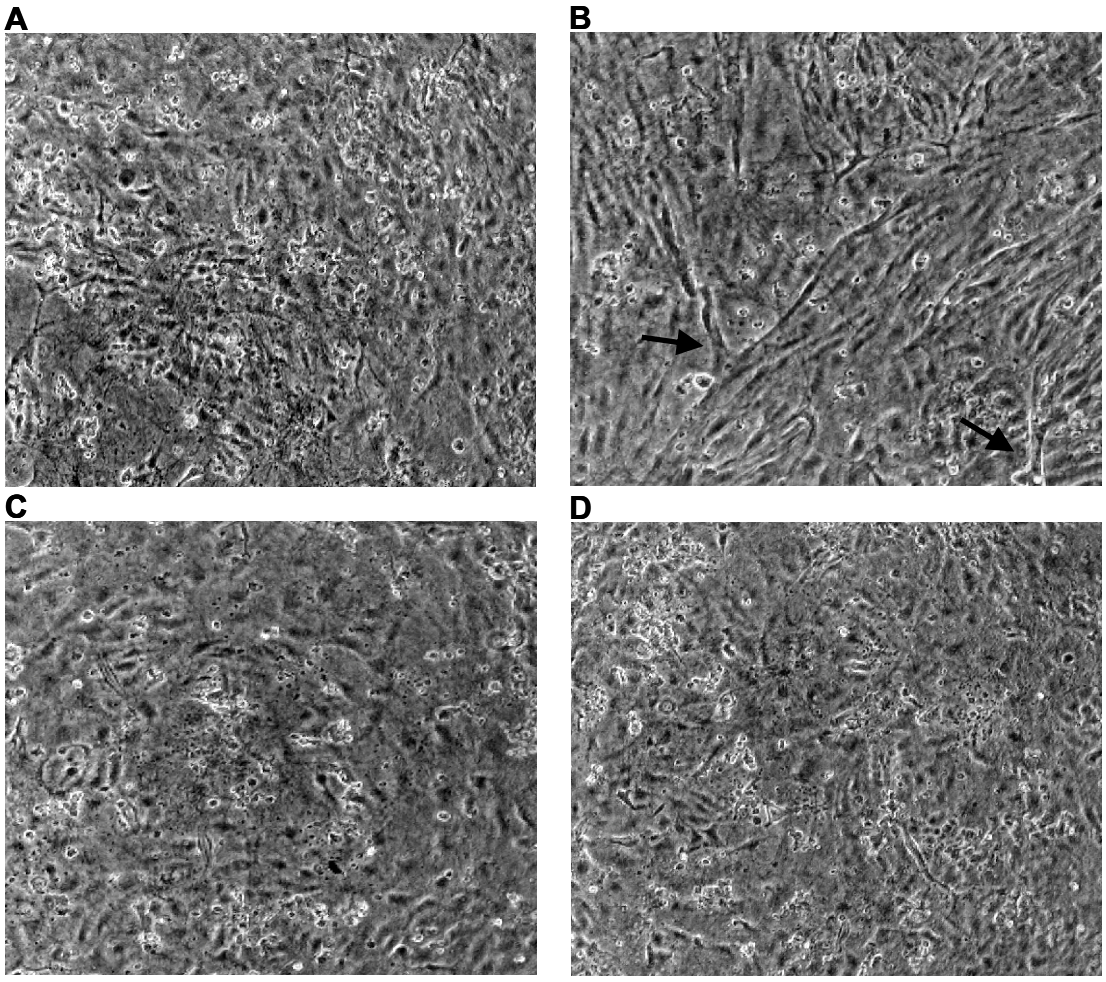


Figure 42: Effect of ORM and anti-PAI-1 antibody on endothelial tube formation. In comparison to untreated HDMECs, as negative control (A), and VEGF-A treated cells, as positive control (B), ORM (C) did not effect endothelial tubes, while treatment of antibody against PAI-1 formed several endothelial tubes (D), but it is not a considerable effect. Wild type HDMECs were stimulated with 100 ng/ml ORM protein and 15 ng/ml of anti-PAI-1 antibody.

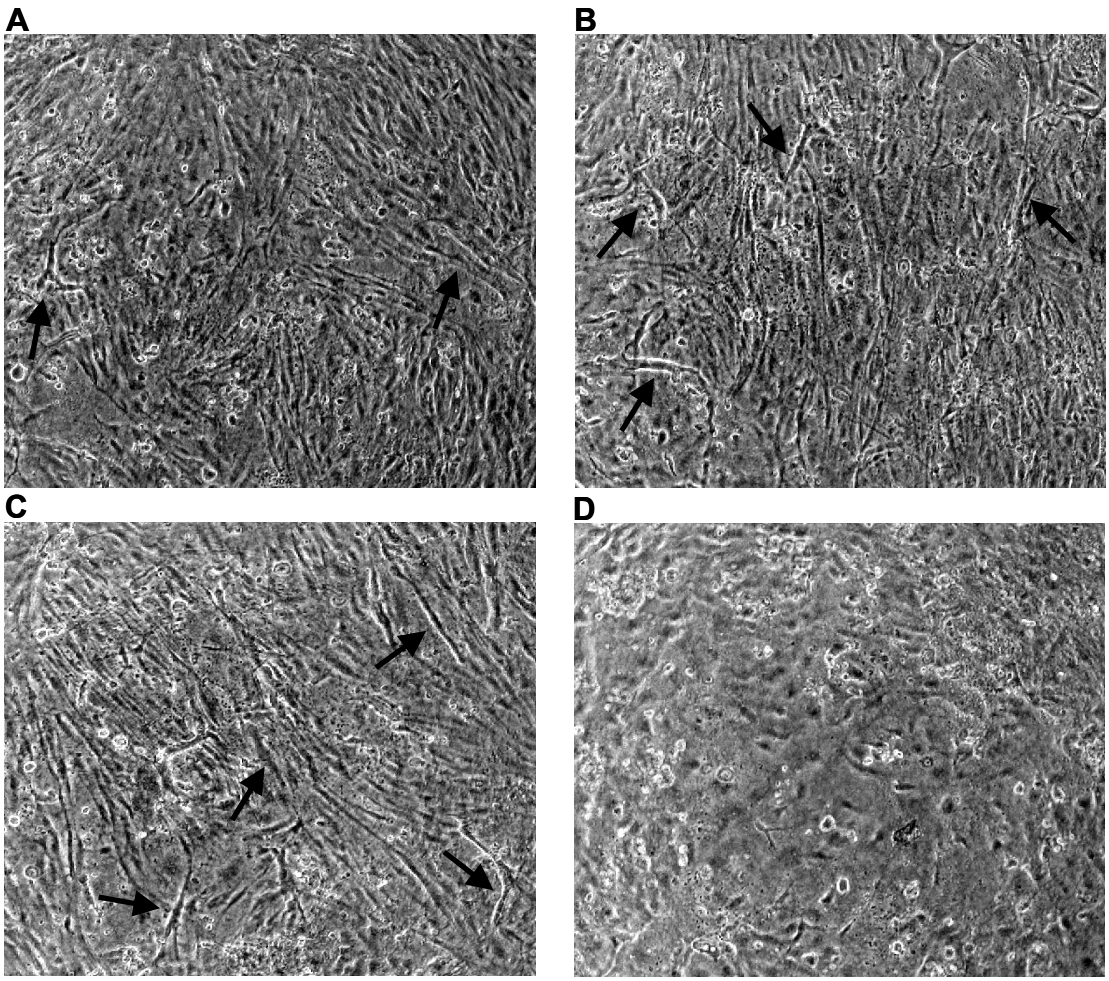


Figure 43: Effect of ORM and anti-PAI-1 at VEGF-induced endothelial tubes. By combined application of VEGF-A and ORM protein, at two different concentrations as 100 (A)and 300 ng/ml (B), the numbers of VEGF-induced endothelial tubes are significantly increased in correlation with applied ORM concentration. The treatment with VEGF-A plus anti-PAI-1 antibody induces a higher increase of VEGF-induced endothelial tubes in comparison to ORM application (arrows, C). Combined application of 300 ng/ml of ORM and anti-PAI-1 antibody does not effect endothelial tube formation (D).

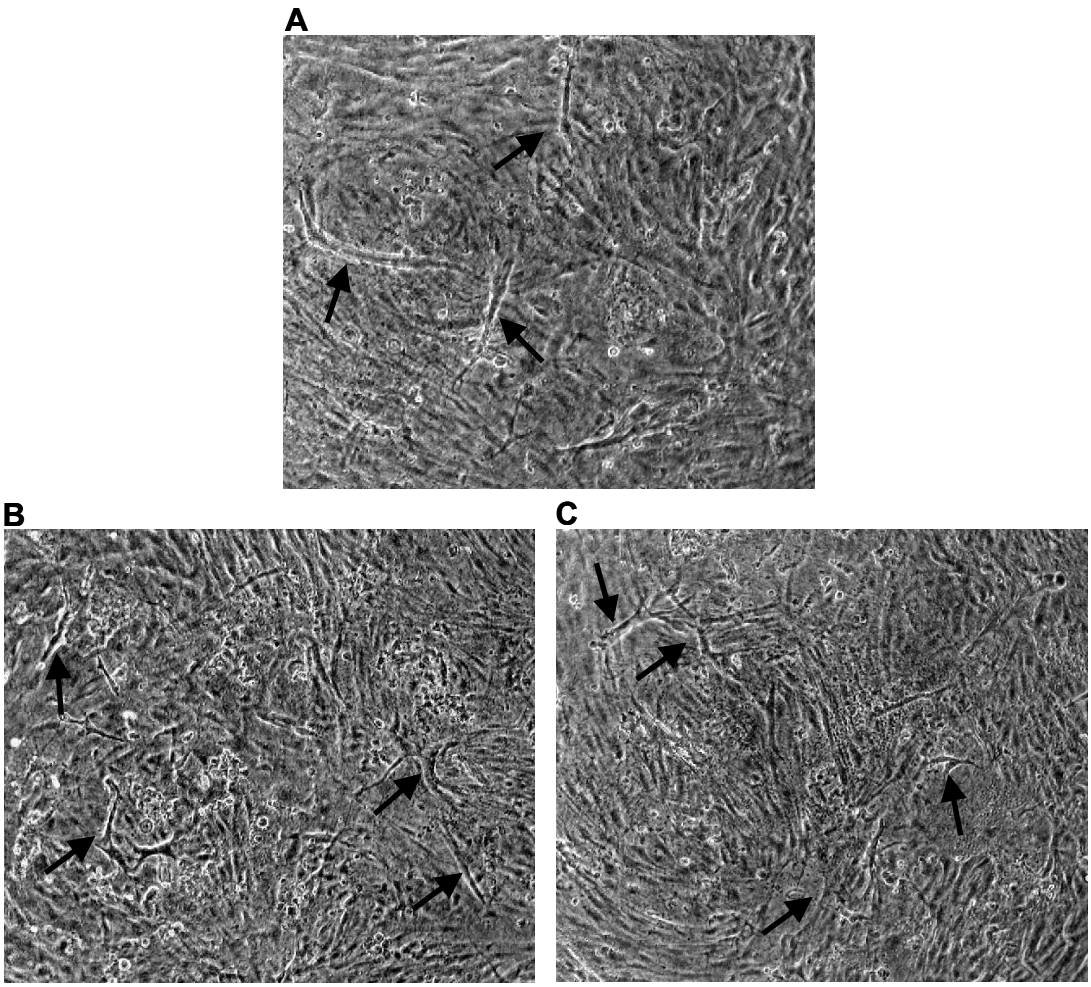


Figure 44: Effect of VEGF, ORM and anti-PAI-1 combination on endothelial tube formation. VEGF-induced endothelial tubes are exteremly increased by combined application of ORM, as 100 ng/ml (arrows, A) and 300 ng/ml concentration (arrows, B,C) with anti-PAI-1 antibody. Increase of VEGF-induced endothelial tubes correlates with ORM concentration.

4.7 Immunolocalization of ORM in in-vitro induced endothelial tubes

Endothelial tubes on collagen gel which were formed by wild type HDMECs after treatment with stimulating factors such as ORM, VEGF-A and anti-PAI-1, were fixed using Bouin´s fixative over night and embedded in paraffin. On paraffin sections of 5-7 μm thickness obtained from paraffin embedded tissue block of *in-vitro* induced endothelial tubes immunhistochemistry was performed using polyclonal anti-ORM antibody. Untreated endothelial cells and tubes on collagen gel revealed a weak staining for ORM (Fig. 45A) in comparison to endothelial tubes induced by VEGF-A (Fig. 46A). There is no immunostaining in control sections exposed only to secondary antibody (Fig. 45B; Fig. 46B). Endothelial tubes induced by treatment with anti-PAI-1 alone exhibited stronger immunostaining for ORM (Fig. 47A) in comparison to tubes induced by combined application of VEGF and anti-PAI-1 (Fig. 47B).

