MULTIPLEX xMAP IMMUNOANALYSIS
AND EXAMPLES OF ITS APPLICATION

Doctoral thesis

Plzeň, 2009

Mgr. Jindra Vrzalová
MULTIPLEXOVÁ xMAP IMUNOANALÝZA
A PŘÍKLADY JEJÍHO VYUŽITÍ

Dizertační práce

Plzeň, 2009

Mgr. Jindra Vrzalová
Děkuji mému školiteli prof. MUDr. O. Topolčanovi CSc. za jeho užitečné rady a hodnotné vedení a všem kolegům za jejich ochotnou spolupráci a pomoc s laboratorními i klinickými problémy.
Zvláštní poděkování patří mé rodině a přátelům za jejich trpělivou podporu.

I would like to thank my supervisor prof. MUDr. O. Topolčan CSc. for his valuable advice and skilled leadership and all colleagues for their willing help and cooperation in laboratory and clinical tasks.
Special thanks belong to my family and friends for their patient support.
MOTTO: 3

1 AIM OF THE STUDY 3

2 INTRODUCTION 4

3 THEORETICAL PART 6

3.1 HISTORY OF IMMUNOANALYSIS 6
3.2 PROTEOMICS 7
3.3 MULTIPLEX PROTEOMIC TECHNOLOGIES 9
  3.3.1 IMMUNOASSAY TECHNOLOGIES 10
  3.3.1.1 Planar microarrays on chips 10
  3.3.1.2 Arrays in plastic microwell 11
  3.3.1.3 Bead arrays 11
  3.3.2 2D ELECTROPHORESIS 13
  3.3.3 MASS SPECTROMETRY 14
3.4 XMAP TECHNOLOGY 15
  3.4.1 HISTORY OF XMAP TECHNOLOGY 15
  3.4.2 PRINCIPLE OF XMAP TECHNOLOGY 16
  3.4.3 MULTIPLEX IMMUNOASSAY REACTION 18
    3.4.3.1 Reagents 20
  3.4.4 ASSAY PRINCIPLES FOR NUCLEAR TRANSCRIPTION FACTOR ASSAY 20
  3.4.5 ASSAY PRINCIPLE FOR MULTIPLEX RNA QUANTIFICATION 20
  3.4.6 miRNA QUANTIFICATION TECHNOLOGY 21
  3.4.7 ASSAY PRINCIPLES FOR GENETIC RESEARCH 22
  3.4.8 XMAP TECHNOLOGY ADVANTAGES AND BENEFITS: 23
  3.4.9 APPLICATION RANGE 24
    3.4.10 FUTURE VISION 24
3.5 APPLICATION OF XMAP MULTIPLEX IMMUNOANALYSIS 25
  3.5.1 OPHTHALMOLOGY 25
  3.5.2 CANCER MONITORING 26
  3.5.3 NEONATAL SCREENING – DRIED BLOOD SPOT ANALYSIS 29
  3.5.4 SEPSIS 30
  3.5.5 ENDOCRINOLEG 31
  3.5.6 GYNAECOLOGY AND OBSTETRICS 31
  3.5.7 AUTOIMMUNITY 33

4 EXPERIMENTAL PART 34

4.1 ANALYTICAL PART 37
  4.1.1 WHICH BLOOD SAMPLE TYPE IS ADEQUATE? 37
  4.1.2 COMPARISON WITH OTHER IMMUNOANALYTIC METHODS 43
    4.1.2.1 Comparison with another multiplex bead assay: Flow Cytomix 43
    4.1.2.2 Comparison with conventional nonmultiplex immunoassay methods 46
  4.1.3 QUALITY CONTROL: 49
    4.1.3.1 Commercial controls 49
Motto:

The only way of finding the limits of the possible is by going beyond them into the impossible.

Jediným způsobem, jak zjistit meze možného, je odvážit se alespoň malý kousek za hranice nemožného.

A.C. Clarke

1 Aim of the study

The task of my work was to study the possibilities of the modern multiplex method - Luminex xMAP technology on the field of immunoanalysis with focus on measurement of cytokines, growth factors, adhesive molecules and other novel biomarker levels in various biologic fluids for clinical research and its possible use in clinical practise with focus on cancer applications.
2 Introduction

Laboratory results are an important part of diagnostic and treatment monitoring process. Markers of disease and pathophysiologic status of organism e.g. hormones occur in very small concentrations in biologic fluids. Thanks to development of immunoanalysis even proteins in pg/mL and lower concentrations are nowadays routinely measured in clinical laboratories.

Diseases are not simplex processes and so measuring of one protein for one disease is not sufficient and on the other hand measuring of protein panels one by one protein, even with up-to-date automatic instrumental assays, is very expensive, biologic material and time consuming task. That’s why nowadays many modern analytical tools for genomic, proteomic and metabonomic studies come forward and the research is now focusing on omics and multiplex point of view. The omics studies are not only designated for obtaining of vast amounts of data, but much more for introduction of novel philosophy in research - omics mean to study the multiplex pathways and processes by multiplex tools. Multiplexing is necessary for research and clinical monitoring of net physiological systems with complex interrelationships and complex effect on organism e.g. cytokines, chemokines and growth factors. Nowadays there already exist technical properties for multiplex analyses, but there is a lack of experience with their use and a lack of knowledge in multiplex data handling and multiplex statistical methods in clinical research and routine. That is why I focused my work on one of the modern multiplex technologies - xMAP Luminex technology

My thesis is divided into two major sections – theoretical and experimental:
While many works describe the basic principles of the immunoassay, the theoretical part of this thesis describes only shortly history of immunoassay development resulting in modern multiplex technologies. There are briefly discussed major highlighting up-to-date proteomic technologies. The principle of xMAP technology, used for experiments, is described in detail. The last part of the theoretical section is dedicated to the illustration of the possible use of the xMAP multiplex immunoanalysis on fields closest to rapid and innovative application in routine.
Experimental section includes two separated parts. In first experimental part, analytic experiments are described including comparisons of the xMAP with other methods, quality control data and comparison of the results for different sample blood types. In second experimental part, results of three different pilot clinical experiments are shown as examples of the use of xMAP technology in clinical oncology research in our laboratory.
3 Theoretical part

3.1 History of immunoanalysis

The principle of an immunoassay was invented by R. S Yalow and S. A. Berson. Yalow was awarded the 1977 Nobel Prize for Medicine for “the development of the RIA for peptide hormones”. The basic idea for immunoassay was an observation that the binding of labeled insulin to a fixed concentration of antibody is a quantitative function of the amount of insulin present. This observation provided the basis for the radioimmunoassay of plasma insulin. In the first immunoanalytic studies iodine-131 (β and γ radiation) was used for the labeling. However, investigations and analysis, which lasted for several years and which included studies on the quantitative aspects of the reaction between insulin and antibody and the species specificity of the available antisera, were required to translate the theoretical concepts of radioimmunoassay into the experiments which led first to the measurement of plasma insulin in rabbits following exogenous insulin administration and finally in 1959 publication (Yalow R. S. and Berson S.A., 1959) of the measurement of insulin in unextracted human plasma.

(Yalow R. S., 1977)

The second milestone of the immunoassay history was the development of noncompetitive methods and methods using two antibodies, so called sandwich methods.

In 1968, Miles and Hales published their first results of an immunoradiometric technique with radioactive labeled antibodies rather than labeled antigen for measuring insulin in human plasma (Miles L.E.M. and Hales C.N. 1968). The Uppsala group had developed a so-called (radio)immunosorbent technique in which antibodies were insolubilized by coupling them to cellulose or Sephadex beads.

Between 1966 and 1969, the group in Villejuif reported their successful results of coupling antigens or antibodies with enzymes such as alkaline phosphatase (EC 3.1.3.1), glucose oxidase (EC 1.1.3.4), and others. This started an expansion of new techniques for detection of immunoanalytic reaction. Engvall and Perlmann published their first paper on ELISA in 1971 and demonstrated quantitative measurement of IgG in rabbit serum with alkaline phosphatase as the reporter label. In the same year, van Weemen and Schuurs published their innovative
work on EIA and reported that it was possible to quantify human chorionic gonadotropin concentrations in urine. They used the enzyme horseradish peroxidase (EC 1.11.17), coupled by means of glutaraldehyde, as the reporter label. Colour enzyme reactions were followed by labeling with fluorophores or fluorescence polarization. The use of chemiluminiscence as a label for immunoassay was first described in 1976 by Schroeder et al. and it is used in major of nowadays immunoassay instruments. Novel detection techniques combined with better avidity of antibodies have enabled improvement of sensitivities of assays. Further technical advances led to first automated pipetting devices (Micromedics; Hamilton), multichannel pipettes (Lab Systems) and microtiter plate readers and washers (Fig. 1), and in the 1980s fully automated test instruments were manufactured by Boehringer-Mannheim and Abbott, among others. Such automated systems have come to stay in medical laboratories. (Lequin R. M., 2005; Wu A.H.B., 2006)

Till today the immunoassay has become the accepted gold standard for single protein measurement, but up-to-date milestone of immunoassay world is to enter into the era of multiplexing.

3.2 Proteomics

After the completion of human genome sequencing project the science has emerged into the proteomics era. It was shown that the rate of expression is not straight correlated to protein level because of further regulation steps in translation and that from genome or transcriptome data it is not possible to deduce the protein functional status, that’s why the proteome is in the centre of the interest. Proteomics is the study of the function of all expressed proteins. The traditionally held view of proteomics as simply cataloging and developing lists of the cellular protein repertoire of a cell is now changing, especially in the sub-discipline of clinical proteomics. Clinical proteomics, as a new and most exciting sub-discipline of proteomics, involves the bench-to-bedside clinical application of proteomic tools. The most relevant information archive to clinical applications and drug development involves the elucidation of the information flow of the cell; the "software" of protein pathway networks and circuitry. Tremendous progress has been made in the past few years in generating large-scale data sets for protein-protein interactions, organelle composition, protein activity patterns and protein profiles in cancer patients. Cancer, as a model disease, provides a fertile environment to study the application
of proteomics at the bedside. The promise of clinical proteomics in conjunction with novel technologies is that we will detect cancer earlier through discovery of biomarkers, of the next generation of drug targets and imaging biomarkers, and further that we will apply this knowledge to patient-tailored therapy. Further technological improvements, organization of international proteomics projects and open access to results are needed for proteomics to fulfil its potential.
(Tyers M., 2003; Krieg R.C., 2002)

In 2001, The Human Proteome Organisation (HUPO) was founded as an international scientific organization representing and promoting proteomics through international cooperation and collaborations by fostering the development of new technologies, techniques and training. Several proteome databases were established. The Swedish Human Protein Atlas (HPA) program, founded by the Knut and Alice Wallenberg Foundation, has been set up to allow for a systematic exploration of the human proteome using Antibody-Based Proteomics. This is accomplished by combining high-throughput generation of affinity-purified (mono-specific) antibodies with protein profiling in a multitude of tissues/celltypes assembled in tissue microarrays. The program is run by Proteome Resource (HPR) Center located in Stockholm and Uppsala, Sweden. (www.proteinatlas.org)

Another database, The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics is dedicated to the analysis of protein sequences and structures as well as 2-D polyacrylamide gel electrophoresis (www.expasy.org). Further, The Human Protein Reference Database (HPRD) represents a centralized platform to visually depict and integrate information pertaining to domain architecture, post-translational modifications, interaction networks and disease association for each protein in the human proteome. All the information in HPRD has been manually extracted from the literature by expert biologists who read, interpret and analyze the published data. (Mishra G. 2006, www.hprd.org)

Protein-protein interaction databases have become a major resource for investigating biological networks and pathways in cells. Each of these databases has their own unique features with a large variation in the type and depth of their annotations; e.g: BIND, DIP, HPRD, IntAct, MINT, MIPS, PDZBase and Reactome databases. (Mathivanan S. 2006)
3.3 **Multiplex proteomic technologies**

Multiplexed measurement is logical for biological discovery with proteins because they constitutively function within networks, pathways, complexes and families. The activity of an individual protein is dependent not only on its abundance, but also on the effects on interacting, modifying, antagonistic and synergistic proteins. Cytokine biology provides an example of the complexity of protein networks and inadequacy of monoplex measurement. In living systems, effects on target –cell activities are a dynamic aggregate of multiple agonist and antagonist cytokines, associated modifier proteins, ligands and receptor antagonists.... But the protein multiplex measurements meet technical challenges – diverse physico-chemical properties of proteins, their instability, the interferences in immunoassays, high diversity in dynamic range of protein concentration levels – their abundance ranges from grams to tenths of picograms per mL. Furthermore, serum protein abundance can change by as much as 10000 fold on stimulation. (Kingsmore S.F., 2006)

Goals of multiplexed measurement:

1. study of protein abundance – “descriptive” proteomic
2. study of protein networks, pathway modelling of physiological as well as pathophysiological processes
3. biomarker validation
4. clinical diagnostic
5. looking for therapy targets

Reasons for multiplexed type of measurement:

1. reduce of time and labour for “multiprotein” studies – gain large data in a short time
2. cost-effectiveness
3. valid data for comparison of results among different proteins – similar preanalytic as well as analytic conditions for all proteins
4. sample volume reduction
Multiplex analytical technologies can be divided according to their basic principle into three categories:

1. Immunoassay
2. Separating technologies – two dimensional electrophoresis or chromatography
3. Mass spectrometry
4. The proteomic tools based on combining the latter two principles – e.g. two dimensional electrophoresis with mass spectrometry or high performance liquid chromatography coupled to mass spectrometry

### 3.3.1 Immunoassay technologies

The technologies can be divided according to their format philosophy: forward phase arrays, reverse phase arrays and arrays for protein to protein interaction. On forward phase protein arrays, the sample is incubated on the array, so that different analytes can be detected simultaneously. Reverse phase arrays consists of different samples that are immobilised on a chip. In a single step, a large collection of samples can be screened for the presence or absence of one distinct target protein. This technique is used as well with tiny amounts of tissue or microdissected tissue, and so the protein screening can be very address for the protein occurrence location.

(Poetz O., 2005)

#### 3.3.1.1 Planar microarrays on chips

They have evolved from DNA arrays. Antibodies or antigens are spotted on the planar surface - silicon chips or glass slides with attached nitrocellulose membrane (FAST slides). The spatial location of the specific capture reagent on the microarray defines the identity of analyte measured at that location. Studied proteins are quantified thanks to various signal generation and enhancement – colorimetry, radioactivity, fluorescence, chemiluminiscence, quantum dots, enzyme-linked assays, tyramide signal amplification, rolling circle amplification etc. - as the intensity of the signal on the dedicated spot is read by array scanner.

Advantages – low cost, relatively high number of studied protein; glass slides have the advantage that they are compatible with standard microarray equipment and detection equipment used for DNA chips.
Disadvantages – denaturation of capture molecules on slide, lot-to-lot variability, only comparative results – not quantifying (no calibration curve), no interlaboratory comparison possibility, still lack the power of full automation.

3.3.1.2 Arrays in plastic microwell

Thermo Scientific SearchLight Protein Array Technology is a multiplexing sandwich-ELISA system based on chemiluminescent or fluorescent detection of analytes whose respective capture-antibodies are spotted in arrays within each well of a 96-well microplate. As many as 16 analytes (4 x 4 array in each well) can be measured per well, meaning that 16 cytokines or other biomarkers can be assayed simultaneously with each 50 µl sample. Cytokines that are captured by the arrayed mAbs are detected by using biotinylated mAbs, followed by the addition of a streptavidin-horseradish peroxidase (HRP) conjugate and a chemiluminescent substrate. The light produced from the HRP-catalyzed oxidation of the substrate is measured at each spot in the array by imaging the entire plate with a commercially available CCD camera. For measuring there is needed CCD imaging system with special software. (www.piercenet.com/products; Moody M.D., 2001).

3.3.1.3 Bead arrays

Bead-based systems are an interesting alternative to planar arrays. UltraPlex is a microparticle array technology developed by SmartBead Technologies Ltd. The system comprises aluminium microparticles that bear a unique barcode etched through the metal. A range of molecules can be coupled to the microparticles, which in effect barcodes the molecules and has the capacity to multiplex from two to millions of individual tests simultaneously in the same vessel (as the number of barcodes available is almost unlimited). The UltraPlex reader is an image-capture-based system, based around a fluorescent microscope, the unit accepts standard 96-well plates and reads up to 100 000 microparticles per hour. Transmitted white light is used to read the barcode within each microparticle and a fluorescent light source excites the reporter fluorophore used in the UltraPlex assays. (Pang S., 2005; www.ultraplex.com)
Flow cytometry detection is involved mainly for projects, where the number of studied protein is lower - more focused. The common principle of these assays are polystyrol beads with fluorescent internal dye coupled with antibodies (or antigens) specific to the analytes to be detected. A mix of coupled beads is incubated with the samples to be tested. Proteins are captured from the sample onto the beads and by sandwich technology the second biotin conjugated antibodies are bound as well. Streptavidin-Phycoerythrin (PE) is added and binds to the biotin conjugates. Beads are differentiated by their sizes and/or distinct spectral signature for flow cytometry. There exist two major technologies in this field: FlowCytomix – developed by BenderMedSystems company and xMAP technology developed by Luminex Corporation. The latter will be discussed in separate chapter. In the experimental part the comparison experiment of these two methods is described.

**FlowCytomix**

For FlowCytomix assay two sets of beads with different sizes (4 µm & 5 µm) are used. Each of the two sizes consists of bead populations which are differentiated by varying intensities of an internally fluorescent dye. The dye can be excited with an Argon, He-Ne, or even UV laser, and emits in the far red (690 nm) which is detected in the FL-3/FL-4 channel. The combination of the two different bead sizes and different internal dye intensities makes it possible to distinguish up to 20 bead sets in one fluorescent channel. Streptavidin-PE, which binds to the biotin conjugate, emits at 578 nm and is detected in the FL-2 channel and allows the quantification of the analyte. Data are analysed by special software provided with the kits. (www.bendermedsystems.com/multiple-analyte-detection-flowcytomix)

**Picture 1 An example of measurement by FlowCytomix technology**
Furthermore, Illumina company produced the BeadArray, which consists of a high-density, ordered microwell array that is connected to individual optical fibers. Each fiber is chemically etched to create a 3 μm diameter well and is filled by a single encoded bead that is randomly assembled by simple dipping. This system allows assays with encoded microbeads to be performed without the use of flow cytometer. (Lim C.T., 2007)

3.3.2 2D electrophoresis

Two-dimensional electrophoresis comprises isoelectric focusing as a first dimension, which separate proteins according to their charge (pI), and sodium dodecyl (lauryl) sulfate-polyacrylamide gel (SDS-PAG) electrophoresis as a second dimension, which separate proteins according to their size (Mr), both under denaturing conditions. A pH gradient is applied to a gel and an electric potential is applied across the gel. The result is that the analytes are spread out across a two-dimensional surface rather than along a line. This technique enables to separate 2000 proteins but sample may contain more than 10000 proteins. Unlike, there could be more than one protein at one given spot on the 2-D gel. A lot of methods are applied to dye the proteins on the gel to visualize them, e.g., silver, Coomasie, Sypro Ruby staining. The fluorescent detection has increasingly replaced colorimetric and radiometric detection on gels and blots. Quantitative computer analysis of the image of the gel enables comparing of protein levels. One of the major problems of 2D electrophoresis is protein solubility which causes that e.g. membrane proteins are under-represented on 2D gels from cells.

DIGE: Difference Gel Electrophoresis

In DIGE-based proteomics, the experimental and control samples are derivatized with different fluorophores and are run in the same gel, thereby minimizing technical variation. DiGE has made it possible to detect and quantitate differences between experimental pairs of samples resolved on the same 2D gel by applying up to three different protein samples labeled with fluorescent dyes prior to 2-D electrophoresis. (Lilley K.S, 2004).

Combining of electrophoresis with mass spectrometry enables to characterize the proteins in spots on gel. The spots of the 2-DE gels are excised and a tryptic digestion is performed to generate tryptic peptide mixtures of the proteins that are applied to mass spectrometry for identification of the excised protein.
3.3.3 Mass spectrometry

The technology of choice for proteomic surveys today is shotgun mass spectrometry. Many technology variations exist that differ in specificity, sensitivity, dynamic range, ease of use and cost. All are based on conversion of intact proteins in a sample into peptide fragments, followed by their volatilisation – ion formation, measurement of their mass-to-charge ratio (m/z ratio) and intensity of signal. Then tandem MS is used to identify the peptides within each of mixtures and off-line protein identification by comparison with a database of peptides and their masses. Subtractive algorithms can be applied to the datasets of peptides identified in the samples to recognize peptides that are either unique or more highly abundant in the serum acquired from disease-affected patients versus controls.

Mass spectrometry techniques differ by type of ion source (e.g. SELDI – surface enhanced laser desorption, MALDI – matrix-assisted laser desorption ionization, electrospray ionization - ESI) and by m/z detection (e.g. TOF – time of flight, ion trap). The major progress in proteome study is the technology of quantitative proteomics, which introduced isotopes into an analysis. Proteins from different samples are first labelled with different isotopes, then mixed and analysed. The measurement of either the MS peak intensities or areas can infer relative abundance between protein samples.

The most exciting approach into the plasma proteomics is SELDI technology, based on protein –chip array. Sample is applied to a chip made up of a specific chromatographic surface, then series of washing steps follow, which reduce the complexity of the sample, continuing by application of matrix and then the ions of captured proteins produced by laser impulse are introduced into the m/z detector.