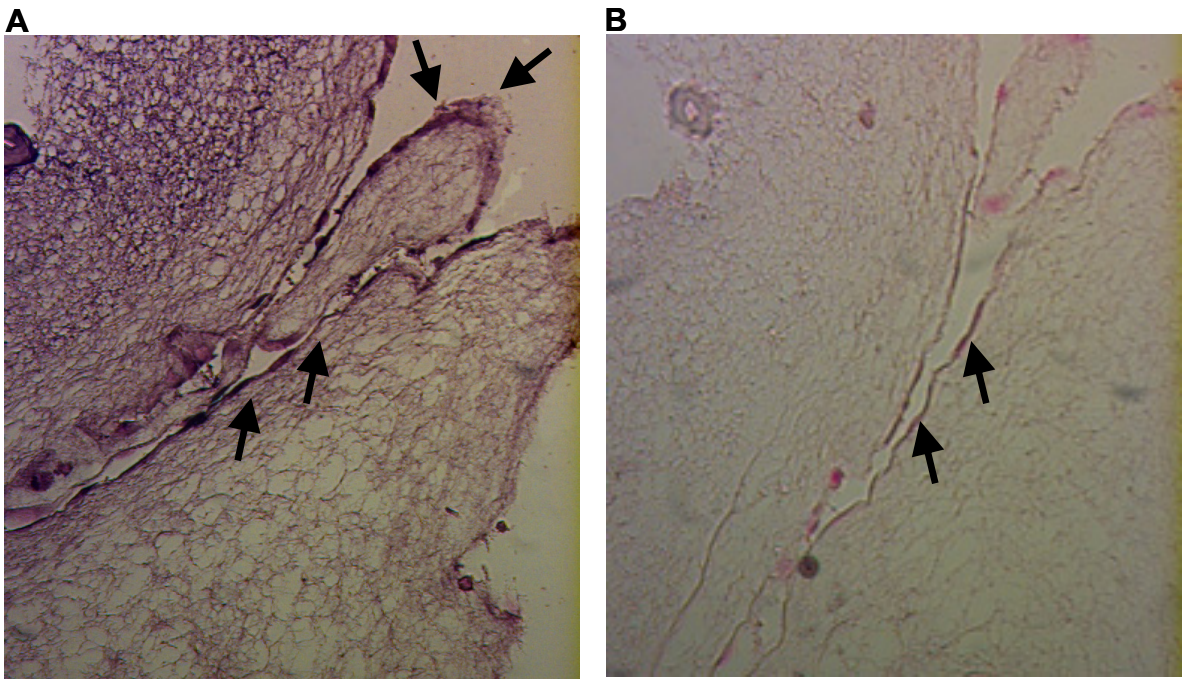


Figure 45: Localization of ORM on wild type endothelial cells after tube formation assay. Unstimulated wild type HDMECs (arrows) in collagen gel revealed a weak staining for ORM (A). In control section, no staining is seen (B).

No staining was detected in control section of endothelial tubes induced by treatment with anti-PAI-1 alone (Fig. 47C). Endothelial tubes induced by application of ORM at 100 ng/ml alone exhibited stronger ORM immunostaining (Fig. 48A) in comparison to endothelial cells treated only with basal medium (Fig. 45A). But the treatment of endothelial cells with ORM at 100 ng/ml and anti-PAI-1 revealed strongest ORM immunostaining on endothelial cells (Fig. 48B,C). Interestingly, in the case of treatment of endothelial cells with the same combination but a higher concentration of ORM as 300 ng/ml ORM immunostaining on endothelial tubes was almost weaker than the use of ORM at 100 ng/ml (Fig. 49A,B). The control section of endothelial tubes treated with ORM at 300 ng/ml and anti-PAI-1 which was exposed only to the secondary antibody did not show ORM immunostaining (Fig. 49C). Immunostaining for ORM was most prominent in sections obtained from endothelial tubes treated with ORM at 300 ng/ml, anti-PAI-1 antiobody and VEGF-A simultaneously (Fig. 49D).

To define exact changing in number and network of endothelial tubes which were induced by treatment with ORM plus anti-PAI-1 plus VEGF-A, during their growing on collagen gel, the length of endothelial tubes were measured. After the cells have been stimulated with the factors mentioned above the photographs of wells containing endothelial collagen gel were taken every day using phase contrast microscope

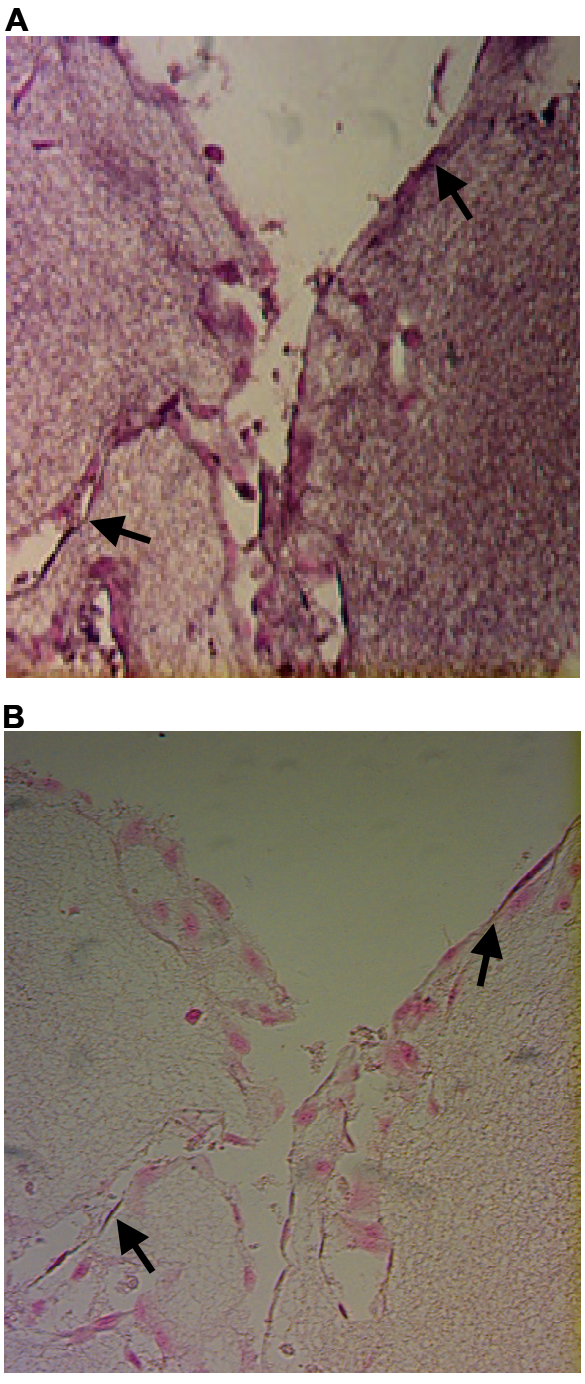


Figure 46: Immunostaining for ORM on VEGF-induced endothelial tubes. There is positive immunostaining for ORM on endothelial cells forming VEGF-induced tubes, which were used as positive control (arrows, A). Control section is negative for ORM (B).

(Zeiss, Jena). The endothelial tubes were marked with arrows and the length of each endothelial tube was measured. Total lengths of all tubes induced by a factor or a combination of factors were determined for each sample and the values were summarized in table 20.

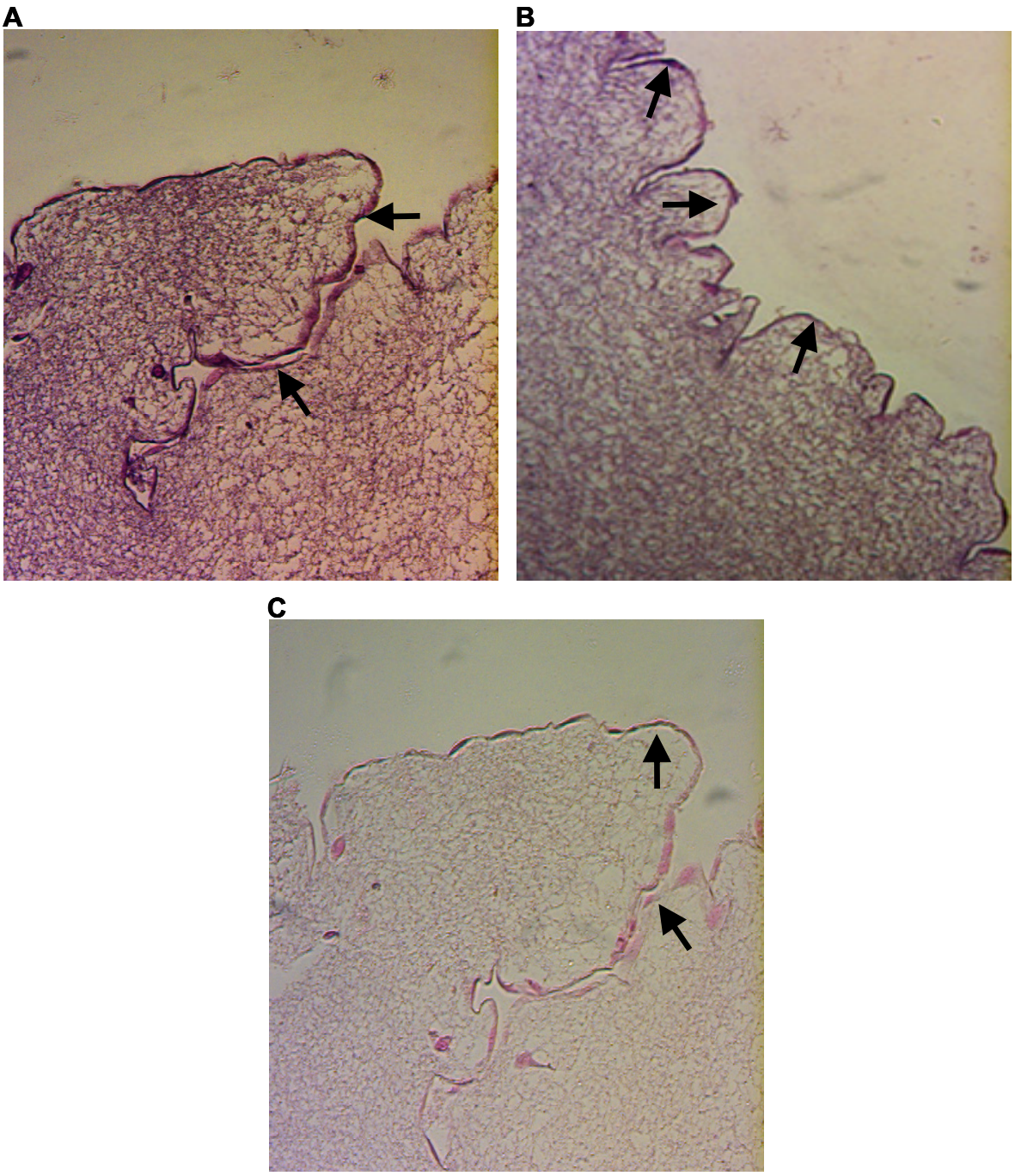


Figure 47: Localization of ORM on endothelial tubes treated with anti-PAI-1 antibody. ORM is strongly positive in endothelial cells forming tubes by blocking of PAI-1 (arrows, A) in comparison to combined application of VEGF-A and anti-PAI-1 antibody (arrows, B). The control section shows no specific staining (C).

The quantification of endothelial tubes as mentioned above revealed that the application of VEGF alone induces endothelial tubes in considerable amount as expected (Tab. 20, column2) while endothelial cells treated with basal medium as a negative control did not form endothelial tubes (Tab. 20, column1).