Mass spectrometry is ideal for rapid, cost effective, initial proteomic surveys of experimental systems. Analytical sensitivity is much lower than ELISA and limits the usefulness of the technology with small or rare proteins such as cytokines in blood samples. Sensitivity can however be enhanced by sample preparation to remove high-abundance proteins. Mass spectrometry is in general semiquantitative and has a high day-to-day, operator-to-operator and machine-to-machine variation. For these reasons, multiplexed protein measurement by mass spectrometry is currently best used for hypothesis-generating studies. An example of this is the discovery of serum protein biomarker candidates.

3.4 xMAP Technology

3.4.1 History of xMAP technology

Flow cytometry was primarily designed for cellular analysis and microspheres have been used as calibrators for flow cytometers. As early as 1977, flow cytometry was applied to the detection of single antigens captured by antibodies coated on the surface of microspheres. These basic applications have been expanded to the simultaneous detection of multiple analytes in the same sample volume by combining individual reactions on size and spectrally distinct sets of microspheres. In 1988, some of the first multiplex immunoassays detected by flow cytometry were introduced by Hugh and colleagues for detection of cytomegalovirus and herpes simplex virus antibodies. (McHugh T.M. 1988; Fulwyler M.J., 1990)

In the beginning, only conventional flow cytometers were used for microspheres data acquiring. In the 1990s the Luminex Corporation (Austin, Texas, USA) coupled a digital signal processor and a Becton-Dickinson benchtop cytometer in so called FlowMetrix System, which enables to detect and process rapidly and in real time the fluorescent signals generated by multiplexed assays with 64 sets of microspheres distinguished by spectral fluorescent code allowing to analyse up to 64 analytes in one assay. This system has been used to perform qualitative and quantitative immunoassays for multiple serum proteins in both capture and competitive inhibition assay formats. The system has also been used to perform DNA sequence analysis by multiplexed competitive hybridisation with 16 different sequence-specific oligonucleotide probes.

This system was replaced by xMAP technology and Luminex 100 instrument specialized only for detection of multiplex bead-based assays.

(Kellar K.L., 2002; Fulton R.J., 1997)
3.4.2 Principle of xMAP technology

Multi-analyte profiling technology (xMAP) is based on a set of 5.6 μm polystyrene microspheres (beads). In this set there are 100 bead populations, which differ by internal spectral codes carried out by different ratios of two internal fluorescent dyes. The multiplex analysis is than enabled by various molecules, which capture the studied analytes from solution, linked to different microsphere populations. The internal code of the bead refers to identity of measured analyte. Because microsphere populations can be distinguish by their spectral addresses, they can be combined in an assay, allowing measurement of up to 100 different analytes in one well. The amounts of the captured analytes are determined by reporter molecule connected with fluorescent molecule.

![Bead differentiation used in xMAP technology](www.panomics.com)

The measurement is performed on special flow cytometer Luminex 100 IS. Microspheres from the assay well are introduced into the instrument, they flow rapidly in a sheat fluid through the instrument and one by one they pass through two different lasers. The fluorescent signal after the excitation by the first laser determines the spectral code of microspheres and so the identity of an analyte, and fluorescent signal after the excitation by the second laser detects the amount of reporter molecule on microspheres, so the quantity of an analyte.
Luminex 100 instrument is a benchtop cytometer with sheath flow rate of 90μl/sec equipped by two lasers:

- Yttrium aluminium garnet (YAG) – 532 nm, 10-15 mW – excitation of reporter fluorochrome with fluorescence wavelength 575nm (Bandwidth 20nm) e.g. R-phycoerythryn (PE – 578 nm emission)
- 635 nm, 9 mW red diode laser – excitation of classification internal dyes of microspheres (emission at 568 and 712 nm)

The cytometer is coupled to XY platform, which enables facilitate reading of 96 well microtiter plates – filter-bottom as well as solid-bottom plates

Picture 3 Luminex 100IS instrument (www.luminexcorp.com)
3.4.3 Multiplex immunoassay reaction

In the case of immunoassays we are using in our laboratory, for protein quantification there is implemented sandwich technology well known from traditional immunoassays. To the sample there are added specific antibodies linked to microspheres. The studied proteins are captured by the antibodies and so bound to microspheres. For reaction special microtiter plates with filtration bottom are used. The unbound material is washed out through the filters in the bottom of the reaction well and the studied proteins bound to microspheres stay in the well on the filter. Second step is the addition of second antibodies linked with biotin. After that the unbound second antibodies are washed out from reaction on filter plate. Finally, the reporter molecule – phycoerythrin – is added and linked by streptavidin to the biotinylated second antibodies. The concentrations of proteins are assessed according to standard calibration curves for each analyte assayed with unknown samples. All other immunoassay principles e.g. competitive format or antigens linked to microspheres for antibody concentration determinations can be implemented.
Picture 5 Antibodies bound to microspheres (picture source www.rndsystems.com)

Picture 6 An example from measurement by XMAP technology - Multiplex analysis of ten cytokines in human seminal plasma in our laboratory

Picture 7 An example of calibration curve
3.4.3.1 Reagents
Several companies offer a growing list of ready-to-use multiplexed sandwich immunoassays based on the xMAP technology, but not all analytes can be multiplexed in immunoanalysis into single panel – there are three major reasons:

- too different concentration levels of analytes, some of them would require different predilution of biologic fluid before analysis
- crossreactivity of some antibodies which has to be avoided
- different requirements for preanalytic conditions for some analytes

3.4.4 Assay principles for nuclear transcription factor assay

The controls and samples are incubated with biotin labeled DNA probes. During this incubation, transcription factors contained in the sample bind to the probes. Then digestion reagent including nuclease is added – transcription factors bound to their DNA probes protect the probes from nuclease, DNA probes without bounded transcription factors are digested. Finally, there are introduced the beads coated with capture DNA sequences designed to bind to the probes through the complementarity and streptavidin-phycoerythrin, which bound to biotin labelled probes.

3.4.5 Assay principle for multiplex RNA quantification

QuantiGene Plex Reagent System enables quantitative measurement of multiple RNA targets directly from cell lysates or tissue homogenates or whole blood without necessity of RNA isolation and reverse transcription or target amplification. This technology is a sandwich nucleic acid hybridization assay combining xMAP with branched DNA signal amplification technology. The RNA transcripts from samples are captured thanks to hybridization to probes on xMAP beads. Signal is amplified by sequential hybridization of the branched DNA and biotinylated label probe. The reaction is detected by streptavidin–phycoerythrin (SAPE) conjugate. The beads are analyzed with a Luminex instrument – amount of SAPE is equal to quantity, bead identity refers to target RNA molecule.

This direct principle of RNA quantification opens a door of possibility to combine multiplex protein and multiplex mRNA quantification in the same reaction well and to measure on the same instrument.

(www.panomics.com)
3.4.6 miRNA quantification technology

The FlexmiR™ microRNA products combine the power of xMAP® technology with Exiqon’s Locked Nucleic Acids™ (LNAs) and include MicroRNA Human Panel, FlexmiR MicroRNA Mouse/Rat Extension Panel, which provide methods that qualitatively and quantitatively measure the expression of up to 100 the miRNA sequences using total RNA in one analysis and sample aliquot without need for RNA size fractionation or amplification.

The FlexmiR technology steps:

- Biotinylate the 3’ ends of total RNA,
- Hybridize the labeled RNA to Tm-normalized LNA capture probes that have been coupled to fluorescently dyed xMAP beads,
- Tag the captured biotinylated miRNAs with streptavidin-phycoerythrin (SAPE)
- Analyze the samples on a Luminex 200.

Locked Nucleic Acids

LNA™ is a conformationally restricted nucleic acid analogue, in which the ribose ring is “locked” with a methylene bridge connecting the 2’-O atom with the 4’-C atom.

Incorporation of LNA monomers into an oligonucleotide capture probe greatly increases the affinity of the oligonucleotide for its complementary RNA target leading to a significant increase in stability and specificity of the duplex.

www.luminexcorp.com
3.4.7 Assay principles for genetic research

There can be used direct hybridisation, competitive hybridisation and a solution-based sequence-specific enzymatic reaction. In direct hybridisation labelled DNA targets are amplified by PCR and directly hybridised to beads with oligonucleotide capture probes specific for each sequence. In comparison, competitive DNA hybridisation is based on competing unlabeled double-stranded PCR amplified targets from sample with added labelled single-stranded oligonucleotide for annealing to the sequence-specific capture probes on beads. The last format involves the incorporation of a specific capture sequence during the enzymatic step that allows hybridisation to a complementary address sequence on the microsphere surface, and includes allele-specific primer extension, oligonucleotide ligation assay and single base chain extension. (Dunbar S.A., 2006)
Picture 10 - Diagram of assay format using solution-based genotyping with microsphere capture. A. Solution-based enzymatic genotyping assay is performed, incorporating the unique capture sequences into the products. B. Address probe-coupled microsphere sets. C. Products are captured onto the microspheres through hybridization of the capture and address sequences, and labeled with streptavidin-R-phycoerythrin (reprinted from Dunbar S.A., 2006)

3.4.8 xMAP Technology Advantages and Benefits:

- reduced cost and labour by multiplexing for complex projects
- smaller sample requirements for complex projects
- liquid reaction kinetics give faster, more reproducible results than with solid, planar arrays
- focused but still flexible multiplexing in the range of 1 to 100 analytes, which meets the needs of a wide variety of applications
- open architecture platform
3.4.9 Application range

Table 1 Summary of xMAP technology applications

<table>
<thead>
<tr>
<th>Protein Profiling</th>
<th>Immunodiagnostics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer Markers</td>
<td>Allergy Testing</td>
</tr>
<tr>
<td>Cardiac Markers</td>
<td>Autoimmune Disease</td>
</tr>
<tr>
<td>Cellular Signaling</td>
<td>HLA Testing</td>
</tr>
<tr>
<td>Cytokines, Chemokines, and Growth Factors</td>
<td>Infectious Disease</td>
</tr>
<tr>
<td>Endocrine - hormones</td>
<td></td>
</tr>
<tr>
<td>Matrix Metalloproteinases</td>
<td></td>
</tr>
<tr>
<td>Metabolic Markers</td>
<td></td>
</tr>
<tr>
<td>Neurobiology</td>
<td></td>
</tr>
<tr>
<td>Transcription Factors/Nuclear Receptors</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Genomic Research</td>
<td>Genetic Disease</td>
</tr>
<tr>
<td>Genotyping –Genes, SNP</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>Expression Profiling - mRNA</td>
<td></td>
</tr>
<tr>
<td>Expression regulation – miRNA</td>
<td>Cytochrome p450</td>
</tr>
<tr>
<td>HLA DNA typing</td>
<td></td>
</tr>
</tbody>
</table>

3.4.10 Future vision

The Luminex company has already introduced new upgrade of xMAP technology, where the beads are magnetic – the assays can be more easily washed and furthermore, the beads can be driven through the instrument by magnetic forces, not by fluid of liquid and so not by principle of flow cytometry. This fact can allow higher throughput of new generation of instruments and as well enable easy automatization of multiplex immunoassay procedures.

The second hot news is a technology that enables to broaden the number of simultaneously multiplexed analytes from nowadays 100 analytes up to 500 in the next generation of assays.
The most advancing is the developing of microversion of the xMAP analysis by combining of multiplexing with microfluidics in microchannels. There exist projects of so called lab-on-chip or micrototal analysis systems. Lab-on-chip would includes all preparation procedures, incubation, detection and analysis into a single chip, no bigger than the size of a microscope slide. These advantages are especially vital to immunoassays that are used for rapid and accurate detection of disease markers to provide point-of-care testing for clinical diagnoses. (Lim C.T., 2007)

3.5 Application of xMAP multiplex immunoanalysis

This review section is focused on examples of possible xMAP technology clinical applications in multiplex immunoassay measurement of disease biomarkers in biologic fluids in humans. The studies, very close to routine clinical use are depicted.

3.5.1 Ophthalmology

Curnow S.J. et al. (Curnow S. J., 2005) has shown that multiplex analysis with multiplex data handling can serve as well in ophthalmology, it is enabled by a small amount of sample required for multiplex measurement. They measured a panel of cytokines in aqueous humour and from spectra of studied cytokines they pointed out by random forest analysis that only IL6, IL8, CCL2, IL13, IL2 and TNFα are required for 100% classification accuracy between noninflammatory control and idiopathic uveitis.

Our laboratory is involved in a project of angiogenic factors (e.g. VEGF, EGF and interleukins) measurement in aqueous humour, which could be applied in future in treatment monitoring and treatment selection for ophthalmodiseases caused by pathologic angiogenesis.

Funding and colleagues used xMAP technology to simultaneously quantitate and compare the concentrations of 17 immune mediators (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, tumour necrosis factor-alpha, interferon-gamma; granulocyte-monocyte colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), CXCL-8, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1beta) in aqueous humour from patients with corneal rejection and patients with a non-inflammatory condition in the anterior chamber. A total of 17 immune mediators were increased in aqueous humour from rejection patients. This result underlines the complex immunological...
interactions of the rejection process and necessity of multiplex laboratory measurement from a small sample volume.
(Funding M., 2006)
In comparable study, Banerjee and colleagues performed measurement of levels of 19 cytokines in vitreous humour of 58 eyes undergoing vitrectomy for a variety of vitreoretinal disorders (IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, TNF, IFN-γ, granulocyte-macrophage-colony-stimulating factor (GM-CSF), and granulocyte-stimulating factor (G-CSF); CCL2, CCL3, CCL4, CCL5, CCL11, and CXCL8; epidermal growth factor (EGF), FGF, and VEGF.
(Banerjee S., 2007).

3.5.2 Cancer monitoring

The ideal tumor marker should be (1) specific for the cancer for which it is tested; (2) not present in any other conditions; and (3) the concentration should change with the amount of malignant tissue present. None of nowadays routinely used tumor markers reach these requirements. More accurate serum tests should be utilized in the screening, diagnosis and staging of cancer patients, and this is an ideal task for multiplex analysis, because it is becoming day by day more clear that there does not exist one ideal marker for one cancer, but that we are forced to deal with panels of markers and their multiparametric evaluation.

There are two major branches of development: first to multiplex the “traditional” tumor markers, not only by analysis, but also by multiparametric data handling, and second to validate novel markers from a family of proteins with tumor biologic activity (cytokines, growth factors, hormones etc. directly in multiplex measurement and handling). These two strategies can be depicted by two tumour panels provided by Novagene company: The WideScreen™ Human Cancer Panel 1 (Tumor Markers) is a panel for simultaneous detection of six tumor-associated human proteins found in biological fluids: AFP (α-fetoprotein), CA 125, CA15-3, CA 19-9, CEA (carcinoembryonic antigen), and prolactin. The WideScreen™ Human Cancer Panel 2 (Growth Factors) is a panel for simultaneous detection of twelve human growth factors in biological fluids: amphiregulin, betacellulin, EGF, EGFR, epiregulin, FGF-basic, HB-EGF, PDGF-BB, PlGF, tenascin C, TGF-a, and VEGF. (http://www.merckbiosciences.co.uk/html/NVG/WideScreen_Human_Cancer_Panel1.html)
The strategy of multiplexing for “traditional” tumor markers was used by Sun et al., who
established a multiplex panel for simultaneously measurement of AFP, carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9, CA 242, and CA72-4. (Sun et al., 2007) Mor and col. used initially microarray analysis to determine 169 proteins in serum from healthy women, women with newly diagnosed and recurrent ovarian cancer. Proteins showing significant differences between controls and cancer patients were further validated by ELISA method. (Mor G., 2005; Visintin I., 2008) On the basis of this study they introduced xMAP multiplex panel specifically designed for ovarian cancer, now commercially available from Millipore Corporation (USA), including leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor and CA125. This panel for serum level measurements was further evaluated in a training set (181 control samples and 113 ovarian cancer) and a test set (181 control samples and 43 ovarian cancer samples), whereas four models were used to combine markers data. The best data combining method and multiplex panel possesses blood biomarker method with 95.3% sensitivity and 99.4% specificity for the detection of ovarian cancer.

Yurkovetsky Z. et al. introduced a large study of 64 biomarkers in serum of patients with endometrial cancer and a control group of healthy women by Luminex technology. 17 markers were elevated and 22 markers were lower in patients in compare to controls. As the strongest discriminative marker for endometrial cancer was observed prolactin providing 98.3% sensitivity with 98.0 specificity. Prolactin was stage and grade independent, but it was not able to satisfactory discriminate endometrial cancer from other cancers. But identified five-biomarker panel (prolactin, growth hormone, eotaxin, E-selectin, and TSH) was able to discriminate the endometrial cancer from ovarian or breast cancer. (Yurkovetsky Z., 2007) Furthermore, Yurkovetsky Z. R. and col. used xMAP technology to simultaneously test 29 cytokines, chemokines, angiogenic and growth factors and soluble receptors in the sera of 179 patients with high-risk melanoma and 378 healthy individuals. 15 serum biomarkers showed differences between melanoma patients and control healthy group. A multivariate bioinformatics Bayesian Network algorithm was used to discriminate between patients with melanoma and healthy subjects. The algorithm was first trained using a training set, and then validated using a test set resulting in 90% sensitivity at 98% specificity with 96.5% of melanoma patients distinguished from healthy individuals. High degrees of discrimination between cancer-afflicted and healthy control groups suggest that serum cytokine profiles may potentially be used for the development of blood-based diagnostic tests for melanoma and for
the prediction of therapeutic benefit among patients treated with adjuvant therapy. (Yurkovetsky Z. R., 2007)

Levels of 55 serum biomarkers including cancer antigens, cytokines and their soluble receptors, growth factors, adhesive molecules, proteases, hormones and other proteins were measured by Nolen and col. pre- and during neoadjuvant chemotherapy for locally advanced breast cancer using eight different multiplexed panels. Biomarkers levels were compared retrospectively with clinical and pathologic treatment responses. Univariate analysis of the data identified several groups of biomarkers that differed significantly among treatment outcome groups early in the course of neoadjuvant chemotherapy. Multivariate statistical analysis revealed multibiomarker panels that could differentiate between treatment response groups (see table 2) (Nolen B.M., 2008)

Table 2 Predictive power of multimarker panels (reprinted from Nolen B.M., 2008)

<table>
<thead>
<tr>
<th>Panel</th>
<th>Timepoint</th>
<th>Response type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Fetoprotein, soluble vascular cell adhesion molecule 1, MMP-9</td>
<td>Pretreatment</td>
<td>Clinical complete response versus no response</td>
<td>83</td>
<td>91</td>
</tr>
<tr>
<td>Tissue plasminogen activator inhibitor 1</td>
<td>Pretreatment</td>
<td>Clinical complete response/clinical partial response versus no response</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>MMP-3, luteinizing hormone, thyroid-stimulating hormone</td>
<td>Pre-cycle 2</td>
<td>Clinical complete response versus no response</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>ErbB2, epidermal growth factor receptor, migration inhibitory factor, MMP-2, CD40 ligand</td>
<td>Pre-cycle 2</td>
<td>Pathologic complete response/pathologic partial response versus no response</td>
<td>85</td>
<td>69</td>
</tr>
</tbody>
</table>

Serum cytokine profiles in breast cancer patients pre and post vaccination by HER2/neu vaccine with GM-CSF were compared to healthy controls by Dehqanzada Z.A: et al. Levels of MCP1, eotaxin, RANTES, GM-CSF, IL1α and IL4 of 22 studied cytokines differed in healthy controls, MCP1 and eotaxin levels were increased in response to vaccination. Differences in serum cytokine profiles may be indicative of an endogenous immune response to the cancer state. (Dehqanzada Z.A., 2007)
3.5.3 Neonatal screening – dried blood spot analysis

Laboratory testing of blood samples from infants is limited by small volume of samples. xMAP technology uses app. 25 µl for analysis for multiple analyte measurement, that is why it is very useful for solving the problem of small sample volumes in infant laboratory medicine. In our laboratory we are using xMAP technology for plasma cytokine and adhesive molecules profiling in infant patients on ventilation.

Newborn screening programs are based on analysis of a single sample of capillary blood collected by heel-prick from babies. The samples are usually stored as dried blood spot on special filter paper.

xMAP technology was used by Bellisario et al. to develop an immunoassay that measures T4 and TSH simultaneously from a single blood-spot sample. It has the flexibility to add other applications to the thyroid tests, including 17-hydroxyprogesterone for congenital adrenal hyperplasia, which would multiplex the endocrinology newborn screening profile into a single test. (Bellisario R., 2000)

Skogstrand et al. introduced a method based on xMAP analysis for measuring of 23 inflammatory and trophic cytokines, triggering receptor expressed on myeloid cells-1 (TREM-1), and C-reactive protein in two 3.2-mm punches from dried blood spot. It opens up new possibilities in the study of diseases, in which neonatal inflammatory reactions are suspected to play important pathogenetic roles. In its present form it is best suited for biobank case–control studies, but after further refinements, it may also have a potential in routine newborn screening. (Skogstrand K., 2005)

xMAP can be used as well in genetic neonatal screening e.g. Pyatt et al. developed neonatal screening method for individuals affected by spinal muscular atrophy by direct DNA analysis to detect the homozygous deletions in exon 7 of the SMN1 gene found in approximately 95% of cases. (Pyatt R.E., 2007)

Furthermore Bellisario R. et al. developed Luminex assay for simultaneously measurement of three HIV antigens in dried blood spot samples. (Bellisario R., 2001) It shows that the multiplex neonatal screening could be very wide-range with use of modern analytic technologies.
3.5.4 Sepsis

Sepsis, a common and often fatal disease, is defined as the presence of infection in the context of systemic inflammatory response syndrome. To reduce sepsis mortality and morbidity, fast and reliable diagnosis is greatly important; however, the complex pathology of the disease makes this difficult. Only C-reactive protein and procalcitonin are routinely used biomarkers but the xMAP technology possesses great possibility to validate new biomarkers from cytokine family or their whole panels. The problem is that commercially available kits, as in the cancer area, do not incorporate all proteins of interest. Kofoed and col. expanded a commercially available xMAP human cytokine panel including IL-1β, IL-6, IL-8, GM-CSF, and TNF-α by in-house 3-plex assay for soluble urokinase plasminogen activator receptor (suPAR), soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and macrophage migration inhibiting factor (MIF). The novel 8-plex assay was fully validated and clinically tested. In plasma collected from 10 patients with bacterial sepsis (confirmed by blood culture) the assay detected significantly increased concentrations of all 8 analytes compared with healthy controls. Furthermore, in a prospective study Kofoed and team showed that combining information from several markers improves diagnostic accuracy in detecting bacterial versus nonbacterial causes of inflammation. Measurements of macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor and soluble triggering receptor expressed on myeloid cells-1 had limited value as single markers, whereas PCT and CRP exhibited acceptable diagnostic characteristics. Observed areas under ROC were 0.72 (95% CI 0.63 to 0.79) for PCT, 0.81 (95% CI 0.73 to 0.86) for CRP, 0.84 (95% CI 0.71 to 0.91) for the composite three-marker test, and 0.88 (95% CI 0.81 to 0.92) for the composite six-marker test.