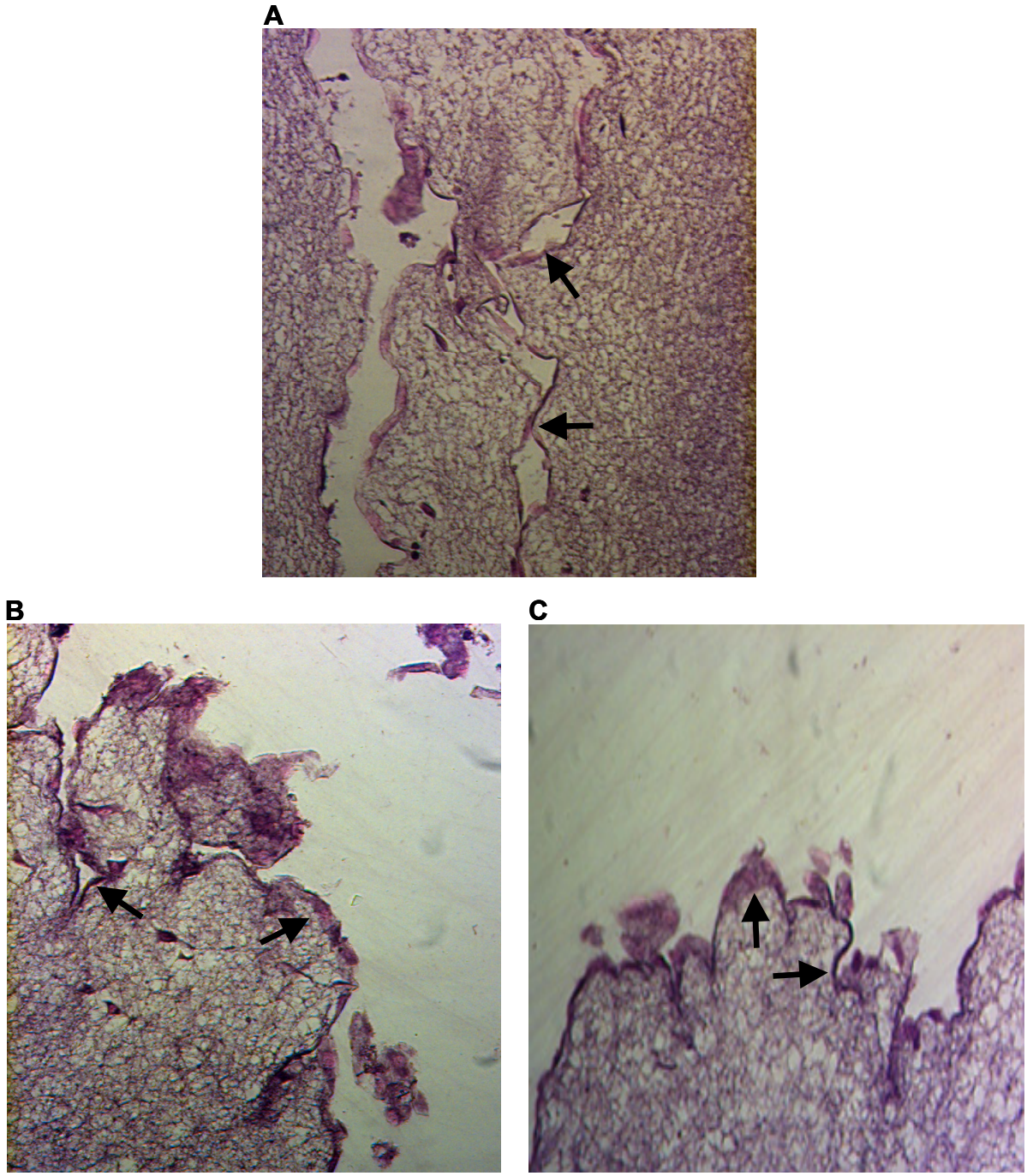


Figure 48: Localization of ORM on endothelial tubes treated with ORM in low concentration and anti-PAI-1 antibody. Endothelial cells forming tubes by ORM stimulation (100 ng/ml) are significantly positive for ORM (arrows, A), while the application of the same ORM concentration with the antibody against PAI-1 shows stronger immunostaining for ORM (arrows, B, C).

The application of anti-PAI-1 alone to endothelial cells was capable to form a few tubes (Tab. 20, column4) but not the individual addition of ORM, while application of ORM at 100 ng/ml plus VEGF enhanced the length of VEGF-induced endothelial tubes significantly (Tab. 20, column5).

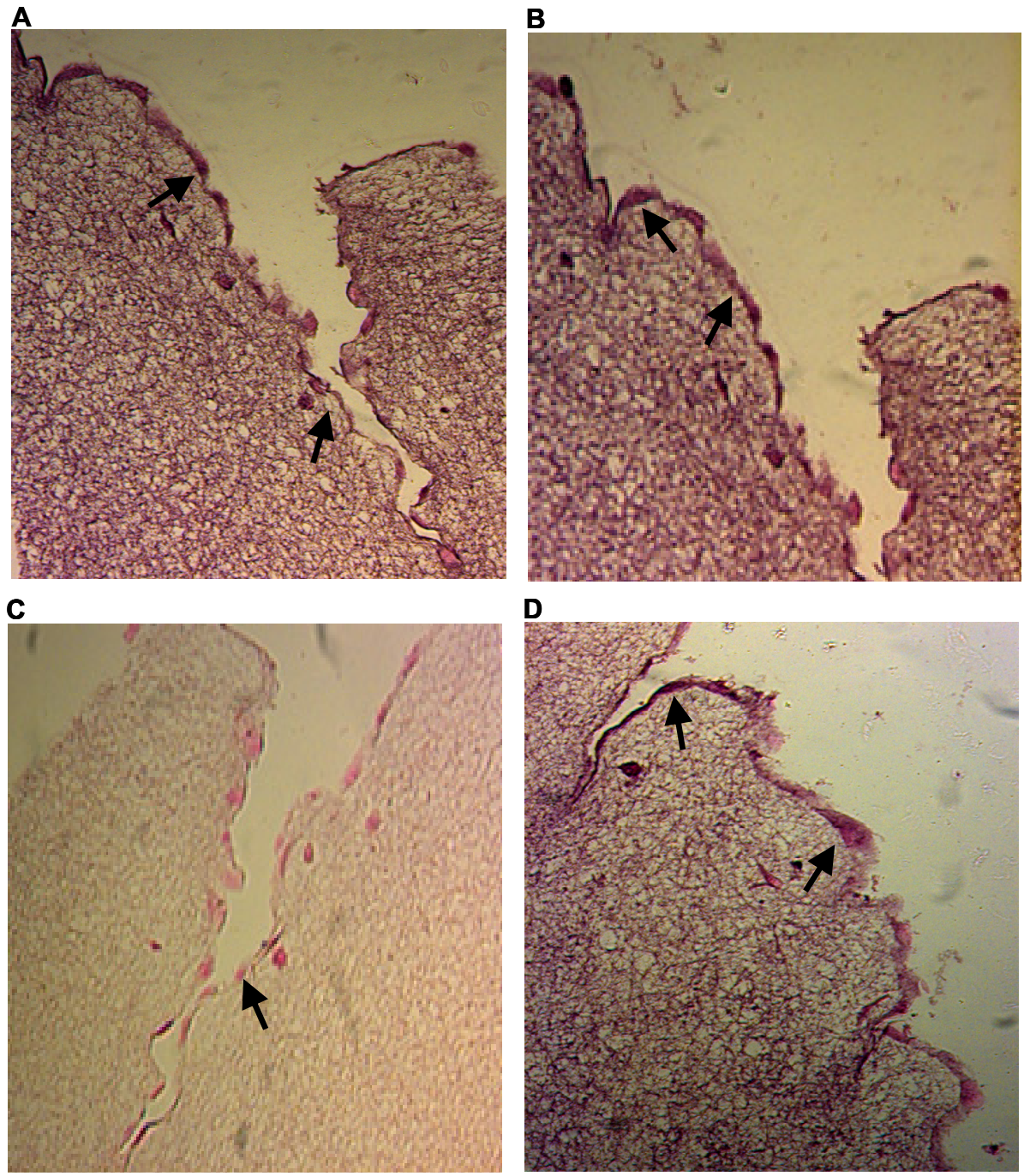


Figure 49: Localization of ORM on endothelial tubes treated with ORM in high concentration with anti-PAI-1 antibody. Interestingly, application of ORM at 300 ng/ml with anti PAI-1 antibody leads to a weaker staining for ORM on endothelial tubes compared to the same combination with low concentration of ORM at 100 ng/ml (arrows, A,B). The control section is negative for ORM (C). The strongest staining for ORM in endothelial tubes is seen by treatment of endothelial cells with VEGF, ORM at 300 ng/ml and anti PAI-1 antibody (arrows, D).

Additional increase of the length of endothelial cells tubes was achieved when VEGF plus ORM (100 ng/ml) plus anti-PAI-1 were applied to endothelial cells on collagen gel (Tab. 20, column9). The most increase of VEGF-induced endothelial tubes was seen by the combined application of VEGF, anti-PAI-1 and ORM in 300 ng/ml concentration (Tab. 20, column8). Also the combined application of ORM (300 ng/ml)

with anti-PAI-1 was capable to induce endothelial tube formation (Tab. 20, column10).

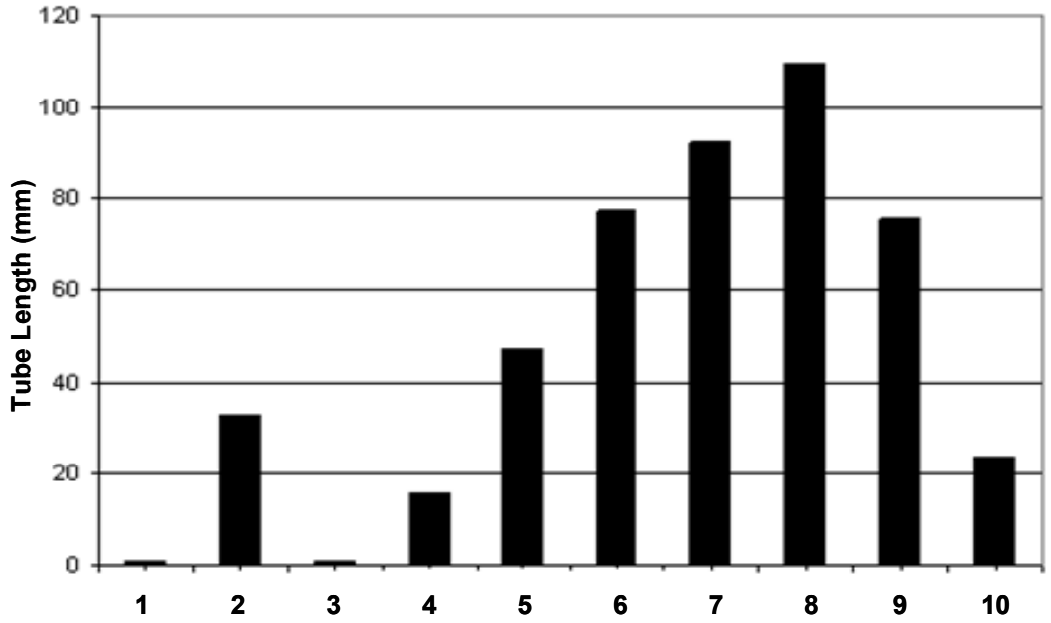


Table 20: Graphical representation of determination of tube lengths. Representation of tube lengths formed by endothelial cells treated only with basal medium (1), with VEGF-A alone (2), with 100 ng/ml of ORM alone (3), with anti-PAI-1 (15 ng/ml) antibody alone (column4), with 100 ng/ml of ORM and VEGF-A simultaneously (5), with 15 ng/ml of anti-PAI-1 antibody and VEGF-A simultaneously (6), with ORM at 300 ng/ml and VEGF-A simultaneously (7), the application of ORM at 300 ng/ml and anti-PAI-1 antibody in combination with VEGF-A (8), with 100 ng/ml of ORM, anti-PAI-1 antibody and VEGF-A simultaneously (9), with 300 ng/ml of ORM and anti-PAI-1 antibody simultaneously (10).

5. Discussion

This study provides the identification of new proteins/glycoproteins in the urine sample of patients with cancer of urinary bladder by combined application of proteomic technology such as 2-DE with subsequent mass spectrometric analyses, conventional protein research techniques such as immunoblotting, immunohistochemical studies on paraffin sections of bladder tissue, genetic approaches including gene silencing via siRNA technique and finally *in-vitro* mechanistic studies such as endothelial tube formations assay on collagen gel. In this study, the optimum conditions were established for appropriate separation of urinary proteins for the application of 2-DE technology on urine samples obtained from healthy persons and patients with bladder cancer of different stage as well as patients on follow-up. Going this way, two urinary proteins, ORM and ZAG, among other proteins found in the urine samples, which were not known to have a functional relation to the stages of bladder cancer and particularly to angiogenesis until now, were identified. This work was particularly focussed on the functions of ORM in endothelial cells. To explore the potential role of ORM in angiogenesis, genetic and mechanistical *in-vitro* studies such as endothelilal capillary formation assay were performed.

5.1 The impact of 2-DE for characterization of urinary proteins

In the last few years, new technologies were developed to identify proteins in body fluids including in urine. Among them a widely used method is the technique of the proteolytic digestion of the entire protein followed by LC coupled with MS/MS (Spahr et al., 2001). Surface-enhanced laser desorpsion/ionization (SELDI)-MS has been utilized to profile urinary proteins as an alternative high-throughput approach (Rogers et al., 2003; Schaub et al., 2004). Among these techniques 2-DE with subsequent MS analysis is the more effective method for generating reproducible results in human proteome pattern and protein identification in body fluids including urine. This method has an advantage over the technique of proteolysis with subsequent MS/MS analysis due to a 2-DE which can show the relative protein expression profiles with molecular weights and pI.