(Kofoed K., 2006; Kofoed K. 2007)

Bozza et al. published a study of plasma levels of 17 cytokines in 60 patients with severe sepsis. Among the 17 cytokines evaluated, IL-8 and MCP-1 exhibited the best correlation with organ dysfunctions on/by day 1; in addition, IL-6, IL-8 and G-CSF concentrations within the first 24 hours were able to predict worsening organ dysfunction or failure of organ dysfunction to improve by/on day 3. In terms of predicting mortality, the cytokines IL-1β, IL-4, IL-6, IL-8, MCP-1 and G-CSF had good accuracy for predicting early mortality (< 48 hours), and IL-8 and MCP-1 had the best accuracy for predicting 28-day mortality; in the multivariate analysis only MCP-1 was independently associated with prognosis.

(Bozza F.A., 2007)
3.5.5 Endocrinology

Arslan and colleagues measured adrenocorticotrophic hormone (ACTH), growth hormone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH) and prolactin using the xMAP method in sera of healthy premenopausal and postmenopausal women. (Arslan A.A., 2008) This panel exists as a commercially available from Millipore company (USA) along with endocrine panel including insulin, C-peptid, glucagon, leptin, amylin and glukagon-like peptide hormone 1. But both these panels are not logically combined from the clinical point of view and furthermore, from the analytical point of view e.g. ACTH measurement requires a very specific preanalytic conditions in contrary to all the others markers in the panel.

T4 and TSH simultaneously measurement from a single blood-spot sample is discussed in appropriate section, but Gonzáles et al. evaluate simultaneous determination of anti-thyroidperoxidase and anti-thyroglobulin antibodies by xMAP technology in comparison to automated enzyme immunoassay. (Gonzáles C., 2005) Combination of such assays could possess multiplex monitoring of thyroid diseases in future.

3.5.6 Gynaecology and obstetrics

Multiplex measurements can be used in gynaecooncology as it is described in related chapter. In this section, major gynaecologic problems considering pregnancy are under spotlight - preterm delivery, preeclampsia and the prenatal diagnosis of chromosomal abnormalities. Multiplex enables to study novel proteins released by the placenta into the maternal circulation and so offers new opportunities to identify genes and their protein products that are key diagnostic markers of diseases and might replace the current screening markers in use for prediction of risk. In the ideal situation, e.g. for Down syndrome, these markers will be sufficiently diagnostic not to require invasive sampling of fetal genetic material in future.

Maternal serum IL-18 and IL-12 levels were measured using xMAP technology by Ekelund et al. in women with symptoms of spontaneous preterm delivery. Low serum levels of IL-18 were shown to be associated with preterm delivery and further increased risk was associated with the combination of low IL-18 and high IL-12. (Ekelund C.K., 2008)

Whitcomb and colleagues measured serum levels of 8 different cytokines during pregnancy. They did not support the theory of cytokine shift to Th2 branch, but they found out that elevated levels of thrombopoietin are associated with increased risk of miscarriage of
clinically recognized intrauterine pregnancy (in less than 140 days of gestation) and should be studied further as a potential early marker of miscarriage. (Whitcomb B.W. 2008)

Levels of 21 cytokines were determined by xMAP technology and by ELISA by Jonsson and col. in sera of women with preeclampsia and compared to levels during normal pregnancies. Preeclamptic women had increased levels of IL6, IL8 and soluble IL4-receptor. (Jonsson Y., 2006)

Menon and col. studied by xMAP technology cytokines concentrations in amniotic fluid and their relations to preterm birth in caucasians and afro-americans (white and black womens). They observed that higher amniotic levels of IL6 are associated in white women with preterm birth with no difference in IL10 levels. No association was found in blacks. (Menon R., 2008 A) Menon’s team published as well studies of TNF-α and its soluble receptors, IL1β and IL8 in amniotic fluids (Menon R., 2008 B; Menon R. 2007).

Menon and his team studied further by xMAP technology salivary concetrations of MMPs in women. MMP7 levels were higher in nonpregnant women compared to samples from all stages of pregnancy. MMP2 and MMP3 levels were lower in the second trimester compare to nonpregnant females. The MMP9 protein was the only MMP that was increased in saliva from preterm premature rupture of the membranes patients. Salivary biomarkers represent a great potential for noninvasive screening methods. (Menon R, 2006)

Using Luminex assay and ELISA technology Nelson and col. measured concentrations of certain neurotrophins, neuropeptides and cytokines in pooled samples (one to three subjects per sample) eluted from archived neonatal blood of children with later-diagnosed autism, Down syndrome, very preterm birth, or term control infants and in blood from healthy adult controls. In infants with Down syndrome, concentrations of IL-8 levels were higher than in controls, whether or not corrected for total protein; NT-3 and CGRP were lower and VIP higher. (Nelson P. G., 2006)

Two mass spectrometry studies show the applicability of multiplex approaches as well to prenatal screening of Down syndrome and other chromosomal aberancies. Let’s hope that studies using xMAP technology will occur as well and that they will consider not only the amniotic fluid, but as well the maternal serum protein profiling as e.g. in the case of preterm birth.
Tsangaris with team published quantitative differences in amniotic fluid detected by mass spectrometry for alpha-1-microglobulin, collagen alpha 1 (I) chain, collagen alpha 1 (III) chain, collagen alpha 1 (V) chain d, and basement membrane-specific heparin sulfate proteoglycan core protein (PGBM). These proteins were increased in cases with Down Syndrome, whereas protein IBP-1 (P08833) was decreased by 40% compared with chromosomally normal fetuses. (Tsangaris G.T., 2006)

Mange and col. obtained the proteomic spectra of amniotic fluids from 52 pregnant women, who underwent amniocentesis for prenatal chromosome analysis between gestational weeks 14 and 17. They found in an univariate analysis 40 protein peaks as potential biomarkers, of which 12 were overexpressed and 28 underexpressed, in aneuploid AF as compared to the control samples. Furthermore, multivariate statistical analysis identified combination of two protein peaks with an AUC of 0.928, a classification accuracy of 96.4%, a sensitivity of 93.3% and a specificity of 100%. (Mange A., 2008)

3.5.7 Autoimmunity

Nifli et al. (Nifli A-P., 2006) compare routinely used indirect microscopic immunoflorescence and ELISA with multiplex Luminex commercially available kits from company: Zeus Scientific AtheNA Multi-Lyte ANA and ANCA test. ANA assay enables quantitative measurement of ANA and simultaneously semi-quantitative detection of IgG class antibodies against SS-A, SS-B, Sm, U1-RNP, Scl-70, Jo-1, Centromere B, ds-DNA and histones in human serum. ANCA tests detects IgG class antibodies against Myeloperoxidase and Proteinase 3. The results indicate that multiplex could replace individual ELISA tests for specific autoantibodies.

Koenig and col. have studied 21 autoantibodies in patients with autoimmune myositis, 8 of the autoantibodies were measured simultaneously by xMAP technology with commercially available kit from INOVA Diagnostics Inc. (USA). It was observed that 55% of patients have more than one type of autoantibody, that implies use of multiplex analysis, and that certain myositis autoantibodies are associated with types of clinical manifestation and last but not least, that some autoantibodies are predictors for therapeutic failure of prednisone alone treatment. (Koenig M., 2007)
4 Experimental part

The measurement of cytokines, angiogenic factors, adhesive molecules and other biomarkers was established in several types of biological materials of human, mouse and rat origin including blood derivates – serum, EDTA and citrate plasma, effusions, aqueous humour, seminal plasma, microdialysates, and cell culture supernatants. A large number of proteins has been already measured by xMAP multiplex immunoanalysis in our laboratory: cytokines, chemokines, markers of leukocytes activity (e.g. IL1, IL2, IL4, IL5, IL6, IL8, IL10, TNFα, MCP, MPO, IFN), adhesive molecules (sICAM, sVCAM, E-selectin), metalloproteinases (e.g. MMP 9, MMP1), growth factors (e.g. VEGF, EGF, TGF), adipokines (leptin, adiponectin), hormones (e.g. PTH, insulin, osteocalcin) and others. Because xMAP is a novel technology the first part of the experimental section of this thesis is dedicated to analytic evaluation.

The application of this method in our laboratory for clinical research is very wide but our major challenge is to study novel biomarkers and novel biomarker strategies in cancer diseases. That is why in clinical experimental part of this thesis three projects with use of xMAP technology in cancer diseases are presented. Further projects undergoing in our laboratory related to cancer are focused on colorectal carcinoma and nonsmall cell lung cancer. As an illustration of wide range of applications some of the miscellaneous projects, for which the xMAP technology was used in our laboratory, are briefly mentioned below.

Since several pathophysiological mechanisms of depression have been postulated in the last decade the xMAP technology was used in our laboratory to study the inflammatory hypothesis of depression. The serum levels of IL-6, IL-8, IL-10 and TNF-α were compared in population samples characterized by a high or low level of self-reported depression. We found lower IL-6 in a group with high score of Zung Self-Rating Depression Scale. Serum levels of all other studied cytokines were not significantly different.

Another performed study was related to rheumatoid arthritis. Currently, the most effective drugs for treatment of rheumatoid arthritis are the TNF α blocking agents. Several markers reflecting immune response, inflammation, disease activity and metabolic changes are currently studied and xMAP technology has a potential to be used in routine clinic for
monitoring of disease activity and progression. In a group of 23 patients with rheumatoid arthritis, serum levels of IL 1β, IL10, IL6, VEGF, MMP9 and adiponectin were measured in our laboratory by multiplex immunoanalysis prior therapy and after 14 -16 weeks of therapy with TNF α blocking agent. The significant decrease after therapy was confirmed for VEGF and adiponectin.

A grant project with focus on adipokine study is in run in cooperation with 3rd Faculty of Medicine of Charles University. In this work we investigated the direct effect of insulin to secretion of cytokines and adipokines in adipose tissue by measurements of cytokine levels in plasma and adipose tissue microdialysates of patients during hyperinsulinemic, euglycemic clamps or before and after diet. The interstitial concentration of proteins in the tissue was evaluated using microdialysis in combination with multiplex xMAP analysis.

We have started projects of monitoring of angiogenic cytokines and growth factors in aqueous humour for better surgery treatment tailoring in patients with proliferative diabetic retinopathy. In a pilot study, it was proven the possibility to detect VEGF, EGF and IL 6 and IL 10 in aqueous humour by xMAP technology.

Furthermore, the possibility to measure cytokine levels in human seminal plasma by xMAP technology was shown. Because seminal plasma composition plays a significant role in the fertilization, levels of IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12(p70), TNFα and IFNγ were measured in seminal plasma of men from infertile couples.

xMAP technology was also used in studies considering the role of inflammation in pathophysiology of atherosclerosis and diabetes and the pathophysiology of pleural effusions. We have used the xMAP technology for monitoring of cytokine blood levels of healthy donors of hemopoetic progenitor cells mobilised by granulopoietic growth factor (G-CSF). The cytokine levels were measured before and after 5-day application of G-CSF. The correlation of cytokine levels and their changes to effectivity of stimulation, considered by number of CD34+ cells in peripheral blood before aferesis, was studied. Our aim is to evaluate a marker of mobilization effectivity for prospective identification of donors with insufficient mobilisation.

Experimental project on mice with monitoring of the stress related cytokine levels after several flight hours in plane was performed as a model of work related stress of pilots. Experimental animals spent about 530 flight-hours in the cockpit of ČSA aircrafts.
Catecholamine and IL10, IL1, IL6, and TNFα levels in the blood samples of mice were measured by means of RIA and by xMAP technology. Many new projects are starting for protein multiplex measurements in blood but also project for mRNA and tissue protein profile determination by xMAP technology have been started.
4.1 Analytical part

4.1.1 Which blood sample type is adequate?

Multiplexing requires a global point of view on preanalytical phase it means not only proper handling (transport, storage and etc.) with samples but as well as proper choice of matrices, which should be analysed. It is important to clearly understand that concentrations of some proteins differ in serum, EDTA plasma or citrate plasma blood derivate. There are no recommendations written in instructions for use in commercial kits. That is why 3 separate experiments were performed to learn the differences among different blood sample types for cytokine analysis.

Methods:

Blood collecting and handling: The blood was collected by venipuncture using appropriate Vacuette collection tubes (Greiner Bio-One, Austria). The blood samples were centrifuged at 640 g for 10 min and aliquots were stored until analyzed in freezer at -75°C.

Analysis: The assays were run using commercially available multiplex kits according to manufacturer's instructions for use. The analysis was performed on Luminex 100 IS instrument and data analysis on Luminex 100 IS software version 2.3.

1) EDTA plasma and serum samples of 10 patients with colorectal cancer were analysed for IL6, IL8 and IL10 by 3 plex kit from Human Cardiovascular panel 1 (Linco Research, USA)

2) Blood samples of 30 patients with lung cancer disease for sICAM-1, sVCAM-1 and MMP9 were analysed by 3-plex kit from Human Cardiovascular panel 3 (Linco Research, USA). For all patients the serum and EDTA plasma samples were compared, for 10 patients there was compared as well citrate plasma sample. The results in serum sample and in EDTA and citrate plasma sample were compared for each analyte.

3) Serum samples and EDTA plasma samples of 7 patients of internal clinic were analysed. By 10 plex kit Human Cytokine 10-plex kit (BioSource, USA) there was measured GM-CSF, IFNγ, IL10, IL1β, IL2, IL4, IL5, IL6, IL8 and TNF α.

Results interpretation: For the first two experiments values obtained in different blood sample types were compared on the base of average, median, minimal and maximal value counted from all results for each analyte in each sample type. There were as well counted
ratios for two blood sample types (s/p index, s/EDTA or EDTA/citrate), their average, standard deviation, median, minimal and maximal values. For the third experiment there were counted portions of samples under the calibration curve in percents for each protein and sample type. All the results are presented for sICAM-1, sVCAM-1 and MMP9 in ng/mL and for all of the other proteins in pg/mL.

**Results:**

Experiment 1): For IL 6 and IL 10 there were not observed any outstanding differences between serum and plasma samples. On the other hand for IL 8 the differences in results were striking. In average more than 7-times higher values were observed in serum in comparison to EDTA plasma for IL 8 and the s/p index shows high standard deviation in comparison with the letter discussed proteins. See Graph 1 and 2 and Table 3.
Graph 1 Comparison of IL6 levels in serum and EDTA plasma samples

Serum/plasma comparison for IL 6

\[ y = 1.2373x - 2.0826 \]

\[ R^2 = 0.9596 \]

Graph 2 Comparison of IL8 levels in serum and EDTA plasma samples

Table 3 Comparison of IL6, IL8, IL10 levels in serum (s) and EDTA plasma (p) samples

<table>
<thead>
<tr>
<th>Protein</th>
<th>IL6</th>
<th>IL8</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Serum</td>
<td>Plasma</td>
<td>s/p index</td>
</tr>
<tr>
<td>Average</td>
<td>16.86</td>
<td>18.77</td>
<td>1.09</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.39</td>
<td>5.60</td>
<td>0.37</td>
</tr>
<tr>
<td>Median</td>
<td>5.38</td>
<td>3.91</td>
<td>1.02</td>
</tr>
<tr>
<td>Minimal value</td>
<td>0.64</td>
<td>0.64</td>
<td>0.73</td>
</tr>
<tr>
<td>Maximal value</td>
<td>68.24</td>
<td>94.07</td>
<td>2.06</td>
</tr>
</tbody>
</table>
Experiment 2) For adhesive molecules the values were nicely correlated in serum and EDTA plasma, the values obtained in citrate were lower than in the two other materials. For MMP9 more than 4 times higher values were obtained in serum than in EDTA plasma. For MMP9 values in serum do not correlate with EDTA plasma (s/p index ranging from 0.59 to 14.37 with average 4.64 and SD 3.44) either with citrate plasma (s/citrate ranging from 0.83 to 35.44 with average 9.97 and SD 10.05). It means that for this protein the results differ in all three tested blood types. For results see Graph 3 and 4 and Table 4, 5, 6.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
<th>MMP9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Serum</td>
<td>Plasma</td>
<td>s/p index</td>
</tr>
<tr>
<td>Average</td>
<td>249.38</td>
<td>242.08</td>
<td>1.09</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>236.44</td>
<td>249.39</td>
<td>0.98</td>
</tr>
<tr>
<td>Minimal value</td>
<td>106.84</td>
<td>47.68</td>
<td>0.78</td>
</tr>
<tr>
<td>Maximal value</td>
<td>723.19</td>
<td>457.76</td>
<td>3.59</td>
</tr>
</tbody>
</table>

Table 4 Comparison of ICAM-1, VCAM-1, MMP9 in serum (s) and EDTA plasma (p) samples

<table>
<thead>
<tr>
<th>Protein</th>
<th>ICAM-1</th>
<th>VCAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Serum</td>
<td>EDTA</td>
</tr>
<tr>
<td>Index</td>
<td>EDTA/cit</td>
<td>Index</td>
</tr>
<tr>
<td>Average</td>
<td>277.80</td>
<td>253.33</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.47</td>
<td>0.26</td>
</tr>
<tr>
<td>Median</td>
<td>224.51</td>
<td>238.02</td>
</tr>
<tr>
<td>Minimal value</td>
<td>112.36</td>
<td>117.26</td>
</tr>
<tr>
<td>Maximal value</td>
<td>723.19</td>
<td>457.76</td>
</tr>
</tbody>
</table>
Table 6 Comparison of MMP9 in serum (s) and EDTA plasma (EDTA p) and citrate plasma (citrate p) samples

<table>
<thead>
<tr>
<th>Protein</th>
<th>MMP9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>serum</td>
</tr>
<tr>
<td>Average</td>
<td>707.55</td>
</tr>
<tr>
<td>Standard deviation</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>719.65</td>
</tr>
<tr>
<td>Minimal value</td>
<td>264.21</td>
</tr>
<tr>
<td>Maximal value</td>
<td>1199.44</td>
</tr>
</tbody>
</table>

Graph 3 Comparison of VCAM levels in EDTA plasma and citrate plasma samples
Graph 4 Comparison of VCAM levels in serum and EDTA plasma samples

Comparison serum / EDTA plasma

\[ y = 0.8447x + 184.18 \]

\[ R^2 = 0.7883 \]

Experiment 3) For this experiment there was chosen kit with not the lowest detection limits available on market at this moment. So quite a high number of samples were under the calibration curve. For 9 of 10 studied proteins in serum all the samples were under quantification limit, for EDTA plasma it was only 5 of 10 proteins. For GM-CSF, IFN\(\gamma\), IL1\(\beta\), IL4 and TNF\(\alpha\) there was observed lower number of results under the calibration curve for samples in EDTA plasma blood derivate in comparison with serum, the highest difference was for GM-CSF.

Table 7 Number of results under the calibration curve for samples in EDTA plasma samples in comparison with serum

<table>
<thead>
<tr>
<th>Samples under the calibration curve (%)</th>
<th>GM-CSF</th>
<th>IFN(\gamma)</th>
<th>IL10</th>
<th>IL1(\beta)</th>
<th>IL2</th>
<th>IL4</th>
<th>IL5</th>
<th>IL6</th>
<th>IL8</th>
<th>TNF(\alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>71.43</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>EDTA plasma</td>
<td>14.29</td>
<td>57.14</td>
<td>100.00</td>
<td>42.86</td>
<td>100.00</td>
<td>85.71</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>28.57</td>
</tr>
</tbody>
</table>
4.1.2 Comparison with other immunoanalytic methods

4.1.2.1 Comparison with another multiplex bead assay: Flow Cytomix

xMAP technology was compared with the analytically closest multiplex method: FlowCytomix. The principle of this technique is described in the theoretical part. Furthermore the possibility to use multiplex technology for cytokine measurement in seminal plasma as a very uncommon biological material was under the scope.

Methods:

Biologic material preparation

The analysis was run with 68 seminal plasma samples of men from infertile couples. Semen samples were collected by masturbation with ejaculation into special sterile jar after a sexual abstinence period at least four days. Semen samples were let to fluid, centrifuged for separating out the sperm cells and aliquoted. Aliquots of seminal plasma samples were stored at -70°C. Before analysis, the aliquots were thawed at laboratory temperature and centrifuged for 2 min. at 10000g in order to remove any particles just before analysis.