5.2 Optimized separation of urinary proteins for performing of 2-DE as a powerful tool for identification of urine proteins

At the beginning of this study the first aim was to view complete protein pattern of urine samples and to predict the function of proteins which are present in urine. To address this aim this study focused on developing an effective sample preparation strategy for 2-DE. Additional sample preparation steps can improve the quality of 2-DE images, but each additional step can results in the selective loss of protein species, which is discussed in several studies (Oh et al., 2004; Thongboonkerd and Malasit, 2005). To avoid loss of proteins, a sample preparation strategy was chosen as simple as possible. The principles of this preparation methods and of the 2-DE are described in section of material and methods.

Until now, through extensive studies, a large number of protein spots have been identified, but many of them are charge-isoforms or specific breakdown products of abundant proteins such as albumin, fibrinogen, immunoglobulin, *etc*. However, whether the pattern of these breakdown products shows a disease specific composition or not is an interesting question but needs future analyses. In the literature there are, however, limited proteomic data on human urine due to low abundance of proteins and the complex nature of urine. The majority of proteins which are present in normal urine are originated from cytoplasm and have functional properties similar to plasma proteins (Anderson and Anderson, 2002; Oh et al., 2004). The current certain limitations of proteomic analysis of the urine samples are: (*i*) the total protein amount is low, (*ii*) few proteins present in urine samples in a relatively high amount may lead to difficulties in identification of urinary proteins present only in very low concentrations and the locations of expressed proteins and (*iii*) urine contains high amounts of body filtrates such as water, salts, electrolytes and nitrogenous waste products which disturb 2-DE process.

There are several studies describing methodes and techniques to achieve a higher protein concentration in urine samples such as dye- or solvent-precipitation, ultracentrifugation, lyophilisation and the removal of the proteins abundantly present in urine samples (Marshall and Williams, 1998; Pang et al., 2002; Thongboonkerd et al., 2004). To overcome these difficulties ultracentrifugation technique was applied using a centrifugal filter column to remove the salt, electrolytes, and small molecules (<10 kDa) and as well as to concentrate urine samples. Furthermore, the development of novel methodologies to better prepare the urine samples prior to their

use in 2-DE analyses was a necessary step. Although, considerable studies were published on mapping the human urinary proteome, most of these approaches require many steps for urine preparation prior to its use for 2-DE analyses such as precipitation methods, ultracentrifugation, removal of gylcosaminoglycans, dialysis steps, lyophilisation, gel filtration, even immunosubtraction of the most abundant proteins and/or other pre-fractionation tools (Thongboonkerd and Malasit, 2005).
Recently, after publishing the data presented in this work, a novel method of solid phase based, selective adsorption of proteins for affinity ligands under controlled saturation conditions was described to increase the concentration of low-abundance proteins and to decrease the concentration of high-abundance proteins from same mixture in a single step (Thulasiraman et al., 2005). A further improvement in protein concentration/equalization technique was also recently described using beads coated with hexameric peptide ligand libraries (Castagna et al., 2005) and in that study additional 251 hidden urinary proteins has been reported.
Under consideration of the methodological knowledge reported in literature at that time of the study presented in this work, several techniques were performed at the beginning of the experiments such as different protein precipitation methods (TCA, acetone, etc.) followed by testing the use of different rehydration buffers. We applied ultracentrifugation as a simple method to avoid loss of proteins and alteration in nature of urine. Efficiency of this sample preparation method used for urine samples was enough to obtain a reproducible and high-resolution human urine proteome pattern in 2-D gels. However, for further extended studies on urinary proteins, ultracentrifugation can be combined with the novel techniques mentioned above to improve the protein separation in 2-D gel.

5.3 Urine proteomics enables the identification of proteins related to bladder cancer

Bladder cancer is diagnosed by cystoscopic evaluation, which is expensive and invasive. Urine cytology is the current non-invasive gold standard but it has low sensitivity (Konety and Getzenberg, 2001; Murphy et al., 1984). The search for bladder cancer biomarkers that could potentially replace cystoscopy as a diagnostic and surveillance tool has been complicated by the molecular heterogeneity of this disease. Numerous studies have focused on urine proteomics as a source for a potential biomarker. Vlahou (Vlahou et al., 2001) applied SELDI-TOF-MS to urine

samples to detect transitional cell bladder carcinoma (TCC). Up to date several urinary proteins, which are supposed to be related to bladder cancer, are described as a biomarker for bladder cancer such as urinary calreticulin (Kageyama et al., 2004), calcium-binding protein psoriasin (Celis et al., 2004), nuclear matrix protein 22, bladder tumor antigen, and telomerase (Ramakumar et al., 1999).

After optimization of the techniques of protein preparation and 2-D gel electrophoresis on human urine samples we performed extensive analyses on a large number of urine samples of healthy persons, patient with bladder cancer, and patients on follow-up. Comparison of proteomic map of normal human urine with urine of patients with bladder cancer and patients on follow-up revealed few similarities but significant differences. The major differences were seen especially between urinary pattern of patients and healthy persons. The similarities concerned some protein spots at 55 kDa, which were later identified by mass spectrometric analyses as human serum albumin, and 66 kDa which were present in all urine samples.

5.4 Novel protein spots in urine samples of patients with bladder cancer using 2-DE may be of diagnostic relevance

The comparison of the list of the proteins identified in this study with already published urinary protein databases (Castagna et al., 2005; Celis et al., 1996; Grover and Resnick, 1993; Oh et al., 2004; Pang et al., 2002; Pieper et al., 2004; Rasmussen et al., 1996; Thongboonkerd et al., 2002) revealed similarities regarding the detection of few proteins knowingly being present in the urine abundantly and deriving from blood plasma such as albumin, immunolglobulin, etc. Among a high number of protein spots being identified in silver staining of the 2-DE gel only a small part was also detectable in the CBB staining. These protein spots which have been identified to be either differentially up-regulated or only present in the urine samples of patients with bladder cancer were cut out from the gel and analyzed in mass spectrometric analyses. These analyses led to the reproducible identification and characterization of proteins such as ORM and ZAG.

5.5 ORM is increased in urine samples of patients with bladder cancer

Prior to the discussion of the impact of ORM in bladder cancer it is necessary to give a brief survey about ORM. It was firstly described in 1950 by Schmid (Schmid, 1950;

Schmid, 1953) and belongs to a group of acute phase proteins playing a role in the modulation of the immune response to stress, along with many other functions(Bennett and Schmid, 1980; Schmid, 1975). The relatively high concentration of ORM in the serum of healthy humans is known to rise two- to fivefold in response to different conditions such as acute infection, inflammatory and lymphoproliferative disorders and cancer (Schmid, 1975).

The structure of ORM is well characterized and is composed of a polypeptide chain containing about 45 % carbohydrate including a large amount of fucosylic and sialic acid as mentioned above. Thus, its proposed immunomodulatory activities have been attributed to its glycosylation pattern, since the strongly fucosylated and sialylated ORM glycoforms have the ability to bind E-selectin and to inhibit complement activation (De Graaf et al., 1993). An induced expression of sialyl Lewis X (sLeX) on ORM during acute inflammation has been reported, leading to the speculation that it might influence the E- or P-selectin-mediated influx of sLeX-expressing leukocytes into inflamed areas. It has been suggested that an increased level of sLeX-expressing ORM could have a feedback inhibitory effect on the extravasation of leukocytes, by competition for the E-selectin adhesion molecules (Lasky, 1992). So ORM is thought to modify the permeability of the vascular endothelium, possibly by interacting with the endothelial glycocalyx (Curry and Michel, 1980). Thus far it has been shown that ORM binds to the vascular endothelial cell surface and then causes transcytosis across the cell without passing the intercellular junction (Predescu et al., 1998).

It has been shown that the serum concentration of ORM is elevated in different types of cancers such as breast, lung and ovary cancer (Duche et al., 2000) leading to the assumption that ORM might be produced by cancer cells themselves. The present data here show a significantly increased ORM expression in urinary bladder tumors, particularly in the invasive stages of bladder cancer. Consequently, the detection of large amount of ORM proteins in the urine of bladder cancer patients, as demonstrated in this work, indicates an increased production and secretion of ORM. In the literature, a high number of studies report about ORM glycoform determination in serum diagnosis of different cancer types, such as colorectal (Hansen et al., 1987) and ovarian cancer (Dobryszycka W., 1992). However, only a few studies are available concerning the changes of ORM levels in the urine of patients. It has been

observed to be increased in urine of patients with acute inflammation (Pang et al., 2002) and of normoalbuminuric type2 diabetic patients (Narita et al., 2004).

After being identified in 2-DE of the urine analyses performed in this study also immunoblotting studies revealed an increased level of ORM in the urine samples of patients with bladder cancer and patients on follow-up. To my knowledge this is the first report showing that ORM is significantly increased in urine samples of patients with urinary bladder cancer, particularly in those with invasive tumor stages. The level of ORM has been observed to be increased in urine of patients with acute inflammation (Pang et al., 2002) and of normoalbuminuric type 2 diabetic patients (Narita et al., 2004). Correspondingly the results presented in 2-DE and immunoblotting studies here, the present immunohistochemical results suggest that, in addition to cancer cells, some tissue resident inflammatory cells and, interestingly, endothelial cells of angiogenicly activated blood vessels which are to find in close association to tumor tissue serve as source for this increase of ORM in the urine of bladder cancer patients. This would explain the highest increase in the mean protein amount of ORM at tumor stage pTa to pT1, which marks the switch from a non-vascularized superficial tumor to a tumor type invading the lamina proria where the tumor cells get direct acces to blood vessels with activation of tumor neovascularization. Confirming the previous results presented by Soerensson et al. (Sorensson et al., 1999), the present findings show that human vascular endothelial cells produce ORM endogenously. Thus, the presence of ORM in endothelial cells activated by tumor angiogenesis or probably forming new tumor vessels as demonstrated here does not only reflect the endothelial-bound ORM but also ORM originating from endothelial cells. One of the essential characteristic phenomenons of angiogenically activated blood vessels is the abnormal vascular leakage or permeability (Hashizume et al., 2000). Indeed, ORM has been reported to modify the endothelial permeability, possibly by interacting with the endothelial glycocalyx (Curry and Michel, 1980; Predescu et al., 1998). Serum level of ORM is increased in inflammatory and lymphoproliferative disorders and cancer, such as breast, lung and ovary cancer (Duche et al., 2000; Schmid, 1975) which might be involved the modulation of vascular leakage.

A further aspect of ORM structure is that ORM is a major carrier of sialyl Lewis X (sLeX), particularly during acute inflammation. This finding led to the speculation that via this carbohydrate moiety ORM might be able to bind to E- or P-selectin and might

influence the binding and rolling as well as extravasation of leukocytes into inflamed areas (Lasky, 1992). Similar to ORM, it was recently demonstrated that CEACAM1, a highly glycosylated cell adhesion molecule carrying sLeX and Lewis X residues and acting pro-angiogenic, is up-regulated in endothelial cells of new immature blood vessels of bladder cancer in early tumor stages (Oliveira-Ferrer et al., 2004). This structural similarity between both molecules, which are present in angiogenicly activated blood vessels, leads to the assumption that similar to CEACAM1 also ORM might be involved in the regulation of angiogenesis and tumor vascularization. To address the potential role of ORM in endothelial cell biology and angiogenesis, ORM overexpression and ORM gene silencing via siRNA in HDMECs were performed. After testing the efficiency of these experimental manipulations, genetically modified HDMECs were used in in vitro capillary formation assay (endothelial tube formation).

5.6 Endothelial overexpression of ORM as well as endothelial stimulation by ORM support the VEGF-induced endothelial tube formation

Until now it has been shown that ORM binds to endothelial cell surface (Boncela et al., 2001) but it is so far unclear whether ORM plays a role in capillary morphogenesis provided by endothelial cells. The results presented here demonstrate clearly, that the majority of the overexpressed ORM in HDMECs is secreted into the supernatant. This finding suggests that probably ORM produced endogenously by endothelial cells might also be mainly secreted into the extra cellular space and thus, the ORM detected in cultured HDMECs and in endothelial cells of angiogenicly activated tumor blood vessels might mainly result in ORM binding on endothelial cell surface. Also data obtained by endothelial tube assay as presented here support this interpretation. While the use of HDMECs transfected for ORM in endothelial tube assay did not lead to significant differences in comparison to the wild type HDMECs when they were stimulated by VEGF, the simultaneous stimulation of wild type HDMECs by the supernatant of ORM overexpressing HDMECs and VEGF led to a significantly increased length and network of endothelial tubes in comparison to the stimulation of HDMECs by VEGF alone. This effect was partly reversed when endothelial cells on collagen gel were stimulated simultaneously with supernatant of ORM silenced HDMECs and VEGF indicating that the ORM-siRNA used here is to some extent effective in the *in-vitro* mechanistic studies. Since the supernatant of HDMECs transfected for ORM and ORM siRNA

simultaneously was not able to block endothelial tubes induced by application of VEGF completely one can assume that either the ORM-siRNA was not effective enough to block the endogenous production of ORM totally or there are other factors replacing the functional effects of ORM in endothelial tube formation.