Measurement:

Results of analyses of IFNg, IL2, IL10, IL8, IL6, IL4, IL5, IL1b and TNFa were compared. For comparison there were used commercially available ten plex kits – High Sensitivity Human Cytokine Panel (Linco Research, USA) and Human Th1/Th2 10 plex KIT II (Bender MedSystems, Austria). Without possibility of comparison there were in multiplex panels analysed as well TNFβ on Flow Cytomix and IL12 on Luminex. xMAP technology measurement was performed with Luminex 100 instrument (software Luminex 100 IS) and Flow Cytomix technology with Cytomixs FC500 flowcytometer (Beckman Coulter comp., USA) (software BMS FlowCytomix for data analysis).

The calibration ranges for Luminex measurement are not strictly determined for used kit by manufacturer. For our analysis the calibration points 0.13 pg/mL, 0.64 pg/mL, 3.2 pg/mL, 16 pg/mL, 80 pg/mL, 400 pg/mL and 2000 pg/mL were used. For IFN-γ and IL2 the 0.64 pg/mL calibrator was set as the lowest limit of quantification because the 0.13 pg/mL did not meet the requirement of difference at least 2 standard deviations from zero standard point.
Statistical methods:
For results obtained by both compared methods there were counted mean, median, SD, minimal and maximal value and percentage of results under the lowest calibration point (percentage of zero results in the case of FlowCytomix) for each protein. The correlation of both methods was studied by rank-order correlation after exclusion of extreme observations and Spearman correlation coefficient was counted.

Results:
It has been shown that multiplex analysis can be used for cytokine studies in seminal plasma with the great advantage - the use of small sample amount. The results of comparison between multiplex methods are listed below:

1. Statistically significant correlations show methods for four of the studied cytokines. See table 8

Table 8 Correlation of results obtained by xMAP and Flow Cytomix technology

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spearman coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL10</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL1β</td>
<td>0.5744</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL2</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL4</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IL5</td>
<td>0.7921</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL6</td>
<td>0.4951</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>IL8</td>
<td>0.7821</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TNFα</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

2. For TNFα, even if the limits of detections of both methods are sufficient, the results obtained by xMAP and FlowCytomix are very different, even the trends for individual samples roughly differ.

3. Limits of detection were insufficient for IL6, IL2, IL10, IFNγ using the FlowCytomix method. For IL4 the percentages of samples under limits of detection are very high using Luminex as well as FlowCytomix. (Luminex 88%, FlowCytomix 97%). The only analyte, for which the percentage of samples under the calibration curve was higher using Luminex, was IL1β. For percentage of samples under the
calibration curve ranges in compared methods for all studied proteins see Table 9 and Graph.

Table 9 Comparison of percentages of samples under the calibration curve ranges for xMAP and Flow Cytomix technology

<table>
<thead>
<tr>
<th>Samples under LOQ %</th>
<th>IFNg</th>
<th>IL10</th>
<th>IL1b</th>
<th>IL2</th>
<th>IL4</th>
<th>IL5</th>
<th>IL6</th>
<th>IL8</th>
<th>TNFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Cytomix</td>
<td>98.53</td>
<td>100.00</td>
<td>26.47</td>
<td>100.00</td>
<td>97.06</td>
<td>13.24</td>
<td>86.76</td>
<td>0.00</td>
<td>73.53</td>
</tr>
<tr>
<td>Luminex</td>
<td>63.24</td>
<td>19.12</td>
<td>64.71</td>
<td>72.06</td>
<td>88.24</td>
<td>5.88</td>
<td>0.00</td>
<td>0.00</td>
<td>26.47</td>
</tr>
</tbody>
</table>

Graph 5 Percentages of samples under the calibration curve ranges (0% means all samples within calibration curve)
Technical notes

The compared methods are very similar in the assay procedures as well as in the use of filtration microtiterplates for assay performance. The main technical difference is that with the common flow cytometers, as we used for Flowcytomix, it is not possible to use the microtiterplates directly for measurement and so it is necessary to transfer the solution after assay procedure from the microtiterplate into the tubes suited for measurement. This handling is very time consuming and so extended use of Flowcytomix in any laboratory would require flow cytometer equipped by microtitreplate sampler. The Luminex instrument is already suited for aspirating samples directly from microtiterplate, furthermore the Luminex software analyse directly the data from measurement in comparison to Flowcytomix software.

4.1.2.2 Comparison with conventional nonmultiplex immunoassay methods

Results for several analytes obtained by multiplex xMAP technology were compared to conventional immunoanalytic methods in a serie of experiments. Elisa, RIA and automated chemiluminiscence methods were used for comparing measurements in several biologic materials. All specifications of samples and methods including kit manufactures are listed in Table 10 with results of Passing Bablock analysis (including Cusum test for linearity) and Spearman’s coefficients of rank order correlation between methods. Results obtained by multiplex correlate with conventional methods for all tested analytes with Spearman’s coefficients higher than 0.7. The only exclusion was IGFII measurement for which correlation was not so close with coefficient only 0.593. For PTH and adiponectin linearity of correlation was confirm in Cusum test only for values up to 500 pg/mL for PTH and 15000 ng/mL for adiponectin. The correlation was recalculated for values only under these limits.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of samples</th>
<th>Type of material</th>
<th>Biological Material</th>
<th>Method for comparison</th>
<th>Regression equation</th>
<th>Passing and bablock</th>
<th>Rank order correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin</td>
<td>70, 48 (&lt;15000 ng/mL)</td>
<td>Serum</td>
<td>Diabetes mellitus</td>
<td>RIA</td>
<td>Y = 3.3638 + 0.6484 X</td>
<td>Yes</td>
<td>(~&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td>26, 20 (&lt;10000 ng/mL)</td>
<td>Effusions</td>
<td></td>
<td>RIA</td>
<td>Y = 597.7497 + 1.2568 X</td>
<td>Yes</td>
<td>(0.05)</td>
</tr>
<tr>
<td>Leptin</td>
<td>69</td>
<td>Serum</td>
<td>Diabetes mellitus</td>
<td>RIA</td>
<td>Y = -0.4137 + 0.7382 X</td>
<td>No</td>
<td>0.917 0.000</td>
</tr>
<tr>
<td>MMP9</td>
<td>36</td>
<td>Plasma</td>
<td>Colorectal cancer and healthy controls</td>
<td>Elisa</td>
<td>Y = 13.5014 + 1.6623 X</td>
<td>No</td>
<td>0.979 0.000</td>
</tr>
<tr>
<td>PTH</td>
<td>40, 32 (&lt;500 pg/mL)</td>
<td>Plasma</td>
<td>Chronic renal insufficiency (diabetes)</td>
<td>BMA</td>
<td>Y = 40.3829 + 0.5397 X</td>
<td>Yes</td>
<td>(~&lt;0.05)</td>
</tr>
<tr>
<td>PTH</td>
<td>40, 28 (&lt;500 pg/mL)</td>
<td>Plasma</td>
<td>Chronic renal insufficiency (diabetes)</td>
<td>CLIA</td>
<td>Y = 51.5120 + 0.4163 X</td>
<td>Yes</td>
<td>(~&lt;0.05)</td>
</tr>
<tr>
<td>CA125</td>
<td>39</td>
<td>Serum</td>
<td>Ovarian carcinoma and ovarian benign cyst</td>
<td>CLIA</td>
<td>Y = -0.0050 + 0.0259 X</td>
<td>No</td>
<td>0.721 0.000</td>
</tr>
<tr>
<td>IGFII</td>
<td>15</td>
<td>Serum</td>
<td>Ovarian carcinoma and ovarian benign cyst</td>
<td>RIA</td>
<td>Y = 5511.476 + 12.3266 X</td>
<td>No</td>
<td>0.593 0.027</td>
</tr>
<tr>
<td>Osteokalcin</td>
<td>24</td>
<td>Serum</td>
<td>Cancer patients</td>
<td>RIA</td>
<td>Y = 1170.2054 + 397.1244 X</td>
<td>No</td>
<td>0.710 0.001</td>
</tr>
<tr>
<td>IL6</td>
<td>17</td>
<td>Tissue culture supernatant</td>
<td>Adipose tissue</td>
<td>Elisa</td>
<td>Y = 151.7838 + 0.3749 X</td>
<td>No</td>
<td>0.914 0.000</td>
</tr>
</tbody>
</table>
Graph 6 Bland-Altmann graphs and correlation for results of MMP9, PTH and IL6 obtained by xMAP technology and conventional immunoanalytic method
4.1.3 Quality control

The internal quality control of the multiplex analyses is routinely performed in our laboratory by the mixed (or multiplexed) commercial control samples included in kits with stated target ranges for all analytes in the panel. These controls are commonly provided in two levels. For long term quality control assessment we have used two types of control samples - the commercial control samples included in the kits and pooled samples. We have used two pooled samples, called high and low, obtained by pooling of patients sera and effusions into. Effusions were introduced into the pool because of higher levels of studied proteins. The pools were aliquoted and aliquots were stored at temperature below –70°C. One aliquot was thawed before each analysis. No freeze/thaw cycles were allowed.

4.1.3.1 Commercial controls

There were assessed commercially available controls supplied as a part of kits Linco Research (USA) Human CVD1, HumanCVD3 and Human Adipokine Panel, which we are using most commonly for analyses. Kit controls were analysed in each batch measurement in dublets up to tetraplets. For longterm quality control consideration there were used mean and standard deviation of all multiplicates of control sample results obtained in analysis in one batch.

Number of analyses for every analyte was considered (see in table – number of control runs) and only with more than five control runs were considered. Furthermore there was calculated mean intraassay coefficient of variation as a mean of standard deviation of multiplets within each run.

Relative standard deviation (=coefficient of variation) for each analyte was calculated from mean results of controls in runs for longterm reproducibility of results among analyses.
Table 11 Quality control results for selected lots of commercial controls

<table>
<thead>
<tr>
<th>Control lot / Analyte</th>
<th></th>
<th>ICAM</th>
<th>MMP9</th>
<th>MPO</th>
<th>VCAM</th>
<th>tPAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 1 lot CVD1-102</td>
<td></td>
<td>16</td>
<td>16</td>
<td>5</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td></td>
<td>9.54</td>
<td>9.30</td>
<td>12.44</td>
<td>8.17</td>
<td>3.81</td>
</tr>
<tr>
<td>CV % interassay</td>
<td></td>
<td>9.07</td>
<td>19.16</td>
<td>17.59</td>
<td>12.77</td>
<td>10.44</td>
</tr>
<tr>
<td>control 2 lot CVD1-202</td>
<td></td>
<td>16</td>
<td>16</td>
<td>5</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td></td>
<td>9.99</td>
<td>9.63</td>
<td>15.71</td>
<td>8.38</td>
<td>6.94</td>
</tr>
<tr>
<td>CV % interassay</td>
<td></td>
<td>12.92</td>
<td>8.93</td>
<td>18.76</td>
<td>10.25</td>
<td>12.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control lot / Analyte</th>
<th>IL10</th>
<th>IL6</th>
<th>IL8</th>
<th>TNFa</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 1 lot CVD3-102</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td>9.61</td>
<td>8.85</td>
<td>6.80</td>
<td>6.68</td>
<td>13.25</td>
</tr>
<tr>
<td>CV % interassay</td>
<td>14.64</td>
<td>16.82</td>
<td>12.64</td>
<td>14.12</td>
<td>28.71</td>
</tr>
<tr>
<td>control 2 lot CVD3-202</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td>8.29</td>
<td>5.74</td>
<td>15.63</td>
<td>12.13</td>
<td>8.89</td>
</tr>
<tr>
<td>CV % interassay</td>
<td>15.09</td>
<td>16.03</td>
<td>24.48</td>
<td>42.09</td>
<td>18.24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control lot / Analyte</th>
<th>IL6</th>
<th>TNFa</th>
<th>ICAM</th>
<th>MMP9</th>
<th>VCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>control 1 lot HADIP 105</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td>8.57</td>
<td>9.02</td>
<td>7.14</td>
<td>11.49</td>
<td>10.93</td>
</tr>
<tr>
<td>CV % interassay</td>
<td>15.57</td>
<td>16.37</td>
<td>7.60</td>
<td>14.85</td>
<td>8.34</td>
</tr>
<tr>
<td>control 2 lot HADIP 105</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean intraassay CV %</td>
<td>9.60</td>
<td>8.79</td>
<td>13.52</td>
<td>12.90</td>
<td>10.94</td>
</tr>
<tr>
<td>CV % interassay</td>
<td>13.10</td>
<td>12.51</td>
<td>19.22</td>
<td>4.66</td>
<td>4.29</td>
</tr>
</tbody>
</table>

Graph 7 Quality control results of commercial controls for ICAM and VCAM (con1 - control level 1, con 2 –control level 2) lot CVD1-102
Graph 8 Quality control results of commercial controls for MMP9, MPO, tPAI – (con1-control level 1, con 2 –control level 2)

Graph 9 Quality control results of commercial control level 1 (con 1) for IL10, IL6, IL8, TNFα and VEGF
Graph 10 Quality control results of commercial controls for ICAM and VCAM (con1 - control level 1, con 2 –control level 2) lot HADIP 105

Graph 11 Quality control results of commercial control level 1 (con 1) lot HADIP 105 for IL6, TNFα and MMP9
4.1.3.2 Pool control
We have used two pooled samples, called high and low, obtained by pooling of patients sera and effusions into. Effusions were introduced into the pool because of higher levels of studied proteins. The description of pool control samples as high and low is only sorting nomenclature, while it is not possible to predict final concentration of all studied proteins in pool. Pool controls were analysed in monoplets up to doublets in every batch. Results, evaluated in the same way as the kit controls (described above), and minimal and maximal value are shown in table. Results over the calibration curve were set as the highest calibration point value (for IL6 10000pg/mL). Furthermore graphs for analytes were constructed.

Table 12 Quality control results of pool control sample high

<table>
<thead>
<tr>
<th>Pool control - high</th>
<th>IL10</th>
<th>IL6</th>
<th>IL8</th>
<th>VEGF</th>
<th>MMP9</th>
<th>NT-proBNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of measurements</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>2 836.73</td>
<td>10 000.00</td>
<td>9 003.08</td>
<td>621.08</td>
<td>995.29</td>
<td>5 624.38</td>
</tr>
<tr>
<td>CV %</td>
<td>4.65</td>
<td>0.00</td>
<td>16.96</td>
<td>10.72</td>
<td>14.16</td>
<td>48.79</td>
</tr>
<tr>
<td>min.</td>
<td>2 676.50</td>
<td>10 000.00</td>
<td>6 540.13</td>
<td>530.71</td>
<td>895.66</td>
<td>16.76</td>
</tr>
<tr>
<td>max.</td>
<td>3 022.90</td>
<td>10 000.00</td>
<td>10 000.00</td>
<td>709.27</td>
<td>1 094.91</td>
<td>11 232.00</td>
</tr>
<tr>
<td>Mean CV% intraassay</td>
<td>5.68</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 13 Quality control results of pool control sample low

<table>
<thead>
<tr>
<th>Pool control -low</th>
<th>IL10</th>
<th>IL6</th>
<th>IL8</th>
<th>TNFa</th>
<th>VEGF</th>
<th>ICAM</th>
<th>MMP9</th>
<th>MPO</th>
<th>VCAM</th>
<th>NT-proBNP</th>
<th>tPAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of measurements</td>
<td>5</td>
<td>13</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>10</td>
<td>2</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>80.16</td>
<td>2 414.27</td>
<td>34.32</td>
<td>4.72</td>
<td>143.9</td>
<td>102.9</td>
<td>243.21</td>
<td>61.20</td>
<td>849.28</td>
<td>989.54</td>
<td>53.04</td>
</tr>
<tr>
<td>CV %</td>
<td>7.12</td>
<td>41.35</td>
<td>22.38</td>
<td>22.26</td>
<td>17.17</td>
<td>17.35</td>
<td>20.60</td>
<td>7.34</td>
<td>12.39</td>
<td>29.43</td>
<td>5.39</td>
</tr>
<tr>
<td>min.</td>
<td>73.66</td>
<td>693.73</td>
<td>27.26</td>
<td>3.24</td>
<td>122.4</td>
<td>4</td>
<td>70.32</td>
<td>196.86</td>
<td>58.02</td>
<td>717.77</td>
<td>783.62</td>
</tr>
<tr>
<td>max.</td>
<td>86.48</td>
<td>4 131.70</td>
<td>47.51</td>
<td>6.20</td>
<td>181.5</td>
<td>8</td>
<td>128.7</td>
<td>369.92</td>
<td>64.37</td>
<td>1 077.02</td>
<td>1 195.46</td>
</tr>
<tr>
<td>Mean CV% intraassay</td>
<td>11.32</td>
<td>10.43</td>
<td>11.75</td>
<td>4.87</td>
<td>10.17</td>
<td>6.05</td>
<td>6.72</td>
<td>10.41</td>
<td>7.20</td>
<td>3.83</td>
<td></td>
</tr>
</tbody>
</table>
Graph 12 Quality control results of pool control sample high for IL6, IL10, IL8 and VEGF

Graph 13 Quality control results of pool control sample low for ICAM and VCAM
Graph 14 Quality control results of pool control sample low for IL 6

Graph 15 Quality control results of pool control sample low for MMP9
4.1.4 Discussion

The planning of multiplex study has to be really multiplex and have to start already with precise choice of used material, proper handling (transport, storage and etc.) with samples with respect to all of the simultaneously measured analytes and the right choice of kits with regard to concentration levels of proteins in different biologic materials. According to our experience, 100 proteins analysed in one well is only theory using commercial available kits. In this time, it is not possible to analyse simultaneously proteins differing roughly in concentration levels in one well: e.g. adhesive molecules and MMP9 require preanalytical sample dilution and so cytokines, occurring often in very low concentration, can not be analyse in one well with the former ones.

Our experiments show the necessity of right choice of sample blood type, not only because of differences in obtained values but as well for protein detectability in material. This fact is widely understood for single method but it has got a new dimension with start of multiplexing. Information on differences in analysis of proteins in different sample types can be very important for planning multiplex research, but it is not many complex studies on this topic. The more complex data we have found are published in article on web pages of Rules Based Medicine company including results from experiment, where 78 proteins were measured in different blood sample types: EDTA plasma, citrate plasma, heparine plasma and serum. Results obtained in our experiments agree with data published by Rules Based Medicine. (http://www.rulesbasedmedicine.com/). Precise choice of used material is crucial, but unfortunately often compromise is necessary. The material can be chosen either as the sample type suitable for the most of the proteins or the sample type suitable for the proteins of the most interest in study. The blood sample should be consistent throughout the whole study without any doubt.

When we compared two studied multiplex technologies for cytokines measurement in seminal plasma, xMAP technology appears to us more convenient, mostly because of better detection limits. This is probably not caused by technologic differences but only by the existence of the high sensitive kits for xMAP technology, which are not available for FlowCytomix. Lash et al. published comparison of xMAP technology with two other multiplex cytokine analysis systems: SearchLight and FAST Quant. These two technologies are multiplex sandwich ELISA utilises standard ELISA combined with microspot technology. Levels of IL1, IL2, IL6, IL8, and IFNγ did not differ in their study among assay systems. Levels of IL 12 were not detected by Luminex, and were lower in SearchLight in
comparison to Fast Quant. Levels of IL4, IL10 and TNFα differ between assays, but there did not appear to be any systematic differences between the technologies. (Lash G.E., 2006) These findings agree with our results showing that there are no systematic differences among immunoassay multiplex technologies but only differences in detection limits and for some analytes results differences (TNFα) caused by use of different antibodies.

In our study, results obtained by multiplex correlate with conventional methods for all tested analytes with Spearman’s coefficients higher than 0.7. The only exclusion was IGFII measurement for which correlation was not so close with coefficient only 0.593. This lower comparability for IGFII could be caused by preanalytic requirement to separate this protein from its binding protein in procedure, which differs between kits. Even if the multiplex correlate with other methods, the values obtained by multiplex and conventional methods are not similar. However this fact is common problem when comparing nonmultiplex immunoanalytic methods. It is likely caused by use of different antibodies in assays. Liu et al. measured levels of leptin, insulin, C-peptide, monocyte chemoattractant protein-1 (MCP-1), eotaxin, IL8, TNFα, and IL6 with multiplex xMAP panels from 3 different manufacturers and compared the results with ELISA assays. Correlation between the Luminex multiplexed assays and ELISAs was observed to be good for leptin (Linco), insulin (Linco), MCP-1 (Biosource and Upstate), and eotaxin (Biosource) with correlation coefficients of 0.711–0.895; fair for eotaxin (Upstate) and C-peptide (Linco) with correlation coefficients of 0.496–0.582; and poor for TNFα, IL-8, and IL-6 (Linco, Biosource, Upstate, and R&D) with correlation coefficients of -0.107 to 0.318. (Liu M.Y., 2005). Multiplex results for IL6 levels in 225 samples were compared to ELISA analysis also by Ray et al. The reported degree of agreement by the concordance correlation coefficient was 84.5%. Multiplex results were found to be 2.36-fold higher than ELISA values on the average. After adjusting for this mean difference, the 95% empirical limits of agreement for the ratio of individual sample values were 0.33, 2.65. (Ray C.A., 2005). Khan et al. compared Luminex kits and ELISA for IL8 measurement. Concentrations measured by ELISA were similar to the values obtained by Luminex if kits from the same manufacturer were used (for R&D Systems kits r=0.96, P=0.0037 and for BioSource kit r=0.92, P=0.0037) (Khan S.S., 2005)

In presented study, the correlation is linear only up to some limit value for some analytes. This fact is mainly caused by the calibration curve character and could limit the applicability of multiplex for some analytes for samples with very high concentration. Such limits have to be discussed in future from the point of view of dynamic biologic ranges of measured proteins in clinical practice.
When planning the multiplex experiment it is very important to think over the expected concentrations of studied proteins. Above all e.g. cytokines are in healthy human blood samples in very low concentration levels. It is necessary to choose only some proteins from the commercially available panels and it is important to use the analytical system (kit manufacturer) with the lowest possible detection limits. Khan et al. compared Luminex kits from three different manufacturers. The trends in cytokine concentration values followed a similar pattern among all multiplex kits. Linco manufacturer was observed to have lowest detection limits. (Khan S.S., 2005)

Last but not the least analytic problem is the quality control of multiplex analysis. While no special recommendations for multiplex exists there are mostly implied the rules for convenient immunoassay methods. In our analyses the intrassay impression (counted as mean CV% of results for samples measured in doublets) was generally lower than 15% as it is stated in instructions for use by manufacturers. Liu et al. also reported that within- and between-run imprecision values were generally <15% for the multiplex xMAP method (Liu M.Y., 2005). No rough violation in intraassay quality assessment was observed in presented study. In interassay quality control some inconvenient results were found in presented study, but there are caused by obtained isolated outlying control levels, which can distort the results, while only a few measurements from each lot (of reagents and controls) are available in a clinical research with diversiform projects. „Home made“ pool samples are not a satisfactory solution, while there is not certified stability and target values. We consider a monitoring of interassay imprecission as a very problematic task and we are performing further studies. We would welcome any system of external quality control for multiplex measurement of proteins, while large data sets could be better interpreted among laboratories. Such a system is missing for cytokines, adhesive molecules and many other molecules even for convenient immunoassay methods
4.2 Clinical part

4.2.1 Test of Ovarian Cancer Multiplex xMAP Technology Panel

4.2.1.1 Introduction
Ovarian cancer has been termed the “silent killer” with an incidence of 10.94 and a mortality of 7.28 per 100,000 inhabitants in the Czech Republic in 2005 (according to the Institute of Health Information and Statistics of the Czech Republic). Unfortunately medical science is still ill-equipped to diagnose this disease in its initial stages, which results in a high mortality. Nowadays, there is no marker established for screening or early diagnostics, for treatment monitoring and follow up CA125 is in use. But the latest developments in ovarian cancer therapy - the introduction of novel anticancer drugs based on anti-CA125 antibodies will exclude the possibility of therapy monitoring only using CA125 serum levels, because according to our experience these drugs interfere in the CA125 immunoassays. The use of CA125 is also affected by a very frequent non-specific elevation of the marker in a number of physiologic and pathologic processes. CA-125 has been shown to be elevated in women with cirrhosis, congestive heart failure, in presence of ascites, and has been reported to vary by age, race, smoking history, obesity, and the presence of benign gynecologic conditions (endometriosis, hysterectomy, hormone therapy use) and other malignancies besides ovarian cancer. (Johnson C.C., 2008)

For these reasons the development of novel biomarkers is necessary. More than ten years ago a marker combination was shown to be more effective than single marker for pelvic mass management. (Woolas R.P., 1995) The introduction of novel multiplexed immunoanalytic assays has enabled new era of multiparametric panel studies. One of such analytical tools is bead-based Multi-analyte profiling technology (xMAP).

In this study a commercially available xMAP multiplex panel has been tested for the measurement of serum levels of tumour biological activity markers, specifically designed for ovarian cancer including leptin, prolactin (PRL), osteopontin (OPN), insulin-like growth factor II (IGFII), macrophage migration inhibitory factor (MIF) and CA125. It was tested in a group of ovarian cancer patients and patients with benign ovarian disease as a control, and
results were compared to levels of non-multiplexed serum tumour markers: CA125, TPS, thymidine kinase (TK), mononuclear and novel tumour marker HE4.

4.2.1.2 Methods

Patient groups
The multiplex panel was studied in a group of 19 women with ovarian cancer of FIGO stages 3 or 4. As a benign control group 20 patients with benign ovarian cysts were enrolled. The age of the patients varied from 16 to 94 years. Median age in control group was 42.5 years (range 16-68) and median age in cancer group was 57 years (range 44-94).

The peripheral blood was drawn using VACUETTE® Z Serum Sep tubes (Greiner Bio-One, Austria) and allowed to clot. Sera were separated by centrifugation at 1700g for 10 min. and all specimens were immediately aliquoted and frozen. Samples were stored at -80°C. No more than 1 freeze-thaw cycle was allowed before an analysis. Before a multiplex analysis the aliquots were centrifuged for 5 min. at 10000g to remove any clots or particles.

Biomarker measurement
Serum levels of MIF, prolactin, CA125, leptin, osteopontin and IGF-II were measured by xMAP technology using commercially available kit Beadlyte® Human Cancer Biomarker Panel (Millipore-Upstate, USA) and Luminex 100 instrument (Luminex Corp., USA). For the assay, 50µL of serum diluted 1:6 for 5-plex and 1:20 acid/ethanol treated serum for IGF-II monoplex were used. The multiplex analysis was run in duplicate.

Serum levels of single tumour markers were measured by routinely used immunoassays: thymidine kinase (radioenzymoimmunoassay, Immunotech-Beckman Coulter, CR, USA), TPS (IRMA, IDL BIOTECH AB, Sweden), mononuclear (IRMA, IDL BIOTECH AB, Sweden) and HE4 (Elisa, Fujirebio, Japan). CA125 levels were measured for comparison by the routinely used chemiluminiscence automated immunoassay on DxI 800 instrument (Beckman Coulter, USA). Results of CA125 obtained by the multiplex method are stated in the article as CA125Luminex.

Statistical methods
A descriptive statistic was calculated for all markers. Wilcoxon test was used for a comparison of marker levels between groups. The significance was set for p-value lower than 0.05. For all markers ROC analysis was performed; the area under ROC curves (AUC), sensitivity for 95% specificity and a related cut-off were calculated. Correlations of patient age to marker levels were calculated without respect to the cancer or the benign diagnoses.

Because the multiplex is not a routine method, mean coefficients of variation –CV% (relative
standard deviation in percents) were calculated for all multiplexed analytes. Results under the calibration curve ranges were stated as the value of the lowest calibration point. For a comparison of CA125 analytical methods, rank order correlation was performed (Spearman correlation coefficient was calculated).

4.2.1.3 Results
Results for all markers are shown in Table 14. Considering the biological activity markers included in the multiplex panel only CA125Luminex, osteopontin and IGF-II differed significantly between groups. Osteopontin levels were higher in the cancer group. On the contrary IGF-II levels were significantly decreased in the cancer group. Serum levels of MIF, prolactin and leptin did not significantly differ between groups. Levels of all non-multiplexed tumour markers were significantly higher in the cancer group compared to the benign control. A significant difference in age between groups was observed \( (p = 0.0007) \), the benign group being younger with a median age of 42.5 years contrary to the cancer group with a median age of 57 years, but no correlation between age and serum levels was found for any of markers.

All studied markers can be ordered by decreasing area under ROC curve as follows: HE4, CA125, monototal, TPS, CA125 Luminex, IGF II, TK, OPN, MIF, leptin and PRL. Mean coefficient of variation for doublets in multiplex analysis was best for MIF and worst for OPN. CV% for MIF (2.74%), PRL (3.59%) and leptin (2.49%) were under 5%, for IGF-II (7.99%) under 10%. CV% for CA125Luminex (11.24%) and OPN (14.44%) were slightly higher but still under 15%.

For two markers from multiplex panels some non-measurable values were obtained. For some samples levels under the calibration curve occurred. In CA125Luminex analysis 9 results (7 from benign group, 2 from cancer group) were under the lowest standard. In OPN analysis 16 results (12 from benign group, 4 from cancer group) were under the calibration curve.

CA125 levels analysed by multiplex measurement correlated with routine immunoassay - correlation coefficient 0.72087 \( (p < .0001) \), but discrepant values were observed. CA125 measured by routine method possessed more than 20-times higher values than CA125 values obtained by multiplex measurement.
Table 14: Summary results for all studied markers in benign and cancer group, their comparison by Wilcoxon test and ROC analysis – sensitivity in percent at 95% specificity, related cut-off and area under ROC curve (AUC)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Characteristic</th>
<th>MIF</th>
<th>PRL</th>
<th>CA125 LUMINEX</th>
<th>OPN</th>
<th>Leptin</th>
<th>IGF-II</th>
<th>HE4</th>
<th>CA125</th>
<th>TK</th>
<th>TPS</th>
<th>Monototal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pg/mL</td>
<td>Ng/mL</td>
<td>IU/mL</td>
<td>Ng/mL</td>
<td>ng/mL</td>
<td>pM</td>
<td>IU/mL</td>
<td>IU/L</td>
<td>IU/L</td>
<td>IU/L</td>
<td>IU/L</td>
</tr>
<tr>
<td>Benign</td>
<td>Median</td>
<td>372.61</td>
<td>8.10</td>
<td>0.25</td>
<td>0.15</td>
<td>11.06</td>
<td>2649.38</td>
<td>49.25</td>
<td>10.95</td>
<td>5.90</td>
<td>30.00</td>
<td>53.30</td>
</tr>
<tr>
<td></td>
<td>5-95% kvantil</td>
<td>58.80</td>
<td>2.56</td>
<td>0.19</td>
<td>0.15</td>
<td>3.50</td>
<td>1869.51</td>
<td>35.65</td>
<td>4.60</td>
<td>2.50</td>
<td>10.00</td>
<td>0</td>
</tr>
<tr>
<td>Cancer</td>
<td>Median</td>
<td>596.32</td>
<td>11.54</td>
<td>4.00</td>
<td>2.35</td>
<td>7.48</td>
<td>978.99</td>
<td>212.00</td>
<td>18.00</td>
<td>148.00</td>
<td>198.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-95% kvantil</td>
<td>63.97</td>
<td>0.33</td>
<td>0.19</td>
<td>0.15</td>
<td>1.27</td>
<td>118.45</td>
<td>41.70</td>
<td>7.30</td>
<td>5.10</td>
<td>41.00</td>
<td>93.20</td>
</tr>
<tr>
<td>p-value</td>
<td>Wilcoxon test</td>
<td>0.0973</td>
<td>0.6657</td>
<td>0.0011</td>
<td>0.0027</td>
<td>0.4611</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>0.0011</td>
<td>0.0002</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Sensitivity %</td>
<td>(95% specificity)</td>
<td>31.579</td>
<td>5.263</td>
<td>68.421</td>
<td>47.368</td>
<td>21.053</td>
<td>63.158</td>
<td>89.474</td>
<td>78.947</td>
<td>52.632</td>
<td>76.471</td>
<td>47.059</td>
</tr>
<tr>
<td>Related cut-off</td>
<td>1347.67</td>
<td>51.27</td>
<td>1.47</td>
<td>2.91</td>
<td>3.45</td>
<td>1804.60</td>
<td>63.80</td>
<td>20.00</td>
<td>16.70</td>
<td>92.00</td>
<td>232.20</td>
<td></td>
</tr>
<tr>
<td>ROC (AUC)</td>
<td></td>
<td>0.647</td>
<td>0.705</td>
<td>0.836</td>
<td>0.804</td>
<td>0.592</td>
<td>0.805</td>
<td>0.952</td>
<td>0.897</td>
<td>0.917</td>
<td>0.841</td>
<td>0.908</td>
</tr>
</tbody>
</table>
Graph 16 Comparison of ROC curves for all studied markers for discriminating between cancer and benign patients

Graph 17 Correlation of results for CA 125 obtained by xMAP technology (CA125-Lu) and chemiluminescence method (CA125)
4.2.1.4 Discussion

The commercially available multiplex panel used was developed following proteomic studies by Mor et al. They initially used the microarray analysis to determine 169 proteins in serum from healthy women and women with newly diagnosed and recurrent ovarian cancer. Proteins showing significant differences between controls and cancer patients were further validated by ELISA method (Mor G., 2005). On the basis of this study they introduced the multiplex panel, and it was tested with four different multiparametric result interpretation systems for ovarian cancer detection. The best multiparametric result handling method in conjunction with multiplex panel possesses in their study blood biomarker method with described 95.3% sensitivity and 99.4% specificity for the detection of ovarian cancer (Visintin I., 2008). For multiparametric statistical testing of 6-plex a patient cohort of at least 500 probands is necessary which is why it was not possible to test the multiparametric data handling.

Contrary to Mor et al. results, in this study 3 of the 6 multiplex markers do not reach any significant differences between the cancer and benign groups. This could have been caused by a very narrow selection of benign disease in the control group, i.e. only patients with benign ovarian cysts were enrolled. Leptin and prolactin have been discussed as tumour markers for several years, however no difference in their levels between groups was observed. Leptin is a member of adipose tissue metabolic pathways and alterations of serum leptin levels in obesity and in obesity-related syndromes are known. Unfortunately, no data on body mass indexes or weights of patients enrolled into the study were available and so it was not possible to exclude the difference of these factors between the studied groups, which could have influenced the results. Thorpe et al. have shown that prolactin levels are affected by collection conditions and proposed that prolactin elevations in comparison to healthy group, traditionally being explained by cancer disease, can be a consequence of surgical treatment of cancer patients. On the contrary, levels of MIF and CA125 have been reported to be unaffected (Thorpe J.D., 2007).

The commercially available multiplex panel turned out to be non-satisfactory and incomplete from the point of view of marker choice. Many other biomarkers are arising from proteomic studies. Havrilesky et al. developed and demonstrated the utility of several multimarker algorithms for ovarian cancer screening and treatment monitoring encompassing HE4, MMP7, serine leukocyte protease inhibitor-1 (SLP1), receptor for Urinary-type plasminogen activator (PLAU-R), mucin 1, inhibin, glycodelin, PAI-1 and CA125 (Havrilesky L.J., 2008).
Many other novel markers and panels of markers are being studied: e.g. haptoglobin, mesothelin, IL8 and anti-IL8 antibodies, transthyretin, APOA1, YKL40, total inhibin, vascular smooth muscle growth-promoting factor (VSGP/F-spondin), sIL-2Ralpha and others. (Gagnon A., 2008; Nossov V., 2008; Tsigkou A., 2007; Pyle-Chenault R. A., 2005; Palmer C., 2008; Sedlaczek P., 2002)

According to the presented results HE4 protein seems to be the most promising single marker. The results correlate with HE4 studies indicating that HE4 is better than CA125 in distinguishing patients with malignant from those with benign ovarian disease at high specificity (Hellström I., 2003). It does not show satisfactory sensitivity as a single marker, but could be very useful as a member of a multiplex panel. Palmer et al. used multiplexed measurement of CA125, HE4 and mesotelin in their study along with another single assayed markers, but any marker combination did not possess significantly better results than CA125. (Palmer C., 2008)

IGF II and OPN should be further studied in detail whether they are measured by common single assays or in multiplex. In concordance with our results, a simultaneous decrease of IGF II and an increase of OPN in ovarian cancer was published. (Visintin I., 2008; Mor G., 2005). Furthermore, significantly lower free IGF-II serum levels were observed in breast tumour patients than in healthy controls contrary to those described in serum IGF-II up-regulation specific for cervical cancer (Singer C.F., 2004; Mathur S.P., 2005). Significantly higher osteopontin levels in plasma in patients with epithelial ovarian cancer compared to healthy controls, benign ovarian disease and patients with other gynecologic cancers have been observed in non-multiplex studies e.g. by Kim et al (Kim J.H., 2002). Osteopontin in serum is bound to the complement factor H and serum levels were shown to be increased in prostate, breast and lung cancer in measurements performed after a disruption of the binding complex by Fedarko et al (Fedarko N.S., 2001). Novel studies, including the multiplex measurements, are not interested in the serum binding of OPN and pre-assay complex disruption.

The benign ovarian cyst disease occurs in younger patients contrary to ovarian cancer, which is why the control group in this study is not exactly age-matched to the cancer group. Because no correlation between age and marker levels was observed this age difference between groups is considered acceptable.
The discrepant values of CA125 obtained by multiplex measurement and by routine immunoassay, even if correlated, impress that different antibodies are used in assays and/or that further validation with international standards is necessary for the multiplex assay. Multiplex is a powerful tool in multimarker studies and in the future for multiparametric cancer disease diagnostics and monitoring, however we are still in the era of looking for the right marker combinations. Several studies, in which xMAP technology was used for cancer biomarker development were published. A large study of 64 biomarkers in serum of patients with endometrial cancer and a control group has been introduced, resulting in an establishment of five-biomarker panel (prolactin, growth hormone, eotaxin, E-selectin, and TSH) for discriminating the endometrial cancer from ovarian or breast cancer. (Yurkovetsky Z., 2007). xMAP technology was used as well to simultaneously test of 29 biomarkers in the sera of patients with melanoma. Fifteen serum biomarkers showed differences between melanoma patients and control healthy group. A multivariate bioinformatics Bayesian Network algorithm was used for discriminating between patients and controls and yielded 90% sensitivity at 98% specificity. (Yurkovetsky Z.R., 2007). Futhermore levels of 55 serum biomarkers were measured by Nolen et al. pre- and during neoadjuvant chemotherapy for locally advanced breast cancer using eight different multiplexed panels. Multivariate statistical analysis revealed multibiomarker panels that could differentiate between treatment response groups. (Nolen B.M., 2008) From the analytical point of view the tested commercially available multiplex panel fulfils all the requirements for further clinical research or in future clinical routine use – satisfactory coefficients of variations and calibration curve ranges for measuring parameters in serum. However, from the clinical point of view it is recommended that at least the novel HE4 tumour marker, and preferentially also non-specific markers for tumour mass or growth such as TPS, monototal or TK, are introduced into the panel. The novel multiplex panel will be further tested on a larger ovarian cancer cohort and in patients with other cancer and non-cancerous diseases.
4.2.2 Markers of biologic activity in metastatic process

4.2.2.1 Introduction
The metastatic progression of cancer disease is a cause of worse prognosis and mostly death of cancer patients. The development of metastatic disease is a complex process. For dissemination tumour cells use a variety of motility mechanisms, chemokine gradients, and proteinases to enter and exit the circulation. Since new treatments in cancer now allow to inhibit specific oncogenic pathways, it will be important to be able to predict the prevalence of the drug target within dissemination disease sites, to select patients for therapy and to assess response (Eccles S.A., 2007) The rapidly developing multiplex analytic technology opens the doors for multimarker blood monitoring of multiplex cancer processes. One of such tool can be bead based xMAP technology.

Metastatic spread of cancer to the bones is observed in many malignancies but is mostly related to multiple myeloma, breast, kidney, prostate and lung cancer. Bone metastases result in a number of complications in patients including bone pain, pathologic fractures, spinal cord compression, and hypercalcemia followed by decrease of quality of life. (Selvaggi G., 2005) It was found that some genes expressed by tumour cells were common for metastases to all sites e.g. osteopontin), whereas others were selectively expressed if the cell lines had a predilection to growth in a given tissue. The vicious cycle related to bone metastasis develops when factors secreted by or expressed on tumour cells (e.g. Parathyroid hormone-related peptide) activate osteoblasts and osteoclasts in the bone microenvironment to produce cytokines (e.g. Receptor activator for nuclear factor κB ligand – RANKL); bone remodeling and osteolysis causes release of growth factors (e.g. TGFβ and IGFI), which then stimulate tumour cell growth and motility and further release of parathyroid hormone related peptide. (Eccless S.A., 2007; Virk M.S., 2007)

In past, there were no therapeutic possibilities for bone metastatic disease, nowadays there exist not only palliative therapies but as well curative treatment leading to stabilized status and furthermore application for bone metastasis development prevention has already occurred. Imaging methods as conventional measurements of skeletal health and treatment response in metastatic bone lesions are imprecise and can only detect changes after the damage has occurred. Tools that are simple and can rapidly and sensitively detect changes in
bones during cancer are needed. The science and clinical utility of biochemical markers of bone metabolism are still evolving; therefore, they are not yet an established surrogate measurement for clinical efficacy. (Coleman R., 2008)

Our study was focused on testing commercially available multiplex Human Bone Panel introduced last year by Millipore corp. (USA) for the measurement of serum levels of markers: osteoprotegerin (OPG), osteopontin (OPN), osteocalcin (OC), parathormon (PTH) and leptin by xMAP technology. Our aim was to study the possibility of a detection of tumour induced bone disease (bone metastases) by serum tests, to setup the reference serum levels for parameters included in multiplex panel, and to compare serum bone markers nowadays routinely used in monitoring of several bone diseases: N-terminal propeptide of type I procollagen (PINP), N-propeptide of type III procollagen (PIIINP), bone-specific alkaline phosphatase (ostase, known as well as bALP), C-terminal telopeptid of type I collagen (ICTP), C-terminal cross-linking telopeptide of type I collagen (CTx, beta-CrossLaps) and 25-hydroxyvitamin D were assessed in cancer groups.

4.2.2.2 Methods

Patient cohorts
We studied 24 cancer patients with metastatic disease. Cancer cases were divided into a group 1 with occurrence of bone metastases (13 patients) and into the group 2 (11 patients) without any bone metastases. A control group of 20 healthy blood donors referred as group 0 was studied as well.

The peripheral blood was drawn using VACUETTE® Z Serum Sep tubes (Greiner Bio-One, Austria) and allowed to clot. Sera were separated by centrifugation and all specimens were immediately aliquoted and frozen. Samples were stored at -80°C. Before a multiplex analysis the aliquots were centrifuged for 5 min. at 10000g to remove any clots or particles.

Biomarker measurement
Serum levels of osteoprotegerin, osteopontin, osteocalcin, parathormon and leptin were measured by multiplex xMAP technology (Luminex 100 instrument) with use of Human Bone Panel A (Millipore Linco corp., USA) in cancer and healthy group. The multiplex analysis was run in duplicates. Routinely used serum bone markers: PINP, ICTP and PIIINP (all RIA, Orion Diagnostica, Finland), ostase (automated CLI, DxI UniCell 800, Immunotech - Beckman Coulter comp., USA), CTx (ECLIA, Elecsys, Roche, USA) and 25-hydroxyvitamin D (RIA, IDS, UK) were assessed in cancer groups. Osteocalcin levels
were measured for comparison by routinely used immunoassay (RIA, CisBio International, France), as well.