5.7 ORM interaction with PAI-1 influences the VEGF-induced endothelial tube formation

To gain more insight into the function of ORM in capillary morphogenesis, further *in vitro* endothelial tube formation studies were performed to investigate the role of the interaction between ORM and PAI-1. Recently it has been shown that ORM and PAI-1 form a complex on the surface of endothelial cells which obviously influences the migration of endothelial cells (Boncela et al., 2001). These studies show that it is the active form of plasminogen activator inhibitor-1 (PAI-1) which constitutes the complex with ORM. The building of this complex stabilizes the active form of PAI-1 suggesting that ORM-PAI-1 complex would increase the inhibitory effect of PAI-1. The ORM-PAI-1 co-localization was found in thymosin β4 (Tβ4)-activated but not in quiescent HUVECs (human umbilical cord vein endothelial cells). ORM may influence the adhesive properties of endothelial cells as well as their ability to generate plasmin (Boncela et al., 2001). The role of the urokinase type plasminogen activator (uPA) in angiogenesis is widely described in the literature (Andreasen et al., 2000; Binder et al., 2007; Boncela et al., 2001; Choong and Nadesapillai, 2003; Chorostowska-Wynimko et al., 2004a; Chorostowska-Wynimko et al., 2004b). While the generation of plasmin from plasminogen by action of uPA or tPA is essential for the regulation of the activity of the coagulation system, it is well documented that uPA plays a crucial role in the remodelling of the extra cellular matrix including the vascular basement membrane normally underlying the endothelial cell layer and enclosing the pericytes in capillaries (Andreasen et al., 1997). A well construction of the basement membrane is of importance for the structural integrity of the vascular wall and also for migration activity of endothelial cells which is an essential step during the new formation of blood vessels by angiogenesis (Folkman and D'Amore, 1996). The pro-angiogenic effects mediated by uPA are blocked by PAI-1 (Andreasen et al., 2000; Binder et al., 2007) which has been shown to interact with uPA and uPAR (Andreasen et al., 1997; Binder et al., 2007). This interaction shows a dual character as demonstrated in figure 50: (*i*) The interaction of PAI-1 with uPA and uPAR leads

to the internalization of uPAR and its subsequent recycling and translocation to the membrane at the cellular tips which result in an increase of endothelial migration and angiogenic activity. (*ii*) The presence of ORM results in the building of a complex with PAI-1 at the endothelial cells surface which stabilizes the activity of PAI-1 leading to the blockage of uPAR internalization and recycling which consequently result in the suppression of endothelial migration and angiogenesis (Andreasen et al., 1997).

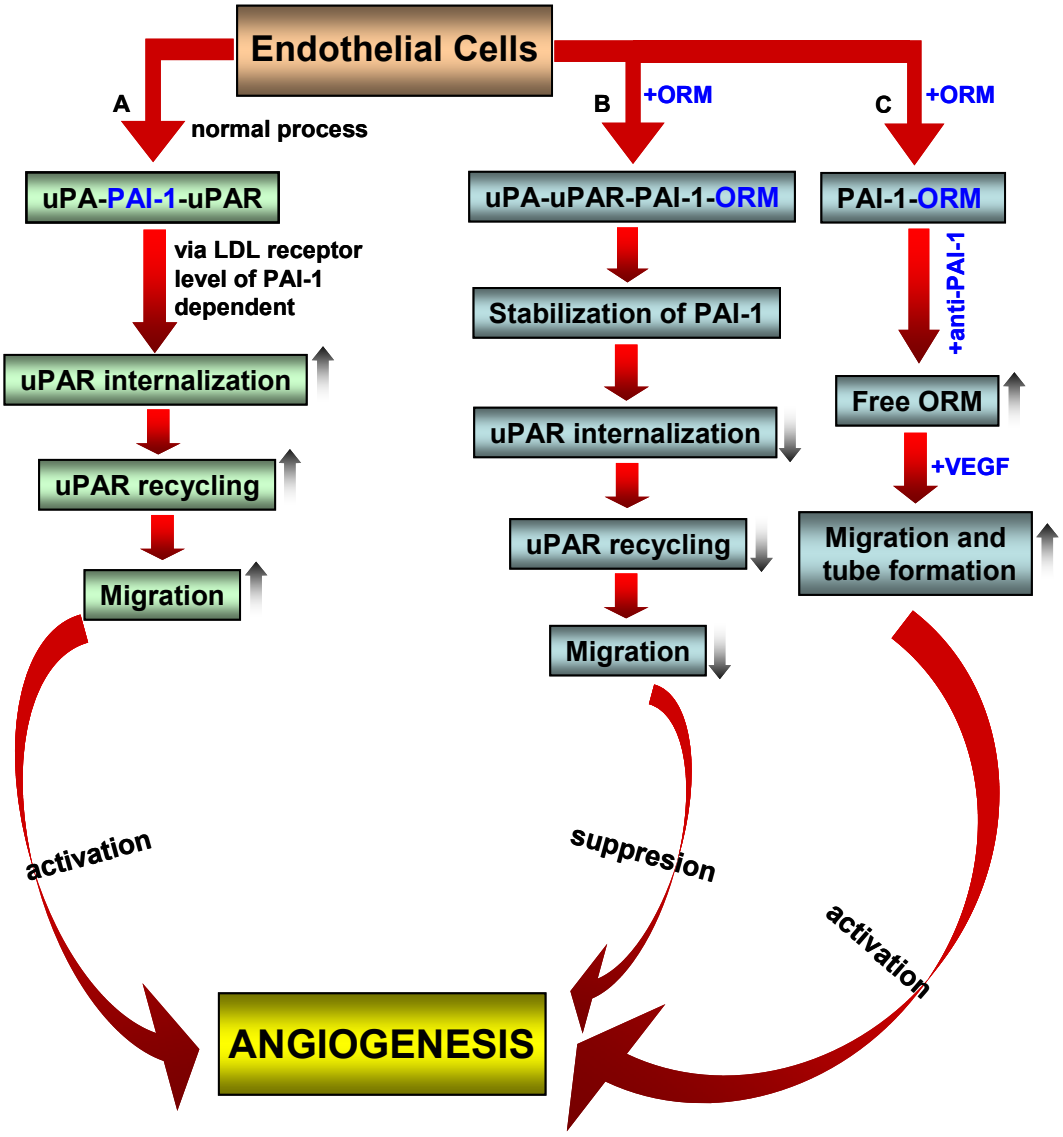


Figure 50: Important interactions within the uPAR-uPA-PAI-1 systems and the role of ORM. Normally uPA and uPAR interaction leads to internalization of uPAR and then recycling of uPAR resulting in activation of endothelial migration and angiogenesis (A). In the presence of ORM there is a complex of ORM and PAI-1 which stabilizes PAI-1 and suppresses then uPAR internalization and recycling. This results in inhibition of endothelial migration and angiogenesis (B). The additional application of anti-PAI-1 which blocks the complex building of PAI-1 and ORM increases the amount of free ORM acting with VEGF-A together proangiogenic and increases the endothelial migration and capillary formation, thereby, promotes angiogenesis (C).

However, the additional application of the anti-PAI-1 antibody as used here in endothelial tube formation assay blocks the building of ORM-PAI-1 complex with probably two consequences which increase the angiogenic activity of endothelial cells: (*i*) The blockage of complex building between ORM and PAI-1 results in enhanced uPAR internalization as described above, and (*ii*) the inhibition of ORM-PAI-1-complex increases the amount of free ORM which obviously act pro-angiogenicly when applied in combination with VEGF and enhances the VEGF-induced capillary morphogenesis *in-vitro*. Thus, it is conceivable to postulate a role of the interaction between ORM and PAI-1 in the vascular morphogenesis.

5.8 Zinc-alpha-2-glycoprotein is increased in urine samples of patients with bladder cancer particularly in the invasive stages

This study demonstrates for the first time that ZAG is detectable in an increased amount in the urine samples of patients with superficial as well as invasive tumors of urinary bladder. Interestingly, our immunohistochemical studies demonstrate that presence of ZAG at the luminal surface of normal transitional epithelium of urinary bladder switches to the basal side once a papillary tumor of the stage pTa is observed. Since in our studies the strongest immunostaining for ZAG was found in tumor cells invading the lamina propria of the bladder wall, we suggest a relationship between tumor invasion and ZAG, which needs further studies. In contrast to ORM, the strongest increase of ZAG is seen from the superficial tumor stage pT1 to the invasive tumor stage pT2, confirming our assumption that the ZAG enhancement is associated with the development of an invasive tumor and not with angiogenesis, whereas ORM increase seems to be related to angiogenic activation as discussed above. The exact mechanisms involved here need further studies on a higher number of tumor tissues and urine samples. ZAG is a soluble protein present in the serum, and has also been found in the cytoplasm of normal secretory epithelial cells, including those of breast, prostate, and liver, as well as in salivary, bronchial, gastrointestinal, and sweat glands (Burgi and Schmid, 1961; Tada et al., 1991). Increased expression of ZAG occurs in several types of malignant tumors (Brysk et al., 1997; ez-Itza et al., 1993) and the serum level of ZAG has been used as a cancer marker. Although the biological functions of ZAG remain largely unclarified, a lipidmobilizing factor purified from murine adenocarcinoma, MAC16, leading to cachexia, and from the urine of patients with cancer cachexia has been shown to be

identical to ZAG (Todorov et al., 1998). More recently, it was shown that overexpression of ZAG in adipocytes enhances tumor-associated cachexia in mice (Bing et al., 2004).

6 Conclusion

In summary, the present results demonstrate that the combined use of proteomics technology with molecular cell biology approach and mechanistic *in-vitro* assays serve as a powerful tool to detect new proteins and factors related to disorder as shown here for human urinary bladder cancer and to clarify their role in the pathogenesis of the disorders. Specifically, the findings here show that ORM plays a clear role in the VEGF-induced capillary morphogenesis which might be of relevance for tumor vascularization and anti-angiogenic tumor therapy.

7 Literature

Agirbasli,M. (2005). Pivotal role of plasminogen-activator inhibitor in vascular disease. Int. J. Clin. Pract. *59*, 102-106.

Alam,T., An,M.R., Mifflin,R.C., Hsieh,C.C., Ge,X., and Papaconstantinou,J. (1993). trans-activation of the alpha 1-acid glycoprotein gene acute phase responsive element by multiple isoforms of C/EBP and glucocorticoid receptor J. Biol. Chem. *268*, 15681-15688.

Anderson,N.L. and Anderson,N.G. (2002). The human plasma proteome: history, character, and diagnostic prospects. Mol. Cell Proteomics., 845-867.

Andreasen,P.A., Egelund,R., and Petersen,H.H. (2000). The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol. Life Sci. *57*, 25-40.

Andreasen,P.A., Kjoller,L., Christensen,L., and Duffy,M.J. (1997). The urokinase-type plasminogen activator system in cancer metastasis: a review. Int. J. Cancer *72*, 1-22.

Asahara,T. and Kawamoto,A. (2004). Endothelial progenitor cells for postnatal vasculogenesis Am. J. Physiol Cell Physiol *287*, C572-C579.

Banks,R.E., Dunn,M.J., Hochstrasser,D.F., Sanchez,J.C., Blackstock,W., Pappin,D.J., and Selby,P.J. (2000). Proteomics: new perspectives, new biomedical opportunities., Lancet *356*, 1749-1756.

Baumann,H. and Gauldie,J. (1990). Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. Mol. Biol. Med. *7*, 147-159.

Baumann,H., Prowse,K.R., Marinkovic,S., Won,K.A., and Jahreis,G.P. (1989). Stimulation of hepatic acute phase response by cytokines and glucocorticoids. Ann. N. Y. Acad. Sci. *557*, 280-95, discussion.

Baumann,H., Richards,C., and Gauldie,J. (1987). Interaction among hepatocyte-stimulating factors, interleukin 1, and glucocorticoids for regulation of acute phase plasma proteins in human hepatoma (HepG2) cells J. Immunol. *139*, 4122-4128.