**Statistical methods**
A descriptive statistic was calculated for all markers, Wilcoxon test was used for a comparison of marker levels between groups. The significance was set for P value lower than 0.1. For the purposes of the scoring system the normal values for multiplex markers were set as a 95 percentil of healthy group, for non-multiplex markers there were used cut off values (see in Table III) derived from cut offs valid in our routine laboratory. Each value above normal level was scored by 1 point. Two scoring systems for better discrimination of group 1 and group 2 were created by empirical choice of analytes. Scoring system I – points for osteoprotegerin, osteopontin, PIIINP, ICTP and ostase were counted up; in scoring system II - points for osteoprotegerin, osteopontin, PIIINP, PINP and ostase were counted up. Because the multiplex is not a routine method, mean coefficients of variation – CV% (relative standard deviation in percents) were calculated for all multiplexed analytes. Results under the calibration curve ranges were stated as the value of the lowest calibration point. For a comparison of osteocalcin analytical methods Passing Bablock analysis (including Cusum test for linearity) was performed and Spearman’s coefficients of rank order correlation between methods was calculated.

### Results
All results for multiplexed markers are shown in table 15 and for nonmultiplexed markers in table 16. Significantly higher levels of osteoprotegerin and significantly lower levels of osteocalcin in both cancer groups compare to control group were found. No significant difference of any of multiplexed markers was found between group 1 and 2. Considering the nonmultiplexed markers significantly higher levels of PIIINP and ostase in group 1 compare to group 2 were observed. Interestingly, 3 of 4 patients with multiple bone metastases have values above set normal value both for osteoprotegerin and osteopontin in comparison to all other cancer patients, where only one of these markers was positive.

Osteocalcin levels obtained by multiplex measurement correlate with routine immunoassay - Spearman’s coefficient: 0.710 with P value 0.001. Regression equation obtained by Passing Bablock for osteocalcin methods: $Y = 0.1153 (95\% CI : -1,1537 to 0,9909) + 0.2977 (95\% CI : 0,2268 to 0,3962) X$ with no significant deviation from linearity.
Mean coefficients of variation for doublets in multiplex analysis were under 7% for all multiplexed markers except for osteopontin. For osteopontin worse accuracy was observed: mean CV% 16.9% but with median of 8.2%, caused by number of samples with CV% over 15%. For leptin no samples with CV% over 15% were observed, for OPG one sample and for OC two samples with CV% over 15% occurred.

No samples out of calibration ranges for osteocalcin and osteoprotegerin were observed with recommended serum dilution, for leptin and PTH three samples under calibration curve and nine samples for osteopontin occurred.

Table 15 Summary of results for multiplexed markers (group 0 – healthy controls, group 1 – bone metastatic cancer group, group 2 – non-bone metastatic cancer group) and comparison of groups by Wilcoxon test

<table>
<thead>
<tr>
<th>Group</th>
<th>OPG (pg/mL)</th>
<th>OPN (pg/mL)</th>
<th>OC (pg/mL)</th>
<th>PTH (pg/mL)</th>
<th>Leptin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Median</td>
<td>262</td>
<td>679</td>
<td>7838</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Kvantil</td>
<td>5%</td>
<td>113</td>
<td>244</td>
<td>4080</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%</td>
<td>639</td>
<td>32596</td>
<td>13304</td>
</tr>
<tr>
<td>1</td>
<td>Median</td>
<td>631</td>
<td>1978</td>
<td>5329</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Kvantil</td>
<td>5%</td>
<td>218</td>
<td>244</td>
<td>1017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%</td>
<td>2417</td>
<td>128232</td>
<td>22821</td>
</tr>
<tr>
<td>2</td>
<td>Median</td>
<td>504</td>
<td>1742</td>
<td>5195</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Kvantil</td>
<td>5%</td>
<td>268</td>
<td>260</td>
<td>1723</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%</td>
<td>954</td>
<td>6918</td>
<td>15594</td>
</tr>
<tr>
<td>Group difference Wilcoxon test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0x1</td>
<td>0.0001</td>
<td>0.1174</td>
<td>0.0554</td>
<td>0.7682</td>
<td>0.9120</td>
</tr>
<tr>
<td>0x2</td>
<td>0.0011</td>
<td>0.1498</td>
<td>0.0142</td>
<td>0.7853</td>
<td>0.1391</td>
</tr>
<tr>
<td>1x2</td>
<td>P&gt;0.1</td>
<td>P&gt;0.1</td>
<td>P&gt;0.1</td>
<td>P&gt;0.1</td>
<td>P&gt;0.1</td>
</tr>
</tbody>
</table>
Table 16 Summary of results for non multiplexed markers (group 1 – bone metastatic cancer group, group 2 – non-bone metastatic cancer group) and comparison of groups by Wilcoxon test

<table>
<thead>
<tr>
<th>Group</th>
<th>PIIINP (µg/L)</th>
<th>ICTP (ng/mL)</th>
<th>VitD (nmol/l)</th>
<th>PINP (µg/L)</th>
<th>Ostase (µg/L)</th>
<th>CTx (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.5</td>
<td>11.0</td>
<td>41.7</td>
<td>49.0</td>
<td>18.4</td>
<td>0.460</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>3.5</td>
<td>3.9</td>
<td>17.5</td>
<td>35.1</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>95%</td>
<td>73.1</td>
<td>75.0</td>
<td>74.8</td>
<td>250.0</td>
<td>121.0</td>
</tr>
<tr>
<td>2</td>
<td>4.8</td>
<td>8.2</td>
<td>42.0</td>
<td>47.0</td>
<td>8.9</td>
<td>0.420</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>2.2</td>
<td>4.6</td>
<td>17.1</td>
<td>30.3</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>95%</td>
<td>15.4</td>
<td>21.4</td>
<td>147.9</td>
<td>77.7</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Group difference Wilcoxon test

1x2 P<0.05 P>0.1 P>0.1 P>0.1 P<0.1 P>0.1

Table 17 Cut off values for markers and percents of samples out of set reference ranges in group of bone metastatic patients - 1 and in group of non-bone metastatic patients - 2

<table>
<thead>
<tr>
<th>Score system</th>
<th>OPG pg/mL</th>
<th>OPN pg/mL</th>
<th>OC pg/mL</th>
<th>PTH pg/mL</th>
<th>Leptin pg/mL</th>
<th>PIIINP ug/L</th>
<th>ICTP ng/mL</th>
<th>VitD nmol/l</th>
<th>PINP ug/L</th>
<th>Ostase ug/L</th>
<th>CTx ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut off value</td>
<td>&gt;639</td>
<td>&gt;32596</td>
<td>&gt;13304</td>
<td>&gt;46</td>
<td>&gt;17645</td>
<td>&gt;6.4</td>
<td>&gt;6.0</td>
<td>&lt;23</td>
<td>&gt;22</td>
<td>&gt;100</td>
<td>&gt;0.6</td>
</tr>
<tr>
<td>Group 1</td>
<td>46% 6/13</td>
<td>23% 3/13</td>
<td>23% 3/13</td>
<td>8% 1/13</td>
<td>8% 1/13</td>
<td>54% 7/13</td>
<td>69% 9/13</td>
<td>31% 4/13</td>
<td>38% 5/13</td>
<td>31% 4/13</td>
<td>38% 5/13</td>
</tr>
<tr>
<td>Group 2</td>
<td>36% 4/11</td>
<td>0% 0/11</td>
<td>9% 1/11</td>
<td>18% 2/11</td>
<td>0% 0/11</td>
<td>27% 3/11</td>
<td>64% 7/11</td>
<td>18% 2/11</td>
<td>9% 1/11</td>
<td>0% 0/11</td>
<td>27% 3/11</td>
</tr>
</tbody>
</table>
Graph 18 Box plots for osteoprotegerin, P3NP and ostase for control healthy group (group 0), bone metastatic cancer group (group 1) and nonbone metastatic cancer group (group 2)
4.2.2.4 Discussion

Most promising from multiplexed markers turned out to be osteoprotegerin, which agree with literature sources, differs significantly in comparison to healthy group not between cancer groups. OPG is a soluble decoy receptor for RANKL and so prevents RANKL binding to RANK and subsequent activation of osteoclast activity. OPG also inhibits apoptosis of tumour cells by inhibiting of TRAIL (TNF-related apoptosis-inducing ligand) and provides them a survival advantage.(Holen I., 2006) Furthermore OPG has been found to stimulate angiogenesis, while RANKL is an angiogenic inhibitor. (McGonigle J.S., 2008). Increased serum OPG levels in comparison to healthy controls were observed in prostate cancer, bladder carcinoma, colorectal and pancreatic cancer and were reported to be associated with several other organ systems and pathologies e.g. endometriosis, periodontal disease, thyroid disease and coronary heart disease. (Holen I., 2006; Lipton A., 2002; Narita N., 2008) Serum OPG levels were found to be increased in patients with metastatic bone disease in prostate cancer compared with patients with organ confined disease. Serum OPG increases may indicate disease progression/relapse in prostate cancer. (Holen I. 2006; Jung K., 2001; Jung K., 2004) In comparison, when analysing site of metastasis Lipton et al. observed significant elevation of serum OPG levels only in patients with liver and soft tissue metastasis and not in patients with bone or lung metastasis. (Lipton A., 2002) Contrary decreased serum levels of OPG were reported in patients with multiple myeloma and in sarcoma patients compared to controls. (Holen I., 2006; Terpos E., 2003; Lipton A., 2002). Interesting preanalytic information is that OPG levels are higher in serum than in plasma and that the levels are higher in females than in males. (Lipton A., 2002). Controversial is the age dependence of OPG levels – age dependence was reported e.g. by Jung et al. and Narita et al. and age independence by e.g. Lipton et al. (Lipton A., 2002; Jung K., 2004, Narita N., 2008)

Lower levels of osteocalcin in cancer group compare to healthy controls were found in our study, but no difference between bone metastatic group and nonbone metastatic group was found. Osteocalcin is a small γ carboxyglutamicacid-rich peptide one of the major noncollagenous proteins of the bone matrix exclusively synthesized by osteoblasts with a very short biologic half-life. Serum levels were reported to correlate well with osteoblast activity. In advanced untreated metastatic bone disease, serum osteocalcin levels might be low in the presence of high serum BAP levels. The reasons for this metabolic uncoupling between two bone-formation markers are unclear; however, possibilities include proteolytic
cleavage of osteocalcin, changes in gene expression and disturbed osteoid maturation in the presence of active tumor osteopathy. (Racek J., 2006; Seibel M.J., 2005; Fohr B., 2006). In contrary OC levels were found to be significantly higher in breast cancer patients with bone metastasis compared to non-metastatic or soft tissue metastasis. (Salem A. M., 2007) In our opinion the multiplex measurement in conjunction with multimarker data handling can improve discrimination of bone and non-bone metastatic disease in future. For illustration two scoring systems were created. For scoring system 1: score 3 or higher was positive for 46% patients in group 1 (6/13) in comparison to 9% in group 2 (1/11), for score 2 or higher positive 61,5% patients (8/13) in group 1 in comparison to 36% (4/11) in group 2. For scoring system 2: score 3 or higher positive 38% of patients in group 1 (5/13) in comparison to 0% in group 2 (0/11), for score 2 or higher positive 54% of patients in group 1 (7/13) contrary to 9% (1/11) in group 2. We observed that patients with multiple bone metastases have values above set normal value both for osteoprotegerin and osteopontin, this fact could help in discriminating of such patients according to serum tests. Inside the bone osteopontin is produced by both osteoblasts and osteoclasts and has a multiple presumed functions: the attachment of osteogenic cells to the bone matrix, control of mineralization, coupling of bone formation and resorption. Elevated OPN levels were described in patients with epithelial ovarian cancer, breast, lung and prostate cancer and were found to be associated with a shorter survival, larger numbers of sites of metastatic involvement in metastatic breast cancer. (Kim J.H., 2002; Fedarko N.S., 2001; Rodrigues L.R., 2007). In our study, OPN levels were higher in cancer groups with very high 95% kvantil in bone metastatic group, but the differences did not reach statistic significance. Leptin plays a role not only in regulation of body weight and energy balance but as well in vascular remodeling, regulates neoangiogenesis by itself and in concert with VEGF and fibroblast growth factor, acts as a mitogen transforming factor and supresses apoptosis. No significant differences among groups were observed in our study and reported studies on serum leptin levels in cancer produce ambiguous results, which can be caused by the pulsatile blood levels (frequency about 1 pulse per 45 min.) and diurnal pattern. However, the serum leptin levels were reported to be unaltered or significantly decreased in colorectal cancer and advanced non-small-cell lung cancer in contrary to unaltered or significantly increased leptin levels in breast and prostate cancer. For prostate cancer higher leptin levels were associated with more advanced tumors, characterized by larger size and higher grade. These facts imply the possible role of leptin in metastatic site differences among cancer types, that is why it was enrolled in our multiplex study. (Somasundar P., 2004; Garofalo C., 2006).
PTH, the last member of the multiplex panel, is suspected to promote the growth and invasiveness of cancer in bone thanks to increased expression of the PTH receptor in cancer metastases (Schwartz G.G., 2008), but did not differ between groups in our study.

Considering the nonmultiplexed markers significantly higher levels of PIIINP and ostase in bone metastatic group compared to non-bone metastatic group were observed. Only these two markers discriminate the bone and non-bone metastatic group. No significant differences between bone metastatic and non-bone metastatic patients in any of bone resorption marker (CTx and ICTP) levels were found. Serum levels of ostase were reported to correlate closely with osteoblast differentiation and activity. In most cases of advanced metastatic bone disease ostase levels in serum are elevated, reflecting either a strong osteoblastic component or, in lytic lesions, active repair (Seibel M.J., 2005).

According to Demers et al. the best markers for assessing the presence of progression of skeletal metastases appear to be the collagen breakdown products of type I bone collagen (Demers L.M., 2003). In our study we have shown that interest should be focused as well on the propeptide of type III procollagen, which is considered to be a marker of connective tissue metabolism (Fohr B., 2006) and a marker of liver fibrosis. In our study we have observed elevation of the P3NP in bone-metastatic group in comparison to non-bone metastatic group and OPG in bone metastatic group in comparison to healthy group. Because Lipton et al. observed elevation of serum OPG levels only in patients with liver and soft tissue metastasis and not related to bone disease (Lipton A., 2002), observations could be influenced by the liver metastatic status of the patients. That is why, in future studies, liver metastases should be precisely considered in larger cohort studies of bone metastatic disease.

There is no chance to make a diagnostic decision considering the bone metastatic disease from only one marker. The bone metastatic development and spread is a consequence of broken balance among cytokines and other proteins regulating osteoblastic and osteoclastic activity that is why the absolute level of one marker is not so important as a ratios among regulation proteins. The only way how to monitor this net regulation is multiplex analysis which enables to measure all markers under the same conditions. This “simulation” of pathophysiological process cannot be sufficiently performed by measurements of markers one by one but only by a multiplex solution. Bone metastases are classified as osteolytic, osteoblastic or mixed. In many cases osteolytic and osteoblastic processes are involved and in fact it is a process wherein both biological situations coexists (Selvaggi G., 2005). High bone turnover with excess bone resorption is therefore an archetypal feature of most, if not all, bone metastasis. (Seibel M.J., 2005) That is why we do not consider necessary to
discriminate cases into osteolytic and osteoblastic cohorts and we believe that with multiplex and multiparameter system is possible in future to develop universal diagnostic and prognostic system.

Studied cohort of patients is quite small and doubtless investigation on larger cohort is necessary but it seems that would be very useful for oncology to incorporate other bone markers e.g. ostase into the multiplex panel. According to literature we conclude that further incorporating of at least RANKL and TGFbeta (Virk M.S., 2007) in future studies is necessary for complex point of view. Doubtless diurnal regime and biologic variability of novel bone markers have to be studied. Furthermore at earlier stages of the disease process changes in skeletal morphology or radionuclide uptake might be discrete, nonspecific or absent and so unidentifiable by imaging methods. We admit the possibility that in group 2 there could be enrolled patients with bone metastasis in development underdiagnosed by imaging methods. For next studies not only the results of imaging methods at the time of blood sampling but as well results of imaging methods couple of months after the blood sampling should be considered. In perspectives multiplex panel will be studied in conjunction with RANKL, TGFbeta and routine bone markers on a larger cohort of patients. We supposed that large cohort study would enable to set up a multiparameter result interpretation system and to choose the best marker combination for multiplex measurement. Such novel complex clinical laboratory tool could be used for early detection of bone metastatic disease, for treatment efficacy monitoring, for predicting of risk of bone metastases development for better treatment tailoring and even for control of up-to-date possibilities in prevention of metastatic disease occurrence.

The treatment monitoring and tailoring is one of the most desired future function of markers in bone metastatic disease management. For example Lester et al. have evaluated a bone marker directed schedule of treatment with zolendronic acid based on levels of the bone resorption marker, urinary N-telopeptide of type I collagen. Their experience suggests that a tailored approach to bisphosphonates therapy may be a more cost-effective approach than the currently licensed and recommended fixed schedule of intravenous treatment. (Lester J., 2006) The treatment monitoring would enable to improve the cost-effectivity, to reduce the doses by individual tailoring and hopefully to decrease the severity of adverse therapy effects. Nowadays the necessity of proper patient choice for treatment by novel targeted antibodies drugs is coming up. The trouble is that the bone markers are mainly studies separately, e.g. NTx levels in BISMARK study (Coleman R., 2008) or in a group of 2 to 3 markers. The comparability of results among studies is very problematic. Even the conditions
for patient enrolment into a study widely vary. The assessment of a panel of bone markers should be a part of clinical trials of newly introduced drugs, which have a multicentric manner and precisely defined patient cohorts.

Multiplex bone metastasis detection by serum test in future would have advantages of easiness of venous blood sampling, no radiation exposure of patients in comparison to imaging methods, the possibility of regularly monitoring of the therapy efficacy, the monitoring of whole body bone remodelling not only the imaged area, better cost-benefit ratio than single analytical methods and hopefully it can help with creating of multiparametric scoring system with sufficient sensitivity and specificity for clinical practise.
4.2.3 Biomarkers in prostate carcinoma

4.2.3.1 Introduction
Prostate specific antigen (PSA) is the standard diagnostic and prognostic prostate cancer (PCa) serum marker. Unfortunately PSA is not a satisfactory marker for screening and early diagnostics. Its improper use for screening results in a negative biopsy rate of 70-80% and in increased detection and diagnosis of clinically irrelevant tumours. Using current recommended guidelines, the PSA test suffers from both limited sensitivity and specificity to enable efficacious population-based cancer detection. There is an urgent need of new, complementary markers that would increase the diagnostic specificity and differentiation between harmless and aggressive disease. Novel treatment possibilities underline the necessity of optimal staging, preventing of misclassification of patients with undetected micrometastases and the early detection of hormone independent status / the treatment resistance, which could help in adequate and successful treatment of patients. Another problem is that very poorly differentiated tumours, which are the most aggressive, produce disproportionately low serum concentrations of PSA. (Steuber T., 2008; Hernandez J., 2004; van Gils M.P., 2005).

PCa related proteins released into plasma only partially reflects tumour metabolism, but on the other hand, a biopsy results could be non-representative because of polyclonal tumour nature and in comparison body fluids can be easily obtained. (van Gils M.P., 2005) The area of biomarker candidates is wide e.g. Tricoli et al. reviewed 91 molecular markers that display some level of correlation with prostate cancer presence, disease progression, cancer recurrence, prediction of response to therapy, and/or disease-free survival. (Tricoli J.V., 2004)

The aim of our study was to evaluate diagnostic potential of selected biomarkers for PCa diagnostics. There were measured blood levels of nine biomarkers by multiplex or routine immunooanalysis in men, who underwent examination for suspicion of PCa in our hospital. In complement to PSA, there were chosen potential biomarkers: soluble intercellular adhesion molecule I (ICAM), soluble vascular cell adhesion molecule I (VCAM), matrix metalloproteinase 9 (MMP9), interleukin 6 (IL6), vascular endothelial growth factor (VEGF)
insulin like growth factor I (IGF1) and its binding protein 3 (IGFBP3), thymidine kinase (TK) and chromogranin A (CHRA).