Beetham,R. and Cattell,W.R. (1993). Proteinuria: pathophysiology, significance and recommendations for measurement in clinical practice. Ann. Clin. Biochem. *30 (Pt 5)*, 425-434.

Bennett,M. and Schmid,K. (1980). Immunosuppression by human plasma alpha 1-acid glycoprotein: importance of the carbohydrate moiety. Proc. Natl. Acad. Sci. U. S. A *77*, 6109-6113.

Binder,B.R., Mihaly,J., and Prager,G.W. (2007). uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view. Thromb. Haemost. *97*, 336-342.

Bing,C., Bao,Y., Jenkins,J., Sanders,P., Manieri,M., Cinti,S., Tisdale,M.J., and Trayhurn,P. (2004). Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia Proc. Natl. Acad. Sci. U. S. A *101*, 2500-2505.

Bischoff,R. and Luider,T.M. (2004). Methodological advances in the discovery of protein and peptide disease markers J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *803*, 27-40.

Boncela,J., Papiewska,I., Fijalkowska,I., Walkowiak,B., and Cierniewski,C.S. (2001). Acute phase protein alpha 1-acid glycoprotein interacts with plasminogen activator inhibitor type 1 and stabilizes its inhibitory activity J. Biol. Chem. *276*, 35305-35311.

Boucherie,H., Dujardin,G., Kermorgant,M., Monribot,C., Slonimski,P., and Perrot,M. (1995). Two-dimensional protein map of Saccharomyces cerevisiae: construction of a gene-protein index Yeast *11*, 601-613.

Brysk,M.M., Lei,G., Rajaraman,S., Selvanayagam,P., Rassekh,C.H., Brysk,H., Tyring,S.K., and Arany,I. (1997). Gene expression of zinc-alpha 2-glycoprotein in normal human epidermal and buccal epithelia In Vivo *11*, 271-274.

Burgi,W. and Schmid,K. (1961). Preparation and properties of Zn-alpha 2-glycoprotein of normal human plasma J. Biol. Chem. *236*, 1066-1074.

Cai,Z., Chiu,J.F., and He,Q.Y. (2004). Application of proteomics in the study of tumor metastasis Genomics Proteomics. Bioinformatics. *2*, 152-166.

Carmeliet,P., Kieckens,L., Schoonjans,L., Ream,B., van,N.A., Prendergast,G., Cole,M., Bronson,R., Collen,D., and Mulligan,R.C. (1993). Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization J. Clin. Invest *92*, 2746-2755.

Castagna,A., Cecconi,D., Sennels,L., Rappsilber,J., Guerrier,L., Fortis,F., Boschetti,E., Lomas,L., and Righetti,P.G. (2005). Exploring the hidden human urinary proteome via ligand library beads., J. Proteome. Res. *4*, 1917-1930.

Celis,J.E., Gesser,B., Dejgaard,K., Honore,B., Leffers,H., Madsen,P., Andersen,A., Basse,B., Celis,A., Lauridsen,J.B., and . (1989). Two dimensional gel human protein databases offer a systematic approach to the study of cell proliferation and differentiation. Int. J. Dev. Biol. *33*, 407-416.

Celis,J.E., Gromov,P., Ostergaard,M., Madsen,P., Honore,B., Dejgaard,K., Olsen,E., Vorum,H., Kristensen,D.B., Gromova,I., Haunso,A., Van,D.J., Puype,M., Vandekerckhove,J., and Rasmussen,H.H. (1996). Human 2-D PAGE databases for proteome analysis in health and disease: http://biobase.dk/cgi-bin/celis FEBS Lett. *398*, 129-134.

Celis,J.E., Gromova,I., Moreira,J.M., Cabezon,T., and Gromov,P. (2004). Impact of proteomics on bladder cancer research Pharmacogenomics. *5*, 381-394.

Chevallet,M., Santoni,V., Poinas,A., Rouquie,D., Fuchs,A., Kieffer,S., Rossignol,M., Lunardi,J., Garin,J., and Rabilloud,T. (1998). New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis *19*, 1901-1909.

Choong, P.F. and Nadesapillai,A.P. (2003). Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. Clin. Orthop. Relat Res. S46-S58.

Chorostowska-Wynimko,J., Skrzypczak-Jankun,E., and Jankun,J. (2004a). Plasminogen activator inhibitor type-1 controls the process of the in vitro sprout formation. J. Physiol Pharmacol. *55 Suppl 3*, 49-56.

Chorostowska-Wynimko,J., Swiercz,R., Skrzypczak-Jankun,E., Selman,S.H., and Jankun,J. (2004b). Plasminogen activator inhibitor type-1 mutants regulate angiogenesis of human umbilical and lung vascular endothelial cells. Oncol. Rep. *12*, 1155-1162.

Christensen,E.I. (2002). Pathophysiology of protein and vitamin handling in the proximal tubule. Nephrol. Dial. Transplant. *17 Suppl 9*, 57-58.

Christensen,E.I. and Birn,H. (2001). Megalin and cubilin: synergistic endocytic receptors in renal proximal tubule. Am. J. Physiol Renal Physiol *280*, F562-F573.

Christensen,E.I. and Gburek,J. (2004). Protein reabsorption in renal proximal tubule-function and dysfunction in kidney pathophysiology. Pediatr. Nephrol. *19*, 714-721.

Cohen,S.M., Arai,M., Jacobs,J.B., and Friedell,G.H. (1979). Promoting effect of saccharin and DL-tryptophan in urinary bladder carcinogenesis. Cancer Res. *39*, 1207-1217.

Colledge,M. and Scott,J.D. (1999). AKAPs: from structure to function. Trends Cell Biol. *9*, 216-221.

Correc,P., Fondaneche,M.C., Bracke,M., and Burtin,P. (1990). The presence of plasmin receptors on three mammary carcinoma MCF-7 sublines. Int. J. Cancer *46*, 745-750.

Curry,F.E. and Michel,C.C. (1980). A fiber matrix model of capillary permeability. Microvasc. Res. *20*, 96-99.

Davidoff,G., Schultz,J.S., Lieb,T., Andrews,K., Wardner,J., Hayes,C., Ward,M., Karunas,R., and Maynard,F. (1990). Rehospitalization after initial rehabilitation for acute spinal cord injury: incidence and risk factors. Arch. Phys. Med. Rehabil. *71*, 121-124.

De Graaf,T.W., Van der Stelt,M.E., Anbergen,M.G., and van,D.W. (1993). Inflammation-induced expression of sialyl Lewis X-containing glycan structures on alpha 1-acid glycoprotein (orosomucoid) in human sera. J. Exp. Med. *177*, 657-666.

Del,R.M., Fibbi,G., Dini,G., Grappone,C., Pucci,M., Caldini,R., Magnelli,L., Fimiani,M., Lotti,T., and Panconesi,E. (1990). Role of specific membrane receptors in urokinase-dependent migration of human keratinocytes. J. Invest Dermatol. *94*, 310-316.

Dobryszycka W., Katnik, I., (1992). in: Breborowicz J., Mackiewicz, A. (Eds.), Affinity Electrophoresis: Principles and Application, CRC Press, Boca Raton, 211-225.

Duche,J.C., Urien,S., Simon,N., Malaurie,E., Monnet,I., and Barre,J. (2000). Expressi.on of the genetic variants of human alpha-1-acid glycoprotein in cancer. Clin. Biochem. *33*, 197-202.

Dunham,I., Shimizu,N., Roe,B.A., Chissoe,S., Hunt,A.R., Collins,J.E., Bruskiewich,R., Beare,D.M., Clamp,M., Smink,L.J., Ainscough,R., Almeida,J.P., Babbage,A., Bagguley,C., Bailey,J., Barlow,K., Bates,K.N., Beasley,O., Bird,C.P., Blakey,S., Bridgeman,A.M., Buck,D., Burgess,J., Burrill,W.D., O'Brien,K.P., and . (1999). The DNA sequence of human chromosome 22. Nature *402*, 489-495.

Eisenberg,D., Marcotte,E.M., Xenarios,I., and Yeates,T.O. (2000). Protein function in the post-genomic era. Nature *405*, 823-826.

Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K., and Tuschl,T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Ergun,S., Kilic,N., Fiedler,W., and Mukhopadhyay,A.K. (1997). Vascular endothelial growth factor and its receptors in normal human testicular tissue. Mol. Cell Endocrinol. *131*, 9-20.

Ergun,S., Kilik,N., Ziegeler,G., Hansen,A., Nollau,P., Gotze,J., Wurmbach,J.H., Horst,A., Weil,J., Fernando,M., and Wagener,C. (2000). CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. Mol. Cell *5*, 311-320.

Ergun,S., Tilki,D., Oliveira-Ferrer,L., Schuch,G., and Kilic,N. (2006). Significance of vascular stabilization for tumor growth and metastasis. Cancer Lett. *238*, 180-187.

ez-Itza,I., Sanchez,L.M., Allende,M.T., Vizoso,F., Ruibal,A., and Lopez-Otin,C. (1993). Zn-alpha 2-glycoprotein levels in breast cancer cytosols and correlation with clinical, histological and biochemical parameters. Eur. J. Cancer *29A*, 1256-1260.

Fay,W.P., Shapiro,A.D., Shih,J.L., Schleef,R.R., and Ginsburg,D. (1992). Brief report: complete deficiency of plasminogen-activator inhibitor type 1 due to a frame-shift mutation. N. Engl. J. Med. *327*, 1729-1733.

Ferrara,N. (2005). VEGF as a therapeutic target in cancer. Oncology *69 Suppl 3*, 11-16.

Ferrara,N., Gerber,H.P., and LeCouter,J. (2003). The biology of VEGF and its receptors. Nat. Med. *9*, 669-676.

Flaumenhaft,R., Abe,M., Mignatti,P., and Rifkin,D.B. (1992). Basic fibroblast growth factor-induced activation of latent transforming growth factor beta in endothelial cells: regulation of plasminogen activator activity. J. Cell Biol. *118*, 901-909.

Folkman,J. (2003). Fundamental concepts of the angiogenic process. Curr. Mol. Med. *3*, 643-651.

Folkman,J. and D'Amore,P.A. (1996). Blood vessel formation: what is its molecular basis? Cell *87*, 1153-1155.

Folkman,J., Weisz,P.B., Joullie,M.M., Li,W.W., and Ewing,W.R. (1989). Control of angiogenesis with synthetic heparin substitutes. Science *243*, 1490-1493.

Forsgren,M., Raden,B., Israelsson,M., Larsson,K., and Heden,L.O. (1987). Molecular cloning and characterization of a full-length cDNA clone for human plasminogen. FEBS Lett. *213*, 254-260.

Fournier T., Medjoubi-N N., Porquet D., (2000).Alpha-1-acid glycoprotein. Biochim .Biophy. Acta. 1482 (1-2), 157-71.

Gehling,U.M., Ergun,S., Schumacher,U., Wagener,C., Pantel,K., Otte,M., Schuch,G., Schafhausen,P., Mende,T., Kilic,N., Kluge,K., Schafer,B., Hossfeld,D.K., and Fiedler,W. (2000). In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood *95*, 3106-3112.

Gorg,A., Obermaier,C., Boguth,G., Harder,A., Scheibe,B., Wildgruber,R., and Weiss,W. (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis *21*, 1037-1053.

Gorg,A., Postel,W., Weser,J., Patutschnick,W., and Cleve,H. (1985). Improved resolution of PI (alpha 1-antitrypsin) phenotypes by a large-scale immobilized pH gradient. Am. J. Hum. Genet. *37*, 922-930.

Gorg,A., Weiss,W., and Dunn,M.J. (2004). Current two-dimensional electrophoresis technology for proteomics. Proteomics. *4*, 3665-3685.

Grover,P.K. and Resnick,M.I. (1993). Two-dimensional analysis of proteins in unprocessed human urine using double stain. J. Urol. *150*, 1069-1072.

Hajjar,K.A., Hamel,N.M., Harpel,P.C., and Nachman,R.L. (1987). Binding of tissue plasminogen activator to cultured human endothelial cells. J. Clin. Invest *80*, 1712-1719.

Hanahan,D. and Folkman,J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell *86*, 353-364.

Hanash,S.M. (2000). Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: current status. Electrophoresis *21*, 1202-1209.

Hansen,J.E., Iversen,J., Lihme,A., and Bog-Hansen,T.C. (1987). Acute phase reaction, heterogeneity, and microheterogeneity of serum proteins as nonspecific tumor markers in lung cancer. Cancer *60*, 1630-1635.

Haraldsson,B. and Sorensson,J. (2004). Why do we not all have proteinuria? An update of our current understanding of the glomerular barrier. News Physiol Sci. *19*, 7-10.

Harder,A., Wildgruber,R., Nawrocki,A., Fey,S.J., Larsen,P.M., and Gorg,A. (1999). Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. Electrophoresis *20*, 826-829.

Hashizume,H., Baluk,P., Morikawa,S., McLean,J.W., Thurston,G., Roberge,S., Jain,R.K., and McDonald,D.M. (2000). Openings between defective endothelial cells explain tumor vessel leakiness. Am. J. Pathol. *156*, 1363-1380.

Herbert,B. (1999). Advances in protein solubilisation for two-dimensional electrophoresis. Electrophoresis *20*, 660-663.

Herbert,B.R., Molloy,M.P., Gooley,A.A., Walsh,B.J., Bryson,W.G., and Williams,K.L. (1998). Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis *19*, 845-851.

Hoorn,E.J., Pisitkun,T., Zietse,R., Gross,P., Frokiaer,J., Wang,N.S., Gonzales,P.A., Star,R.A., and Knepper,M.A. (2005). Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology. (Carlton.) *10*, 283-290.

Hoving,S., Gerrits,B., Voshol,H., Muller,D., Roberts,R.C., and van,O.J. (2002). Preparative two-dimensional gel electrophoresis at alkaline pH using narrow range immobilized pH gradients. Proteomics. *2*, 127-134.

Ibel,K., May,R.P., Kirschner,K., Szadkowski,H., Mascher,E., and Lundahl,P. (1990). Protein-decorated micelle structure of sodium-dodecyl-sulfate--protein complexes as determined by neutron scattering. Eur. J. Biochem. *190*, 311-318.

Jurgens,M., Appel,A., Heine,G., Neitz,S., Menzel,C., Tammen,H., and Zucht,H.D. (2005). Towards characterization of the human urinary peptidome. Comb. Chem. High Throughput. Screen. *8*, 757-765.

Kageyama,S., Isono,T., Iwaki,H., Wakabayashi,Y., Okada,Y., Kontani,K., Yoshimura,K., Terai,A., Arai,Y., and Yoshiki,T. (2004). Identification by proteomic analysis of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. Clin. Chem. *50*, 857-866.

Kersten,B., Burkle,L., Kuhn,E.J., Giavalisco,P., Konthur,Z., Lueking,A., Walter,G., Eickhoff,H., and Schneider,U. (2002). Large-scale plant proteomics. Plant Mol. Biol. *48*, 133-141.

Konety,B.R. and Getzenberg,R.H. (2001). Urine based markers of urological malignancy. J. Urol. *165*, 600-611.

Krishna,R.G. and Wold,F. (1993). Post-translational modification of proteins. Adv. Enzymol. Relat Areas Mol. Biol. *67*, 265-298.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lamm,D.L. (1994). RE: Editorial: bladder cancer. J. Urol. *152*, 1213.

Lasky,L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science *258*, 964-969.

Lau,A.T., He,Q.Y., and Chiu,J.F. (2003). Proteomic technology and its biomedical applications. Sheng Wu Hua Xue. Yu Sheng Wu Wu Li Xue. Bao. (Shanghai) *35*, 965-975.

Lawrence,D.A. (1997). The serpin-proteinase complex revealed. Nat. Struct. Biol. *4*, 339-341.

Lawrence,D.A., Ginsburg,D., Day,D.E., Berkenpas,M.B., Verhamme,I.M., Kvassman,J.O., and Shore,J.D. (1995). Serpin-protease complexes are trapped as stable acyl-enzyme intermediates. J. Biol. Chem. *270*, 25309-25312.

Lawrence,D.A., Olson,S.T., Palaniappan,S., and Ginsburg,D. (1994). Engineering plasminogen activator inhibitor 1 mutants with increased functional stability 1. Biochemistry *33*, 3643-3648.

Lee,R. and Droller,M.J. (2000). The natural history of bladder cancer. Implications for therapy. Urol. Clin. North Am. *27*, 1-13, vii.

Luttun,A. and Carmeliet,P. (2004). Angiogenesis and lymphangiogenesis: highlights of the past year. Curr. Opin. Hematol. *11*, 262-271.

MacDonald,T.J., DeClerck,Y.A., and Laug,W.E. (1998). Urokinase induces receptor mediated brain tumor cell migration and invasion. J. Neurooncol. *40*, 215-226.

Maisonpierre,P.C., Suri,C., Jones,P.F., Bartunkova,S., Wiegand,S.J., Radziejewski,C., Compton,D., McClain,J., Aldrich,T.H., Papadopoulos,N., Daly,T.J., Davis,S., Sato,T.N., and Yancopoulos,G.D. (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55-60.

Mandriota,S.J. and Pepper,M.S. (1997). Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. J. Cell Sci. *110 (Pt 18)*, 2293-2302.

Marshall,T. and Williams,K.M. (1998). Clinical analysis of human urinary proteins using high resolution electrophoretic methods. Electrophoresis *19*, 1752-1770.

Maunsbach,A.B. (1997). Absorption of I125-labeled homologous albumin by rat kidney proximal tubule cells. A study of microperfused single proximal tubules by electron microscopic autoradiography and histochemistry. 1966. J. Am. Soc. Nephrol. *8*, 323-351.

Merril,C.R., Goldman,D., Sedman,S.A., and Ebert,M.H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science *211*, 1437-1438.

Miles,L.A. and Plow,E.F. (1985). Binding and activation of plasminogen on the platelet surface. J. Biol. Chem. *260*, 4303-4311.

Molloy,M.P. (2000). Two-dimensional electrophoresis of membrane proteins using immobilized pH gradients. Anal. Biochem. *280*, 1-10.

Morrison,A.S., Buring,J.E., Verhoek,W.G., Aoki,K., Leck,I., Ohno,Y., and Obata,K. (1982). Coffee drinking and cancer of the lower urinary tract. J. Natl. Cancer Inst. *68*, 91-94.

Mostofi,F.K., Davis,C.J., Jr., and Sesterhenn,I.A. (1988). Progress in pathology of tumors of the urinary tract. Prog. Clin. Biol. Res. *277*, 1-20.

Mottonen,J., Strand,A., Symersky,J., Sweet,R.M., Danley,D.E., Geoghegan,K.F., Gerard,R.D., and Goldsmith,E.J. (1992). Structural basis of latency in plasminogen activator inhibitor-1. Nature *355*, 270-273.

Murphy,W.M., Soloway,M.S., Jukkola,A.F., Crabtree,W.N., and Ford,K.S. (1984). Urinary cytology and bladder cancer. The cellular features of transitional cell neoplasms. Cancer *53*, 1555-1565.

Narita,T., Sasaki,H., Hosoba,M., Miura,T., Yoshioka,N., Morii,T., Shimotomai,T., Koshimura,J., Fujita,H., Kakei,M., and Ito,S. (2004). Parallel increase in urinary excretion rates of immunoglobulin G, ceruloplasmin, transferrin, and orosomucoid in normoalbuminuric type 2 diabetic patients. Diabetes Care *27*, 1176-1181.

Newman,A. (1998). RNA splicing. Curr. Biol. *8*, R903-R905.

Oh,J., Pyo,J.H., Jo,E.H., Hwang,S.I., Kang,S.C., Jung,J.H., Park,E.K., Kim,S.Y., Choi,J.Y., and Lim,J. (2004). Establishment of a near-standard two-dimensional human urine proteomic map. Proteomics. *4*, 3485-3497.

Oliveira-Ferrer,L., Tilki,D., Ziegeler,G., Hauschild,J., Loges,S., Irmak,S., Kilic,E., Huland,H., Friedrich,M., and Ergun,S. (2004). Dual role of carcinoembryonic antigen-related cell adhesion molecule 1 in angiogenesis and invasion of human urinary bladder cancer. Cancer Res. *64*, 8932-8938.

Pang,J.X., Ginanni,N., Dongre,A.R., Hefta,S.A., and Opitek,G.J. (2002). Biomarker discovery in urine by proteomics. J. Proteome. Res. *1*, 161-169.

Patthy,L. (1985). Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. Cell *41*, 657-663.

Pepper,M.S., Belin,D., Montesano,R., Orci,L., and Vassalli,J.D. (1990). Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. J. Cell Biol. *111*, 743-755.

Pepper,M.S., Ferrara,N., Orci,L., and Montesano,R. (1991). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem. Biophys. Res. Commun. *181*, 902-906.

Petricoin,E.F., Ornstein,D.K., and Liotta,L.A. (2004). Clinical proteomics: Applications for prostate cancer biomarker discovery and detection. Urol. Oncol. *22*, 322-328.

Pieper,R., Gatlin,C.L., McGrath,A.M., Makusky,A.J., Mondal,M., Seonarain,M., Field,E., Schatz,C.R., Estock,M.A., Ahmed,N., Anderson,N.G., and Steiner,S. (2004). Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics. *4*, 1159-1174.

Pisitkun,T., Shen,R.F., and Knepper,M.A. (2004). Identification and proteomic profiling of exosomes in human urine. Proc. Natl. Acad. Sci. U. S. A *101*, 13368-13373.

Predescu,D., Predescu,S., McQuistan,T., and Palade,G.E. (1998). Transcytosis of alpha1-acidic glycoprotein in the continuous microvascular endothelium. Proc. Natl. Acad. Sci. U. S. A *95*, 6175-6180.

Rabbani,F. and Cordon-Cardo,C. (2000). Mutation of cell cycle regulators and their impact on superficial bladder cancer. Urol. Clin. North Am. *27*, 83-102, ix.

Rabilloud,T. (1998). Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis *19*, 758-760.

Rabilloud,T., Adessi,C., Giraudel,A., and Lunardi,J. (1997). Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis *18*, 307-316.

Rabilloud,T., Blisnick,T., Heller,M., Luche,S., Aebersold,R., Lunardi,J., and Braun-Breton,C. (1999). Analysis of membrane proteins by two-dimensional electrophoresis: comparison of the proteins extracted from normal or Plasmodium falciparum-infected erythrocyte ghosts. Electrophoresis *20*, 3603-3610.

Rafii,S., Lyden,D., Benezra,R., Hattori,K., and Heissig,B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy?. Nat. Rev. Cancer *2*, 826-835.

Rakic,J.M., Maillard,C., Jost,M., Bajou,K., Masson,V., Devy,L., Lambert,V., Foidart,J.M., and Noel,A. (2003). Role of plasminogen activator-plasmin system in tumor angiogenesis. Cell Mol. Life Sci. *60*, 463-473.

Ramakumar,S., Bhuiyan,J., Besse,J.A., Roberts,S.G., Wollan,P.C., Blute,M.L., and O'Kane,D.J. (1999). Comparison of screening methods in the detection of bladder cancer. J. Urol. *161*, 388-394.

Rasmussen,H.H., Orntoft,T.F., Wolf,H., and Celis,J.E. (1996). Towards a comprehensive database of proteins from the urine of patients with bladder cancer. J. Urol. *155*, 2113-2119.

Rogers,M.A., Clarke,P., Noble,J., Munro,N.P., Paul,A., Selby,P.J., and Banks,R.E. (2003). Proteomic profiling of urinary proteins in renal cancer by surface enhanced laser desorption ionization and neural-network analysis: identification of key issues affecting potential clinical utility. Cancer Res. *63*, 6971-6983.

Schaub,S., Wilkins,J., Weiler,T., Sangster,K., Rush,D., and Nickerson,P. (2004). Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int. *65*, 323-332.

Schmid,K. (1950). J. Am. Chem. Soc. 72, 2816.

Schmid,K. (1975). inPutman, F.W.Y (Ed.), Plasma proteins, structure, function and genetic control, Academic Press, NewYork. vol.1. 183-288.

Schmid,K. (1953). J. Am. Chem. Soc.75. 68-60.

Schuck,S., Honsho,M., Ekroos,K., Shevchenko,A., and Simons,K. (2003). Resistance of cell membranes to different detergents. Proc. Natl. Acad. Sci. U. S. A *100*, 5795-5800.

Smith,G., Hippisley-Cox,J., Harcourt,S., Heaps,M., Painter,M., Porter,A., and Pringle,M. (2007). Developing a national primary care-based early warning system for health protection--a surveillance tool for the future? Analysis of routinely collected data. J. Public Health (Oxf) *29*, 75-82.

Somiari,R.I., Sullivan,A., Russell,S., Somiari,S., Hu,H., Jordan,R., George,A., Katenhusen,R., Buchowiecka,A., Arciero,C., Brzeski,H., Hooke,J., and Shriver,C. (2003). High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. Proteomics. *3*, 1863-1873.

Sorensson,J., Matejka,G.L., Ohlson,M., and Haraldsson,B. (1999). Human endothelial cells produce orosomucoid, an important component of the capillary barrier. Am. J. Physiol *276*, H530-H534.

Spahr,C.S., Davis,M.T., McGinley,M.D., Robinson,J.H., Bures,E.J., Beierle,J., Mort,J., Courchesne,P.L., Chen,K., Wahl,R.C., Yu,W., Luethy,R., and Patterson,S.D. (2001). Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. Proteomics. *1*, 93-107.