MMP9 (synonyms collagenase type IV or gelatinase B) is a member of a group of zinc metalloproteases degrading the collagens of the extracellular matrix (ECM) in mammals. (Rydlova M., 2007) For prostate tumor invasion or metastasis is critical the disruption of both the basal cell layer and the basement membrane, composed of type IV collagen, laminins, and other molecules. MMP-9 contributes by indirect mechanisms to osteolysis in prostate cancer–bone interaction. MMP-9 may cause the release of ECM-bound vascular endothelial growth factor into a soluble and bioactive form (Bergers G., 2000), thereby favouring the angiogenesis of intraosseous prostate tumours and the subsequent growth of those tumours. That effect ultimately stimulates new osteoclastic activity necessary to gain more space for expansion of the tumours. This indicate an important contribution of MMP-9 to neovascularization of expanding bone metastase and to subsequent bone degradation. (Engsig M.T., 2000)

The somatomedins, or insulin-like growth factors (IGFs), comprise a family of peptides that play important roles in mammalian growth and development. The IGF family involves the combination of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR), six high-affinity binding proteins (IGFBPs 1–6), a large group of IGFBP proteases and a new group of proteins, low-affinity IGFBP-related proteins (IGFBP-rPs). Members of this family form a network of interactions both among themselves and with other GF families and their signalling pathways. IGFI mediates many of the growth-promoting effects of growth hormone, plays an important role in regulation of cell proliferation, differentiation, apoptosis and transformation. IGFI stimulate the Bcl proteins and block the apoptotic pathway. The activity of IGFI is largely neutralized by IGFBPs. Less than 5% of circulating IGF is free and most is bound into a complex consisting of IGFI, IGFII, acid-labile subunit and IGFBP3. Insulin-like growth factor binding protein-3 (IGFBP-3) modulates the effects of IGF-I, and independently induces apoptosis and inhibits cell growth. Importantly, IGFs induce tumor cell motility via a crosstalk between the IGF family and the integrin system and modulate cell–cell attachment via interactions. (Gennigens C., 2006; Goel H.L., 2004; Damsky C.H., 2002; Fornaro M., 2001; Kahn C.R.,1985) From epidemiological studies high circulating IGF-I concentration with an increased risk of PCa, whereas high IGFBP3 concentration associated with a decreased risk (Gennigens C., 2006).
IGF family probably attribute to the fact, that PCa spreads most commonly to the well-vascularized areas of the skeleton, while IGF-I and IGF-II are the most abundant growth factors stored in the skeleton and they are produced with both type of receptors by bone cells (Gori F., 1999). The tropism of PCa cells for bone and their tendency to induce the osteoblastic phenotype is a result of interactions between PCa cells and osteoblasts. Metastatic PCa cells release and activate uPA, which leads to local increase of uPA-mediated proteolysis of IGFBPs and consequently a local increase in IGF bioavailability which, in turn, stimulates the proliferation of osteoblasts and tumor cells, leading to the development of an osteoblastic reaction at the sites of bone metastases from PCa. Like uPA, PSA can also cleave IGFBP-3, thereby rendering IGF-I available for binding to its receptor and stimulating osteoblast proliferation. (Gennigens C., 2006).

ICAM and VCAM are adhesive molecules and their role in many pathophysiologic processes e.g. atherosclerosis and carcogenesis is under the scope. Circulating levels of these molecules were shown to be elevated in patients with advance breast cancer, to reflect tumour progression and metastasis in gastric cancer and to be a prospective factors for early diagnosis of progression in colorectal cancer. (Holubec L., 2005; O’Hanlon D.M., 2002; Alexiou D., 2003).

Angiogenesis has been shown to be an important part of prostate cancer disease thanks to observation of microvessel density in tumours, which has been proven to be higher in PCa tissue compare to BPH and to correlate with VEGF circulating levels. Many potential growth factors are present in the prostate but VEGF is the most prominent angiogenic factor. Prostate itself might be significant source of circulating VEGF, while it is expressed by prostatic glandular cells. VEGF is the most prominent cytokine responsible for endothelial cell differentiation, migration, proliferation, tube formation, and vessel assembly (Fong G.M., 1995). VEGF stimulates angiogenesis but also has many other functions. The interest in VEGF in PCa came from the observation that androgens regulate VEGF expression, not only in the normal prostate, but also in PCa (Mukherjee P., 1999; Stewart R.J., 2001; Stefanou D., 2004) Results considering the correlation between circulating levels of VEGF and PCa are controversial.

Many physiologic functions are attributed to IL-6 including promotion of antipody production from B lymphocytes, modulation of hepatic acute phase reactant synthesis, promotion of osteoclastic-mediated bone resorption, and induction of thrombopoiesis. IL6 is
a mediator of inflammation but was shown to be involved also in modulation of growth and differentiation in many malignant tumors and mediates its activity through the IL-6 receptor complex. In addition to its role as a mediator of morbidity, IL-6 may also act as a growth factor, and protect prostate cancer cells from cell death induced by certain chemotherapeutic agents. Prostate tissue is a source of IL6 and IL6R by itself and IL6 correlate with tumor burden and patient morbidity. (Hirano T., 1992; Taga T., 1989; Smith P.C., 2001)

Chromogranin A, a member of the granin protein family, regulates neuroendocrine activity in cells and acts as a prohormone of multiple peptides with biological activity. It is stored in the dense core secretory granules of most endocrine and neuroendocrine cells and is a marker of neuroendocrine differentiation. (Nobels F.R., 1998) Chromogranin circulating levels were found to be higher in prostate cancer compare to BPH and correlated either to the stage of disease or to the condition of hormone refractories. (Tricoli J.V., 2004, Berruti A., 2001)

Thymidine kinase 1 (EC 2.7.1.21) is a key cytosolic enzyme in one-step the pyrimidine salvage pathway catalyzing the transfer of the terminal phosphate from ATP to 5’ hydroxyl group of thymidine to allow its incorporation into DNA. Thymidine kinase (TK) is a cell cycle dependent marker that increases dramatically during the S-phase of the cell cycle. Determination of thymidine kinase help to monitor of the follow up of solid tumors and hematological malignancies as well as indicating of the efficacy of adjuvant and palliative chemotherapy. (Topolcan O., 2008).

4.2.3.2 Methods

Patient group

Into the study there were enrolled patients with suspect of prostate cancer. These patients underwent routine examinations including transrectal biopsy to determine the diagnosis and venous blood sampling before examination. According to final diagnostic decision based on biopsy results, 66 patients were divided into three groups: 27 patients with benign prostatic hyperplasia (BPH), 9 patients with prostatic intraepithelial neoplasia (PIN) and 30 patients with prostate cancer (PCa). Into the BPH group only patients with freePSA to PSA ratio higher than 15% were enrolled to prevent missclassification of underdiagnosed PCa. Furthermore the PCa group was divided into the subgroups: i) according to stage of disease – group 1 – prostate localised disease represents patients with stage T2b or lower and group 2 represents patients with extended disease (stage T2c or higher); ii) according to Gleason score – group 1 represents patients with Gleason score 6 or lower and group 2 represents patients with Gleason score 7 or higher.
**Biomarker measurement**

Levels of ICAM-1, VCAM-1, MMP-9 in plasma and VEGF, IL-6 in serum were measured by multiplex xMAP technology with commercially available Human CVD 1 and 2 panels from Millipore - Linco Research comp. (USA) and Luminex 100 instrument (Luminex corp., USA). Other markers were measured by routine immunoassay methods: PSA were measured by CLIA method (Beckman Coulter, USA, DxI 800 Instrument), chromogranin A by IRMA (Cis Bio International, France), IGFBP3 by IRMA (Biosource Europe, Belgium), IGFI by RIA (Immunotech Beckman Coulter, Czech R.) and activity of thymidine kinase by REA (Immunotech Beckman Coulter, Czech R.)

**Statistical methods**

A descriptive statistic was calculated for all markers, Wilcoxon test was used for a comparison of marker levels between groups. The significance was set for P value lower than 0.05. ROC analysis was performed for markers with significant difference between diagnostic groups; the area under ROC curves (AUC) and a sensitivity for 95% specificity were calculated.

**4.2.3.3 Results**

In comparison between patients with BPH and patients with PCa we found statistically significant differences in the levels of PSA (P=0.0001), IGFBP3 (P=0.0027) and IGFI (P=0.0148).

We found also statistically significant differences between patients with BPH and PIN in the levels of PSA (0.0271), VEGF (0.0235) and TK (0.0426). IGFBP3 was also significantly higher in patients with PCa in comparison to patients with PIN. The levels of PSA, TK and Chromogranin A were higher in the group of patients with stage of disease higher than T2c. Levels of MMP-9 were increased in the group with Gleason score 7 or higher.

For all results see tables and graphs.

ROC curve analysis for discriminating BPH and PCa possesses for PSA AUC = 0.846 (95% CI = 0.726 to 0.928). IGFBP3 AUC=0.732 (95% CI 0.598 to 0.841). IGFI AUC = 0.688 (95% CI = 0.552 to 0.804). For discriminating of BPH patients from PCa patients there were found sensitivity of 60% for PSA, 40% for IGFBP3 and 30% for IGFI at 95% specificity.
<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Markers</th>
<th>Units</th>
<th>ng/ml</th>
<th>ng/ml</th>
<th>pg/ml</th>
<th>pg/ml</th>
<th>pg/ml</th>
<th>µg/l</th>
<th>ng/ml</th>
<th>ng/ml</th>
<th>IU/L</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BPH</strong></td>
<td>27</td>
<td>Median</td>
<td>ICAM</td>
<td>80.53</td>
<td>970.95</td>
<td>104470</td>
<td>3.11</td>
<td>45.36</td>
<td>5.3</td>
<td>2898</td>
<td>162</td>
<td>4.7</td>
<td>51.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>42.76</td>
<td>603.55</td>
<td>47155</td>
<td>0.64</td>
<td>15.65</td>
<td>0.67</td>
<td>1802</td>
<td>102</td>
<td>2.5</td>
<td>29.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>150.24</td>
<td>1239.19</td>
<td>210347</td>
<td>26.17</td>
<td>153.23</td>
<td>9.42</td>
<td>3729</td>
<td>257.5</td>
<td>9.62</td>
<td>190.7</td>
</tr>
<tr>
<td><strong>PIN</strong></td>
<td>9</td>
<td>Median</td>
<td>ICAM</td>
<td>100.94</td>
<td>914.66</td>
<td>113944</td>
<td>4.39</td>
<td>68.38</td>
<td>8.7</td>
<td>2650</td>
<td>182</td>
<td>7.9</td>
<td>58.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>68.85</td>
<td>609.96</td>
<td>35879</td>
<td>1.62</td>
<td>33.15</td>
<td>1.80</td>
<td>1230</td>
<td>20</td>
<td>2.5</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>245.26</td>
<td>2644.07</td>
<td>192850</td>
<td>8.12</td>
<td>257.40</td>
<td>95.6</td>
<td>3630</td>
<td>128</td>
<td>22.2</td>
<td>128.7</td>
</tr>
<tr>
<td><strong>PCa</strong></td>
<td>30</td>
<td>Median</td>
<td>ICAM</td>
<td>91.61</td>
<td>907.50</td>
<td>118008</td>
<td>2.85</td>
<td>49.34</td>
<td>12.4</td>
<td>3419</td>
<td>209</td>
<td>5.3</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>51.63</td>
<td>590.31</td>
<td>55895</td>
<td>0.6</td>
<td>17.51</td>
<td>2.8</td>
<td>1889</td>
<td>98</td>
<td>2.5</td>
<td>29.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>148.43</td>
<td>1184.06</td>
<td>226715</td>
<td>34.09</td>
<td>367.41</td>
<td>201</td>
<td>5107</td>
<td>400</td>
<td>9.6</td>
<td>142.2</td>
</tr>
<tr>
<td><strong>Localized PCa</strong></td>
<td>15</td>
<td>Median</td>
<td>ICAM</td>
<td>79.60</td>
<td>885.50</td>
<td>99576</td>
<td>3.19</td>
<td>33.15</td>
<td>8.9</td>
<td>3423</td>
<td>194</td>
<td>3.5</td>
<td>47.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>52.14</td>
<td>670.69</td>
<td>53763</td>
<td>0.61</td>
<td>15.91</td>
<td>2.8</td>
<td>2047</td>
<td>124.5</td>
<td>2.5</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>131.29</td>
<td>1162.65</td>
<td>229064</td>
<td>36.29</td>
<td>321.68</td>
<td>105.1</td>
<td>4906</td>
<td>386</td>
<td>7.3</td>
<td>98</td>
</tr>
<tr>
<td><strong>Extended PCa</strong></td>
<td>14</td>
<td>Median</td>
<td>ICAM</td>
<td>92.84</td>
<td>907.50</td>
<td>126932</td>
<td>2.85</td>
<td>64.46</td>
<td>22.8</td>
<td>3181</td>
<td>233</td>
<td>6.3</td>
<td>73.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>54.57</td>
<td>533.36</td>
<td>80663</td>
<td>0.70</td>
<td>17.88</td>
<td>7.2</td>
<td>1394</td>
<td>78</td>
<td>2.5</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>156.86</td>
<td>1354.00</td>
<td>224145</td>
<td>14.44</td>
<td>414.40</td>
<td>688.1</td>
<td>5283</td>
<td>407</td>
<td>25.8</td>
<td>233.0</td>
</tr>
<tr>
<td><strong>PCa GS&lt;6</strong></td>
<td>11</td>
<td>Median</td>
<td>ICAM</td>
<td>100.65</td>
<td>887.54</td>
<td>103657</td>
<td>3.87</td>
<td>49.73</td>
<td>9.4</td>
<td>3535</td>
<td>220</td>
<td>5.3</td>
<td>50.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>56.42</td>
<td>531.63</td>
<td>52910</td>
<td>0.96</td>
<td>19.47</td>
<td>5.8</td>
<td>2586</td>
<td>139</td>
<td>2.5</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>157.94</td>
<td>1375.10</td>
<td>116594</td>
<td>36.87</td>
<td>111.75</td>
<td>76.4</td>
<td>5542</td>
<td>422</td>
<td>9.5</td>
<td>74.9</td>
</tr>
<tr>
<td><strong>PCa GS&gt;7</strong></td>
<td>12</td>
<td>Median</td>
<td>ICAM</td>
<td>75.75</td>
<td>929.32</td>
<td>134579</td>
<td>1.94</td>
<td>35.93</td>
<td>16.5</td>
<td>3073</td>
<td>219</td>
<td>5.7</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5%kvantil</td>
<td>VCAM</td>
<td>52.20</td>
<td>731.37</td>
<td>66698</td>
<td>0.60</td>
<td>14.50</td>
<td>3.1</td>
<td>1305.5</td>
<td>76</td>
<td>2.5</td>
<td>36.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95%kvantil</td>
<td>MMP9</td>
<td>147.87</td>
<td>1179.94</td>
<td>224021</td>
<td>15.70</td>
<td>448.01</td>
<td>749.0</td>
<td>4248.4</td>
<td>339</td>
<td>27.7</td>
<td>226.5</td>
</tr>
</tbody>
</table>
Table 19 Results of comparison among groups by Wilcoxon test - benign prostatic hyperplasia (BPH), with prostatic intraepithelial neoplasia (PIN) and patients with prostate cancer (PCa)

<table>
<thead>
<tr>
<th>P value</th>
<th>ICAM</th>
<th>VCAM</th>
<th>MMP9</th>
<th>IL6</th>
<th>VEGF</th>
<th>PSA</th>
<th>IGFBP</th>
<th>IGF1</th>
<th>TK</th>
<th>CHRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPHxPIN</td>
<td>0.0705</td>
<td>0.729</td>
<td>1</td>
<td>0.1626</td>
<td><strong>0.0235</strong></td>
<td><strong>0.0271</strong></td>
<td>0.9854</td>
<td>0.7562</td>
<td><strong>0.0426</strong></td>
<td>0.5426</td>
</tr>
<tr>
<td>BPHxPCa</td>
<td>0.1549</td>
<td>0.2565</td>
<td>0.4447</td>
<td>0.9667</td>
<td>0.4449</td>
<td><strong>0.00001</strong></td>
<td><strong>0.0027</strong></td>
<td><strong>0.0148</strong></td>
<td>0.4105</td>
<td>0.3700</td>
</tr>
<tr>
<td>PINxPCa</td>
<td>0.4047</td>
<td>0.6648</td>
<td>0.6634</td>
<td>0.1252</td>
<td>0.1566</td>
<td>0.1380</td>
<td><strong>0.0492</strong></td>
<td>0.0923</td>
<td>0.0801</td>
<td>0.9653</td>
</tr>
<tr>
<td>Localized PCa x Extended</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&lt;0.02</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>GS&lt;6 PCa x GS&gt;7</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&lt;0.05</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&lt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>

Graph 19 Box plot graphs for PSA, IGF and IGFBP3 (group 0 – BPH, group 1 – PIN, group 2 – PCa)
4.2.3.4 Discussion

Aim of our study was to compare levels of circulating biomarkers in benign prostatic hyperplasia versus prostate cancer, because introduction of novel biomarkers could help in diagnostic procedure. Various biomarkers are either expressed in the same high quantities in both high-grade prostatic intraepithelial neoplasia and carcinoma or are expressed at levels between those of benign prostate tissue and carcinoma. (DeMarzo A.M., 2003). We admit that a patient cohort with PIN in our study is relatively small but the number of patients reflects the reported frequency of PIN between 5% and 25% in prostate biopsies. (DeMarzo A.M., 2003; Man Y.G., 2008), furthermore the classification of biopsy as PIN is dependent strongly on experience of pathologist. Significantly higher levels of PSA but also VEGF and TK in PIN were observed contrary to BHP. Higher levels of TK as a marker of cell proliferation and VEGF as a marker of angiogenesis in circulation reflect the high rate of
tissue remodelling in PIN, if we can gather from the small PIN cohort. In our study, PSA and IGFI levels increasing sequentially in PIN and further in PCa, even if the statistic significant difference was observed only between BPH and PCa for both and between BPH and PIN for PSA. The IGFBP3 levels were significantly lower in PIN than in PCa, it is the only significant difference between PIN and PCa. This could imply that low IGF increase in PIN, which is not connected with increase of IGFBP3, make IGFI more biologically available and so support the changes in the prostate tissue in PIN. Even if IGFI and its binding protein have a lower sensitivity than PSA, it seems, that they could help in PCa dignostics. Further relations should be studied with PSA, while IGBP3 is inactivated by methylation in prostate cancer but also degraded by PSA itself. (Joshua A.M., 2007) Furthermore IGFBPs can protect IGFI from proteolytic degradation and thus enhance the bioavailability of IGFI in local tissues. (Gennigens C., 2006) These net relations propose the necessity to consider the ratios or even more a type of scoring system for determination of IGF, IGFBP and PSA biologic activity – not only their levels – for PCa diagnostics process. Li et al. found that a high IGF-I to IGFBP-3 molar ratio was associated with an increased risk of prostate cancer. Furthermore, high IGF-I was associated with increased risk of prostate cancer among men with less advanced disease at diagnosis. These results lend support to the hypothesis that IGF-I, or the IGF-I to IGFBP-3 molar ratio, is an important risk factor for prostate cancer. (Li L., 2003). IGF, IGFBP and PSA are great candidates for multiplex analysis but unfortunatelly there is no commercially available multiplex panels including all of them. An example of multiplex solution is combination of biomarkers associated with the rate of prostate cancer progression defined by high-density tissue microarrays in a study of Rhodes et al. From fourteen candidate biomarkers, finally ratio of EZH2 (enhancer of zeste homolog 2, a transcriptional repressor) to E-cadherin was significantly associated with recurrence and could identify patients at high risk for recurrence after radical prostatectomy.(Rhodes D.R., 2003)

One of the crucial points but is the fact that serum levels of IGFI are age dependent with peak in puberta and further decline with age, considerable interindividual variation in serum levels of IGFI and IGFBP3. Blood levels of each individual are relatively constant with no apparent diurnal or circadian variation (Gennigens C., 2006) that is why it is not easy to predict the cut off value and probably further reason for relative interpretation system. Observed relative standard deviations of values in BPH patients in our study were for diagnostic potential markers: IGFI 28,31%, IGFBP3 26,14% and PSA 56,94%. In a comparable study with larger patient cohorts, Nam et al. observed the IGF-I and IGFBP-3 levels in patients with PIN was
significantly higher compared with controls. The IGF-I and IGFBP-3 levels in patients with Pca contrary to control group did not significantly differ. (Nam R. K., 2005) Gennigens et al. reviewed that meta-analysis in population studies has demonstrated an approximate 8% increase of serum IGF-I level in PCa patients and elevated serum IGF-I could be observed in men at least 5 years prior to the diagnosis of PCa (Gennigens C., 2006). Opposite this study Chen et al. did not found association between IGF-I levels and the risk of prostate carcinoma but reported that an increase of the IGFBP3 level was associated with a modest decrease risk in PCa. (Chen C., 2005)

The Gleason score is a powerful prognostic indicator with the major shift between score 6 and 7 and does influence the treatment decision. Tumour scoring 7, with a greater probability of extension of the tumour outside the prostate, indicates active management contrary to wait and see strategy in lower grades. (DeMarzo A.M., 2003) Significantly higher levels of MMP9 were observed in our study in a group with Gleason score 7 or higher contrary to lower grade group. This indicates that MMP9 could be used additionally for treatment diversification and noninvasive grade shift monitoring.

VEGF expression is low in normal prostate tissue, but markedly increased in tumor tissues, and has a positive association with microvessel density, tumor stage and grade, and disease specific survival in patients with prostate cancer. The tissue co-expression of VEGF with MMP-2 and MMP-9 further increases the malignant potential of prostate tumors. Serum levels were reported to increase significantly from those seen in BHP, through organ-confined PCa to metastatic disease. (Charlesworth P.J., 2006). Chevalier et al. has proven in vitro that neuroendocrine cells are a significant source of VEGF in prostatic tumors, and that VEGF directly act on prostate cancer cells in vitro and so that VEGF-A may be more than angiogenic in prostate cancer and hence favor progression by affecting tumor cells. (Chevalier S., 2002). We did not found elevated VEGF levels in patients with Gleason score 7 or higher and nor in a group of patients with T classification higher than T2b compare to lower stages patients so we did not support the suggested use of VEGF for pretherapeutic staging of patients. Delongchamps et al. reviewed that results of studies considering correlation between circulating levels of VEGF and PCa are controversial. The discrepant observations could be caused by use of serum or plasma. (Delongchamps N.B., 2006) We have chosen serum for VEGF measurement, while, according to our knowledge, VEGF levels are higher in serum.