Stein,L.D. (2004). Human genome: end of the beginning. Nature *431*, 915-916.

Sun,W., Li,F., Wu,S., Wang,X., Zheng,D., Wang,J., and Gao,Y. (2005). Human urine proteome analysis by three separation approaches. Proteomics. *5*, 4994-5001.

Tada,T., Ohkubo,I., Niwa,M., Sasaki,M., Tateyama,H., and Eimoto,T. (1991). Immunohistochemical localization of Zn-alpha 2-glycoprotein in normal human tissues. J. Histochem. Cytochem. *39*, 1221-1226.

Tastet,C., Charmont,S., Chevallet,M., Luche,S., and Rabilloud,T. (2003). Structure-efficiency relationships of zwitterionic detergents as protein solubilizers in two-dimensional electrophoresis. Proteomics. *3*, 111-121.

Thongboonkerd,V., Klein,J.B., Jevans,A.W., and McLeish,K.R. (2004). Urinary proteomics and biomarker discovery for glomerular diseases.. Contrib. Nephrol. *141*, 292-307.

Thongboonkerd,V. and Malasit,P. (2005). Renal and urinary proteomics: current applications and challenges. Proteomics. *5*, 1033-1042.

Thongboonkerd,V., McLeish,K.R., Arthur,J.M., and Klein,J.B. (2002). Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int. *62*, 1461-1469.

Thulasiraman,V., Lin,S., Gheorghiu,L., Lathrop,J., Lomas,L., Hammond,D., and Boschetti,E. (2005). Reduction of the concentration difference of proteins in biological liquids using a library of combinatorial ligands. Electrophoresis *26*, 3561-3571.

Thurston,G., Rudge,J.S., Ioffe,E., Zhou,H., Ross,L., Croll,S.D., Glazer,N., Holash,J., McDonald,D.M., and Yancopoulos,G.D. (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat. Med. *6*, 460-463.

Todorov,P.T., McDevitt,T.M., Meyer,D.J., Ueyama,H., Ohkubo,I., and Tisdale,M.J. (1998). Purification and characterization of a tumor lipid-mobilizing factor. Cancer Res. *58*, 2353-2358.

Tsihlias,J. and Grossman,H.B. (2000). The utility of fibrin/fibrinogen degradation products in superficial bladder cancer. Urol. Clin. North Am. *27*, 39-46.

Vassalli,J.D., Baccino,D., and Belin,D. (1985). A cellular binding site for the Mr 55,000 form of the human plasminogen activator, urokinase. J. Cell Biol. *100*, 86-92.

Vlahou,A., Schellhammer,P.F., Mendrinos,S., Patel,K., Kondylis,F.I., Gong,L., Nasim,S., and Wright,J.G., Jr. (2001). Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am. J. Pathol. *158*, 1491-1502.

Wilson,T.G., Pritchett,T.R., Lieskovsky,G., Warner,N.E., and Skinner,D.G. (1991). Primary adenocarcinoma of bladder. Urology *38*, 223-226.

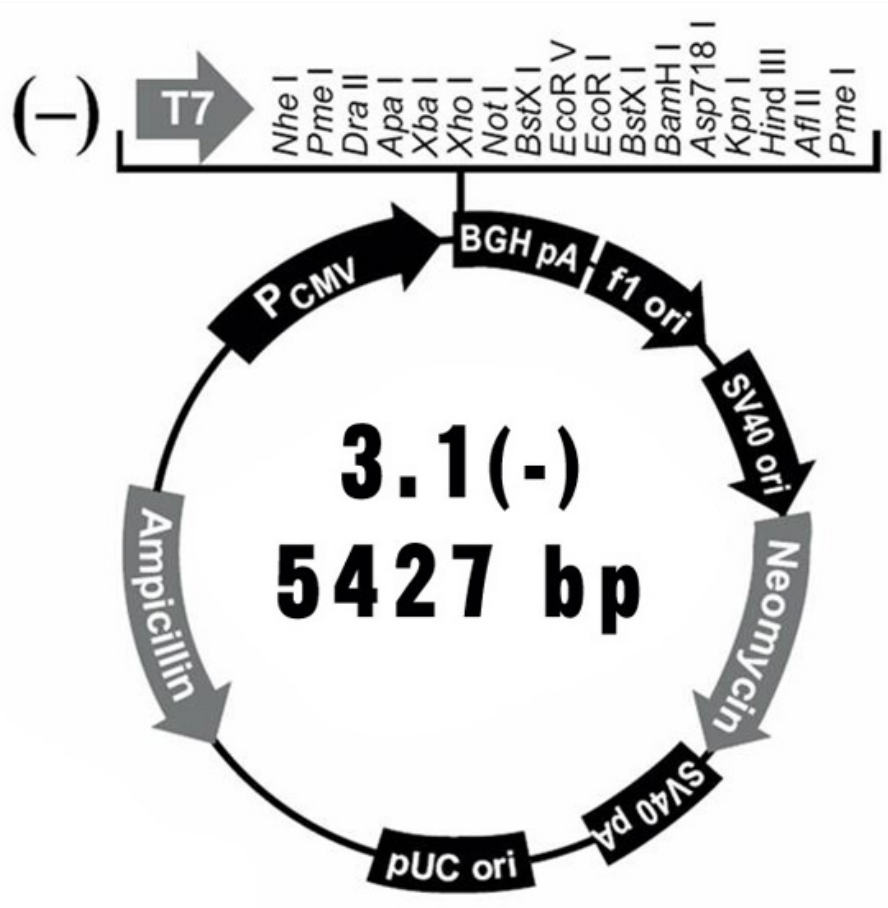
Wynder,E.L. and Stellman,S.D. (1977). Comparative epidemiology of tobacco-related cancers. Cancer Res. *37*, 4608-4622.

Zengin,E., Chalajour,F., Gehling,U.M., Ito,W.D., Treede,H., Lauke,H., Weil,J., Reichenspurner,H., Kilic,N., and Ergun,S. (2006). Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. Development *133*, 1543-1551.

Zhou,H., Yuen,P.S., Pisitkun,T., Gonzales,P.A., Yasuda,H., Dear,J.W., Gross,P., Knepper,M.A., and Star,R.A. (2006). Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int. *69*, 1471-1476.

8 Appendix

8.1 Map of 3.1(-) vector



8.2 Curriculum Vitae

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1990-1993 : High School in Konya, Turkey
1994-1998 : Undergraduate in Chemistry Department, Selcuk University, Turkey
1999-2001 : Graduate (MSc.) in Chem. Dept., Biochemistry Division, Institute of Pure and Applied Science, Hacettepe University, Turkey
2001-2002 : PhD in Chem. Dept., Biochemistry Division, Hacettepe University University, Institute of Pure and Applied Science, Turkey (not completed)
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2002-2006 : Research Scientist in Department of Urology, University Hospital of Hamburg-Eppendorf
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Publications:

1. Tilki D, **Irmak S**, Oliveira-Ferrer L, Hauschild J, Miethe K, Atakaya H, Hammerer P, Friedrich MG, Schuch G, Galalae R, Stief CG, Kilic E, Huland H, Ergun S. CEA-related cell adhesion molecule-1 is involved in angiogenic switch in prostate cancer, Oncogene, 25(36) (2006),4965-74.

2. **Ster Irmak**, Derya Tilki, Jochen Heukeshoven, Leticia Oliveira-Ferrer, Martin Friedrich, Hartwig Huland, Suleyman Ergun, Stage-Dependent Increase of Orosomucoid and Zinc alpha-2 Glycoprotein in Urinary Bladder Cancer, Proteomics 5(16) (2005),4296- 304.

3. L.Oliveira-Ferrer, Derya Tilki, Gudrun Ziegeler, Jessica Hauschild, Sonja Loges, **Ster Irmak**, Ergin Kilic, Hartwig Huland, Martin Friedrich, Suleyman Ergun, Dual Role of CEACAM1 in Angiogenesis and Invasion of Human Urinary Bladder Cancer, Cancer Reseach 64 (2004), 8932-8938.

4. Eroğlu M., **Irmak S.**, Acar A., Denkbaş E.B., Design and Evaluation of a Mucoadhesive Therapeutic Agent Delivery System for Postoperative Chemotherapy in Superficial Bladder Cancer, International Journal of Pharmaceutics, 235 (2002) 51–59.

Presentations (Abstracts Available):

1. **Irmak S**, Tilki D, Oliveira-Ferrer L, Ergün S, Urinary proteomics and the role of orosomucoid (ORM) in vascular endothelial cells and angiogenesis."102nd Annual Meeting of the Anatomische Gesellschaft", 30 March-2 April, 2007, Giessen, Germany. (Oral)
2. **S. Irmak**, D. Tilki, J. Heukeshoven, L. Oliveira-Ferrer, , M. Friedrich, S. Ergün., The detection of orosomucoid in urinary bladder cancer, "17th Symposium Experimentelle Urologie", 17-18 March 2006, Essen, Germany.(Poster)
3. **S. Irmak**, L. Oliveira-Ferrer, J. Heukeshoven, M. Friedrich, H. Huland, S. Ergün., Determination of urinary protein pattern in bladder carcinoma by proteomic analyses., "European Association of Urology(EAU), XIXth Congress, 24-27 March 2004, Vienna, Austria.(Poster)
4. M.Fernando, L. Oliveira-Ferrer, D. Tilki, G. Zeigeler, J. Hauschild, **S. Irmak**, S. Sevinc, E. Kilic, M. Friedrich, C. Wagener, S. Ergün, Dual role of CEACAM1 in angiogenesis and invasion of human urinary bladder cancer., "European Association of Urology(EAU), XIXth Congress, 24-27 March 2004, Vienna, Austria.(Poster)
5. **S. Irmak**, L. Oliveira-Ferrer, J. Heukeshoven, M. Friedrich, H. Huland, S. Ergün., Determination of urinary protein pattern in bladder carcinoma by proteomic analyses., "16th Experimental Urology Congress", 11-13 March 2004, Lübeck, Germany.(Poster)
6. Denkbaş E.B., **Irmak S.**, Özdemir N., Eroğlu M., Acar M., Mitomycin-C Loaded Mucoadhesive Polymeric Carriers to Use in Postoperative Chemotherapy in Superficial Bladder Cancer: *In-vitro* Studies, IX. International Symposium on Biomedical Science and Technology, Antalya, Turkey, 19-22 Eylül 2002. (Poster)

7. Denkbaş E. B.., Kılıçay E., Birlikseven C., **Irmak S.**, Öztürk E.**,** Preparation of magnetic chitosan microspheres and determination of their magnetical properties, "2nd National Congress of Chromatography", Kırıkkale University, 6-8 June, 2001, Kırıkkale. (Poster)

8. Kılıçay E**., Irmak S.**, Baysal M.Y., Denkbaş E.B., Preparation and Characterization of Magnetic Chitosan Microspheres for Enzyme Immobilization, 3rd Mediterranean Basin Conference on Analytical Chemistry, June 4-9, 2000, Antalya, Turkey. (Poster)

Courses, Congress and Summer Schools:

1. ."102nd Annual Meeting of the Anatomische Gesellschaft", 30 March-2 April, 2007; Giessen, Germany.
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3. "15th Annual International CEA Symposium", 21-24 July 2005, Berlin, Germany.
4. "European Association of Urology(EAU), XIXth Congress", 24-27 March 2004, Vienna, Austria.
5. "16th Experimental Urology Congress", 11-13 March 2004, Lübeck, Germany.
6. "2nd National Congress of Chromatography", Kırıkkale University, 6-8 June, 2001, Kırıkkale, Turkey.
7. "Controlled Release Systems", Marmara University, 11 May, 2001, Istanbul, Turkey.
8. "7th National Symposium on Biomedical Science and Technology", Hacettepe University, 25-27 September, 2000, Ankara, Turkey.
9. "Modern and Classical Biochemistry Methods", Summer School, Agean University, 23 August - 2 September 2000, Kuşadası, İzmir, Turkey.
10. "3rd Mediterranean Basin Conference on Analytical Chemistry, MBCAC III", 4-9 June, 2000, Antalya, Turkey.
11. ."1st National Chromatography Congress", 16-18 June, 1999, Kırıkkale University, Kırıkkale, Turkey.
12. ."Certificate of Education", Selçuk University, Education Faculty, 1997, Selçuklu, Konya, Turkey.

Erklärung

Hiermit erkläre ich, gem § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Urinary proteomics and the role of orosomucoid (ORM) in vascularization of bladder cancer“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Ster Irmak befürworte.

Essen, 13. April 2007

Prof. Dr. Süleyman Ergün

Erklärung

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, 13. April 2007

Ster Irmak

Erklärung

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, 13. April 2007

Ster Irmak