PSA, TK and chromogranin A were higher in the group of patients with disease stage higher than T2b. The median values within this group are still under routine cut off values used in
our routine laboratory. Our observations agree with facts reported by Tricoli et al. that chromogranin circulating levels do not accurately distinguish BPH from PCA, but they correlate with tumor stage. In addition, this marker has the capability to detect neuroendocrine cells and thus has the potential to identify androgen-independent disease. Serum chromogranin A levels exhibit a well-documented rise in late-stage disease and demonstrate a wide prevalence range of 32–71%. (Tricoli J.V., 2004)

No changes in IL6 serum levels were found in our study in opposite to studies reporting that IL-6 is elevated in the sera of patients with metastatic PCa and in hormone refractory phenotype and that these levels correlate with tumor burden (Adler H.L., Hoosein N., Twillie D.A., Smith P.C., 2001).

We observed also no changes in adhesive molecules either between diagnostic or prognostic groups. Perabo et al implicate only a limited role of cellular adhesion molecules serum levels in some locally advanced tumors or metastatic disease. (Perabo F., 2001) De Cicco et al. proposed that levels of VCAM should be checked in patients with prostate carcinoma, because distant spread is more likely occured in patients with high levels of VCAM. (De Cicco C., 2008). Because no changes in VCAM or ICAM were found in presented study, it is implied, that on the field of adhesive molecules rather cadherins should be probably studied in future, while soluble N-(neural) cadherin is proposed to be a novel diagnosis/prognostic biomarker showing a correlation with PSA in sera of PCa (De Wever O., 2007).

The results of this study point out that PSA is leading biomarker for diagnostics of prostate cancer. But also other biomarkers can improve diagnostic accuracy and prognosis of patients with prostate cancer in future. From the point of view of multiplex analysis, we proposed that there should be created a novel panel for prostate cancer diagnostic including PSA, members of IGF family, MMP9, chromogranin A, TK and may be other up-coming markers e.g. osteoprotegerin (previous presented study), E-cadherins (De Wever O., 2007), membrane protease hepsin. (Luo J., 2001), spliced form of free PSA, IL4 (Takeshi U., 2005) etc.
5 Conclusions

Presented thesis is focused on methodology of use of multiplex xMAP technology for immunoanalytic measurement of proteins and presentation of three pilot projects with use of xMAP technology for cancer clinical research on the field of potential biomarkers.

Results considering methodology conclusions are listed below:

1. xMAP technology is helpful, because reduces the consumption of biologic material, it is labour and cost-effective

2. Multiplexing requires a global point of view on preanalytical phase
   a. Concentrations of some proteins differ in serum, EDTA plasma or citrate plasma blood derivate
   b. The material should be chosen either as the sample type suitable for the most of the proteins or the sample type suitable for the proteins of the most interest in the study; the blood sample should be consistent throughout the whole study without any doubt.
   c. When planning the multiplex experiment it is very important to think over the expected concentrations of studied proteins. Above all e.g. cytokines are in healthy human blood samples in very low concentration levels. It is necessary to select only some proteins from the commercially available panels and it is important to use the analytical system (kit manufacturer) with the lowest possible detection limits.
   d. In this time, it is not possible to analyse simultaneously proteins differing roughly in concentration levels in one well

3. In comparison of FlowCytomix with xMAP technology for cytokines measurement, xMAP technology appears to us more convenient, mostly because of better detection limits
   a. There are no systematic differences among results obtained by immunoassay multiplex technologies but only differences in detection limits and for some analytes results differences (TNFα) caused probably by use of different antibodies
4. Results obtained by xMAP technology correlate with conventional methods for all tested analytes with Spearman’s coefficients higher than 0.7. The only exclusion was IGFII measurement for which correlation was not so close with coefficient only 0.593.

5. No rough violation in intraassay quality assessment was observed.

6. We consider a monitoring of interassay imprecision as a very problematic task and we would welcome any system of external quality control for multiplex measurement of proteins.

Results from pilot studies reflexing the possibility of use of xMAP technology for oncology biomarker measurement.

7. Ovarian cancer multiplex panel
   a. From multiplexed markers serum levels of CA125, osteopontin and IGF-II differ significantly between group of patients with benign ovarian cysts and ovarian cancer patients.
   b. IGF II and osteopontin should be further studied in detail.
   c. HE4 protein seems to be the most promising single marker.
   d. Larger patient study is necessary for novel biomarker combinations.

8. Bone marker multiplex panel in bone metastasis diagnosis
   a. Most promising from multiplexed markers turned out to be osteoprotegerin, significantly higher in cancer in comparison to healthy group but does not differ between bone metastatic and non-bone metastatic group.
   b. We observed that patients with multiple bone metastases have values above set normal value both for osteoprotegerin and osteopontin, this fact could help in discriminating of such patients according to serum tests.
   c. Lower levels of osteocalcin in cancer group compare to healthy controls.
   d. Only levels of ostase and PIIINP differs significantly between bone metastatic and non-bone metastatic group of patients.
   e. Larger patient study is necessary for novel biomarker combinations, which should includes convention markers of bone diseases in conjunction with osteoprotegerin and osteopontin.
9. Biomarkers in prostate cancer
   f. PSA, IGFI and IGFBP3 serum levels are higher in patients with prostatic carcinoma compare to benign prostatic hyperplasia. These molecules are promising biomarkers and should be in future available in multiplex analysis.
   g. MMP9 is higher in patients with prostate cancer with Gleason score higher than compare to patients with lower Gleason score, and so could be helpful in stratification of prostate carcinoma patients.
   h. Thymidine kinase and chromogranin A levels are higher in prostate carcinoma patients with extended disease.
   i. Larger patient study is necessary for novel biomarker combinations focused on IGF family system in conjunction with PSA, MMP9, thymidine kinase and chromogranin A.

Today the switch from view on multiplex only in area of immunoanalysis or genetics separately to „complex multiplex examination“ is undergoing. Not only measurement of a number of proteins or determination of a number of mutation simultaneously, but as well simultaneous assessment of proteins, mRNA, transcription factors, miRNA molecules and genetic polymorphism or mutations is needed. Nowadays xMAP technology is used for each this category separately, but highlights first methods of simultaneous measurement of mRNA and proteins in the same well, from the same material – without need of prior isolation. Such potential would lead to minimise preanalytic influences and above all falls in extraction and so to standardised comparison of amount of different proteins, their mRNA levels and genetic information status.

The major question of multiplex technologies is their integration into the routine laboratories. xMAP technology seems to be thanks to its universality ideal solution for clinical laboratories of future – as an alternative to vision of centralised laboratories accumulating large number of various instruments, there is a vision of universal technology, which could enable to gain a complex information already in laboratory with preventing of preanalytic and analytic sources of inaccuracy and above all to decrease needed volume of biologic material.

But multiplex is not only a novel analytic philosophy saving time and money, for its complete utilization there is necessary to prepare panels of analytes reflecting the pathophysiologic pathways and diagnostic questions for each clinical unit, which should be used with conjunction of computed evaluation of results, scoring systems or models of neuronal nets. Unfortunately for validation of such complex systems there are required multicentric studies and large patient cohorts.
6 References


De Cicco C., Ravasi L., Zorzino L., Sandri M.T. et al., Circulating levels of VCAM and MMP-2 may help identify patients with more aggressive prostate cancer. Curr Cancer Drug Targets. 8 (3) pp. 199-206, 2008


Delongchamps N.B., Peyromaure M., Dinh-Xuan A.T., Role of vascular endothelial growth factor in prostate cancer, Urology 68(2) pp. 244-8, 2006

Demers L.M., Bone markers in the management of patients with skeletal metastases, Cancer 1;97(3 Suppl) pp. 874-9, 2003

Dunbar S.A., Application of Luminex xMAP™ technology for rapid, high-throughput multiplexed nucleic acid detection, Clinica Chimica Acta 363, 71 – 82, 2006


Fong G.H., Rossant J., Gertsenstein M., Breitman M.L. et al., Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium, Nature 376 (6535) pp. 66-70, 1995


Garofalo C., Surmacz E., Leptin and cancer, J Cell Physiol. 207(1) pp. 12-22, 2006


Gonzáles C., Garcia-Berrocal B., Talaván T. et al., Clinical evaluation of microsphere bead-based flow cytometry assay for the simultaneous determination of anti-thyroid peroxidase and anti-thyroglobulin antibodies, Clinical Biochemistry 38, pp. 966-972, 2005


- http://www.rulesbasedmedicine.com/: Should I Use Serum or Plasma for my MAP study, accessed September 2006
- Chen C., Lewis S.K., Voigt L., Fitzpatrick A. et al., Prostate carcinoma incidence in relation to prediagnostic circulating levels of insulin-like growth factor I, insulin-like growth factor binding protein 3, and insulin, Cancer. 103(1) pp. 76-84, 2005
- Johnson C.C., Kessel B., Riley T.L., Ragard L.R. et al., The epidemiology of CA-125 in women without evidence of ovarian cancer in the Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial. Prostate, Lung, Colorectal and Ovarian Cancer Project Team, Gynecol Oncol 110(3) pp. 383-9, 2008


Kofoed K., Andersen O., Kronborg G., Tvede M. et al., Use of plasma C-reactive protein, procalcitonin, neutrophils, macrophage migration inhibitory factor, soluble urokinase-type plasminogen activator receptor, and soluble triggering receptor expressed on myeloid cells-1 in combination to diagnose infections: a prospective study, Critical Care 11(2), R38, 2007


Lequin R. M., Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA): Clinical Chemistry 51, pp. 2415-2418, 2005


Li L., Yu H., Schumacher F., Casey G. et al., Relation of serum insulin-like growth factor-I (IGF-I) and IGF binding protein-3 to risk of prostate cancer (United States), Cancer Causes Control. 14(8) pp. 721-6, 2003


Lipton A., Ali S.M., Leitzel K., Chinchilli V. et al., Serum osteoprotegerin levels in healthy controls and cancer patients, Clin Cancer Res. 8(7) pp. 2306-10, 2002

Liu M.Y., Xydakis A.M., Hoogeveen R.C., Jones P.H. et al., Multiplexed analysis of biomarkers related to obesity and the metabolic syndrome in human plasma, using the Luminex -100 system, Clin Chem. 51(7):1102-9, 2005


Mathur S.P., Mathur R.S., Gray E.A., Lane D. et al., Serum vascular endothelial growth factor C (VEGF-C) as a specific biomarker for advanced cervical cancer: Relationship to insulin-like growth factor II (IGF-II), IGF binding protein 3 (IGF-BP3) and VEGF-A, Gynecol Oncol 98(3) pp. 467-83, 2005

McGonigle J.S., Giachelli C.M., Scatena M., Osteoprotegerin and RANKL differentially regulate angiogenesis and endothelial cell function, Angiogenesis 2008 Dec 23. [Epub ahead of print]


Menon R., Camargo C., Thorsen P., Lombardi S. et al., Amniotic fluid interleukin –6 increase is an indicator of spontaneous preterm birth in white but not black Americans, American Journal of Obstetrics and Gynecology vol. 198, pp. 77e1-77e7, 2008 (A)


Menon R., Thorsen P., Vogel I., Jacobsson B. et al., Racial disparity in amniotic fluid concentrations of tumor necrosis factor (TNF)-α and soluble TNF receptors in spontaneous preterm birth, American Journal of Obstetrics and Gynecology vol. 198, pp. 533.e1-533.e10, 2008 –Abstract (B)


Mor G., Visintin I., Lai Y., Zhao H. et al., Serum protein markers for early detection of ovarian cancer, Proc Natl Acad Sci USA 102(21) pp. 7677-82, 2005


Narita N., Yuasa T., Tsuchiya N., Kumazawa T. et al., A genetic polymorphism of the osteoprotegerin gene is associated with an increased risk of advanced prostate cancer., BMC Cancer. 6;8 pp. 224, 2008


Pang S., Smith J., Onley D., Reeve J. et al., A comparability study of the emerging protein array platforms with established ELISA procedures: Journal of Immunological Methods 302 , pp. 1 – 12, 2005


Racek J., Klinická biochemie, second revised edition, Prague: Galén, 2005


Sedlaczek P., Frydecka I., Gabrys M., Van Dalen A., Einarsson R., Harłozińska A., et al., Comparative analysis of CA125, tissue polypeptide specific antigen, and soluble interleukin-2 receptor alpha levels in sera, cyst, and ascitic fluids from patients with ovarian carcinoma, Cancer. 95(9) pp.1886-93, 2002

Seibel M.J., Clinical use of markers of bone turnover in metastatic bone disease, Nat Clin Pract Oncol. 2(10) pp. 504-17, 2005

Selvaggi G., Scagliotti G.V., Management of bone metastases in cancer: a review, Crit Rev Oncol Hematol. 56(3) pp. 365-78, 2005


Stewart R.J., Panigrahy D., Flynn E., Folkman J. et al., Vascular endothelial growth factor expression and tumor angiogenesis are regulated by androgens in hormone responsive human prostate carcinoma: evidence for androgen dependent

- Stoll D., Bachmann J., Templin M.F., Joos T.O., Microarray technology: an increasing variety of screening tools for proteomic research, Drug discovery today: Targets 3 (no.1), pp. 24-31, 2004
- Tsigkou A., Marrelli D., Reis F.M., Luisi S. et al., Total inhibin is a potential serum marker for epithelial ovarian cancer, J Clin Endocrinol Metab. 92(7) pp. 2526-31, 2007


Virk M.S., Lieberman J.R., Tumor metastasis to bone, Arthritis Res Ther. 9 Suppl. 1:S5. 2007


Woolas R.P., Conaway M.R., Xu F., Jacobs I.J. et al., Combinations of multiple serum markers are superior to individual assays for discriminating malignant from benign pelvic masses, Gynecol Oncol 59(1) pp. 111-6, 1995


www.proteinatlas.org accessed on 2.8.2008


Yalow R. S., Radioimmunoassay: A Probe for Fine Structure of Biologic Systems - Nobel Prize Lecture, 8 December 1977 accessed at www.nobelprize.org on 2.7.2008


7 Citations of author

7.1 Articles

Article in press:
Test of Ovarian Cancer Multiplex xMAP Technology Panel
J. Vrzalova, M. Prazakova, Z. Novotny, O. Topolcan, M. Casova and L. Holubec
ANTICANCER RESEARCH Volume 29, Issue No. 2, 2009
International Journal of Cancer Research and Treatment
ISSN: 0250-7005

Article in press:
Lower Plasma Levels of Interleukin-6 in a Population Sample with the Symptoms of Depression than in a Population Sample without the Symptoms of Depression
J. Podlipný, Z. Hess, J. Vrzalová, H. Rosolová, J. Beran, B. Petrlová
Manuscript reference: 1695/2008
Physiological Research no 1/2010, volume 58 (online) ISSN 1802-9973

Article in press:
Sperm Antibodies, Intraacrosomal Sperm Proteins, and Cytokines in Semen in Men from Infertile Couples.
Z. Ulcova – Gallova, J.Grubera, J. Vrzalova, K. Bibkova, J. Peknicova,
Z. Micanova, O. Topolcan
Manuscript ID AJRI-09-08-114.R2
American Journal of Reproductive Immunology pISSN: 1046-7408

Article under revision
Effect of hyperinsulinemia and very-low-calorie diet on interstitial cytokine levels in subcutaneous adipose tissue of obese women" by M. Siklova, J. Polak, E. Klimcakova, J. Vrzalova, J. Hejnova, M. Kovacikova, Z. Kovacova, M. Bajzova, L. Rossmeislova, Z. Hnevkovska, D. Langin, and V. Stich,
American Journal of Physiology - Endocrinology and Metabolism pISSN 0193-1849
7.2 Oral presentations

XII International Symposium On Biology and Clinical Usefulness of Tumor Markers.
February 4th – 7th, 2009, Barcelona, Spain

- The test of multiplex panel for ovarian carcinoma. A pilot study, J. Vržalova, O. Topolcan, M. Prazakova, M. Casova, Z. Novotny

XXIX. Imunoanalytické dny - Immunoanalytic days, Špindlerův Mlýn, Czech Republic
11 – 13.5. 2008

- The possible use of multiplex panel for ovarian carcinoma. A pilot study Vržalová J., Topolčan O., Pražáková M., Čásová M., Novotný Z.


- Chromogranin A: Vržalová J., Topolčan O., Holubec L jr, Pražáková M., Pešek M., Kormunda S.

4th International Conference of Postgraduate Medical Students, November 2007, Hradec Králové, Czech Republic

- Assessment of cytokines in human blood serum and plasma by multiplex analysis – technical notes and clinical use: J. Vržalová, tutor: O. Topolčan

XXVIII. Imunoanalytické dny – Immunoanalytic days, April 2007, České Budějovice, Czech Republic

- Sledování sekrece cytokinů in situ v tukové tkáni pomocí mikrodialýzy při hyperinsulinemii a při kontrolních podmínkách - Monitoring of cytokines in situ in fat tissue by microdialysis during hyperinsulinemic status and control conditions: M. Vítková, J. Vržalová, J. Polák, E. Klimčáková, M. Kováčiková, Z. Kováčová, M. Bajzová, O. Topolčan, V. Štich
National student conference of medical research with international participation, November 2006, Plzeň, Czech Republic

6th Cechtuma, May 2006, Prague, Czech Republic
- Multiplex analysis - technical notes and clinical use. Assesment of cytokines in human blood serum and plasma: J. Vrzalová, M. Karliková, O. Topolčan

7.3 Oral presentations – coauthor

14th International Hamburg Symposium on Tumor Markers, 7-9. December 2008, Hamburg, Germany
- The Importance of Monototal for Primary Diagnosis of Patients with Non Small Cell Lung Cancer (NSCLC), O. Topolcan, L. Holubec, J. Safranek, J. Vrzalova, M. Prazakova, M. Casova et. Al

7th Cechtuma 2008, , March 30- April 1, Plzeň, Czech Republic
- HE-4 A New Tumor Marker In Ovarian Cancer (Pilot Study), Z. Novotny, J. Vrzalova, O. Topolcan, L. Holubec, M. Prazakova et al.
- Assessment of Chromogranin A in Lung Cancer Patients, M. Pesek, O. Topolcan, L. Holubec, J. Vrzalova, G. Krakova et al.
- Can Tumor Markers Improve Diagnostic Accuracy in Diifferential Diagnostics of Pleural Effusions?, D. Havel, O. Topolcan, L. Holubec, J. Vrzalova, M. Pesek, M. Casova, M. Prazakova et al.

8th International Conference of Anticancer Research, October 17-22, 2008 Kos, Greece,
- The Importance of Biomarkers in Prostate Cancer Diagnosis, Pilot Study, L. Holubec, J. Vrzalova, J. Klecka, M. Pesta et al.
• HE4 - A New Marker for Ovarian Cancer in Routine?, M. Casova, Z. Novotny, J. Vrzalova, O. Topolcan et al
Abstracts in : ANTICANCER RESEARCH (vol. 28, No 3157-3556, 2008)

XI. KONGRES O ATEROSKLERÓZE – CONFERENCE ON ATEROSCLEROSIS,
December 2007, Špindlerův mlýn, Czech Republic
• Vliv hyperinsulinemie na hladinu adipocytokinů v tukové tkáni sledovaný metodou mikrodialýsy – The effect of hyperinsulinemic status on level of adipokines in fat tissue monitored by microdialysis, Vítková M., Vrzalová J., Polák J., Klimčáková E., Kováčiková M., Kováčová Z., Hejnová J., Bajzová M., Topolčan O., Štich V.

XI. International Symposium on Biology and Clinical Usefulness of Tumor Markers, 21.-24. February 2007, Barcelona, Spain
• Prognostic Factor CA 242 For Colorectal Cancer, O. Topolcan, V. Treska, L. Holubec, J. Vrzalova et al.

7.4 Posters
XII International Symposium On Biology and Clinical Usefulness of Tumor Markers.
February 4th – 7th, 2009, Barcelona, Spain

14th International Hamburg Symposium on Tumor Markers, 7-9. December 2008, Hamburg, Germany

Annual meetings of the American Society of Hematology, December 6-9, 2008, San Francisco, USA
• Lysák D, Jungová A, Vrzalová J, Holubec L, Koza V. Correlation of cytokines levels and adhesion molecules expression with stem cell mobilization efficacy in healthy donors.
8th International Conference of Anticancer Research, 17 - 22 October 2008, Kos, Greece.

- The possible use of multiplex panel for ovarian carcinoma – a pilot study, Vrzalová J., Topolčan O., Pražáková M., Čásová M., Novotný Z.

Abstracts in : ANTICANCER RESEARCH (vol. 28, No 3157-3556, 2008),

The 34th International CLAS Meeting, Coral Springs, USA, 2008


The 11th Central European Lung Cancer Conference, 12-14 June 2008, Ljubljana, Slovenia


XXXII. Brněnské onkologické dny- Days of Oncology in Brno , 17.04. - 19.04.2008 Brno, Czech Republic


The 1st European Lung Cancer Conference, April 2008, Geneva, Switzerland

- The role of Monototal in Relation to Other Cytokeratins for Primary Diagnosis of Patients with Non Small Cell Lung Cancer (NSCLC): Holubec L., Topolcan O., Safranek J., Pražakova M., Vrzalova J., Casova M., Svobodova S, Treska V., Pesek M.

The 35th Meeting of the International Society for Oncodevelopmental Biology and Medicine, ISOBMW 2007, September 2007, Prague

  Abstract in Tumour Biology 28 (suppl 1) 1-144 (2007), ISSN 1010-4283

Planet xMAP Europe, October 2007, Amsterdam, Netherlands


Planet xMAP Europe, October 2006, Amsterdam, Netherlands

- Which blood sample type is adequate?: J. Vrzalová, O. Topolčan
- Adiponectin and leptin – comparison of multiplex and radioimmunoassay methods: J. Vrzalová, O. Topolcan, B. Petrlová, H. Rosolová