New Trends in Laboratory Diagnostics

Tomáš Zima

First Faculty of Medicine
Charles University Prague,
Czech Republic

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Science and Research

- **19th century – own – collect**
  - (museum, collections, e.g. periodical table)
- **20th century – brake**
  - particles
- **21st century – integrate**
  - - omics (proteomics, metabolomics, lipidomics,..)
  - Master
  - System science

New lab diagnostics trends in 21st century

- ✓ lab automation, robotics
- ✓ consolidation
- ✓ integrated IT network organization
- ✓ lab accreditation
- ✓ molecular diagnostics
  - DNA microarray – chips
  - Proteomics
  - Farmacogenetics
- ✓ POCT
- ✓ non-invasive testing
- ✓ image analysis
- ✓ mass spectrometry
- ✓ pacient's ID – el. requisition, barcoding
- ✓ homework - telecommunication

Main topics

- Proteomics
- Pharmacogenomics
- Circulating tumour cells
- Molecular cytogenetics
- Future trends, technologies and labs

Impact of Proteomics on Human Health

Proteomic as diagnostic tool:

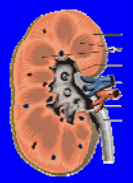
- Proteomics of disease
  - plasma, urine, CSF
- Proteomics for pathogen
- ID proteomics
  - cancer, stem cells..
- Drug impact
  - metabolic, disease progression
- Diagnostic Protein Chip
  - modification chip

PROTEOMICS

- There are the large scale studies of proteins
  - particularly their structure and functions
- The proteome is complex
  - it varies from cell to cell, and is constantly changing through its biochemical interactions with the genome and the environment
- The study of proteomics can lead to a better understanding of the disease process
- To catalog all human proteins is a major challenge for scientists
  - there is an international collaboration to achieve this goal that is being coordinated by the Human Proteome Organization

KEY TECHNOLOGIES used in PROTEOMICS

- One and two dimensional electrophoresis
- X-ray crystallography and magnetic resonance
- Mass spectrometry
  - tandem mass spectrometry
- Affinity chromatography
- X-ray tomography
- Software based image analysis



extraction of protein

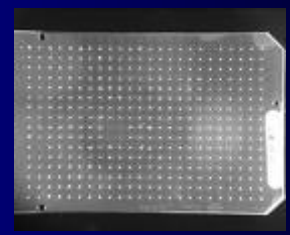
2D PAGE

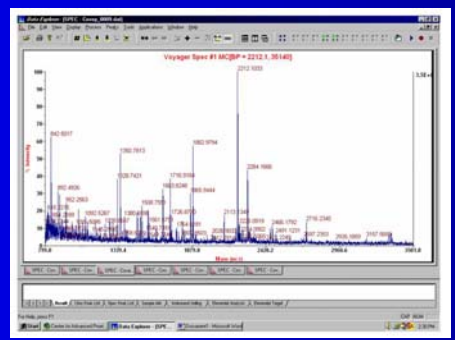
separation of protein

trypsin digestion

- NEW HYPOTHESIS
- NORMAL PHYSIOLOGY
- PATOLOGICAL PHYSIOLOGY
- BIOMARKERS
- DRUGS

Interpretation of results





Mass spectrometry / identification of protein

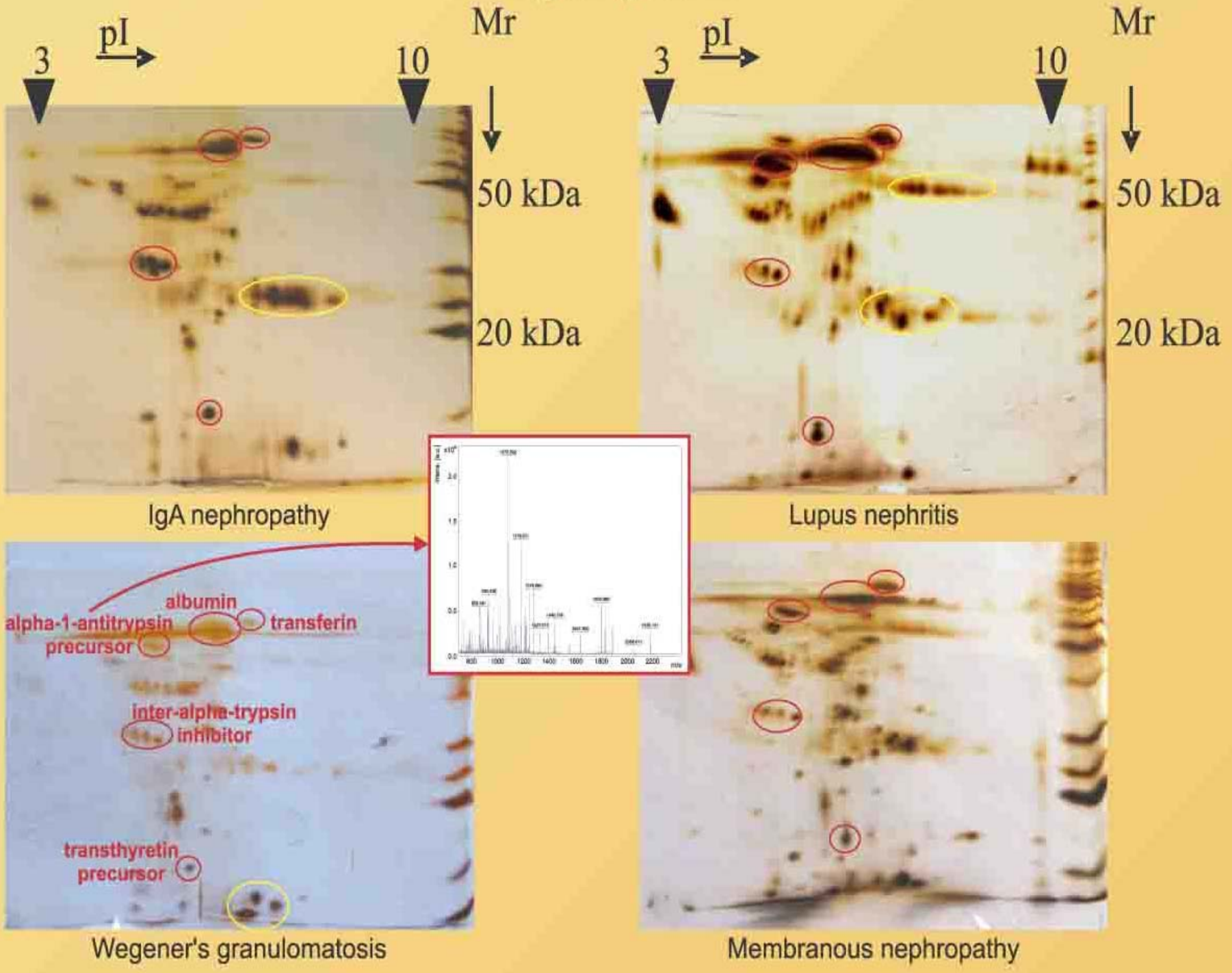
bioinformatics

Purposes of study

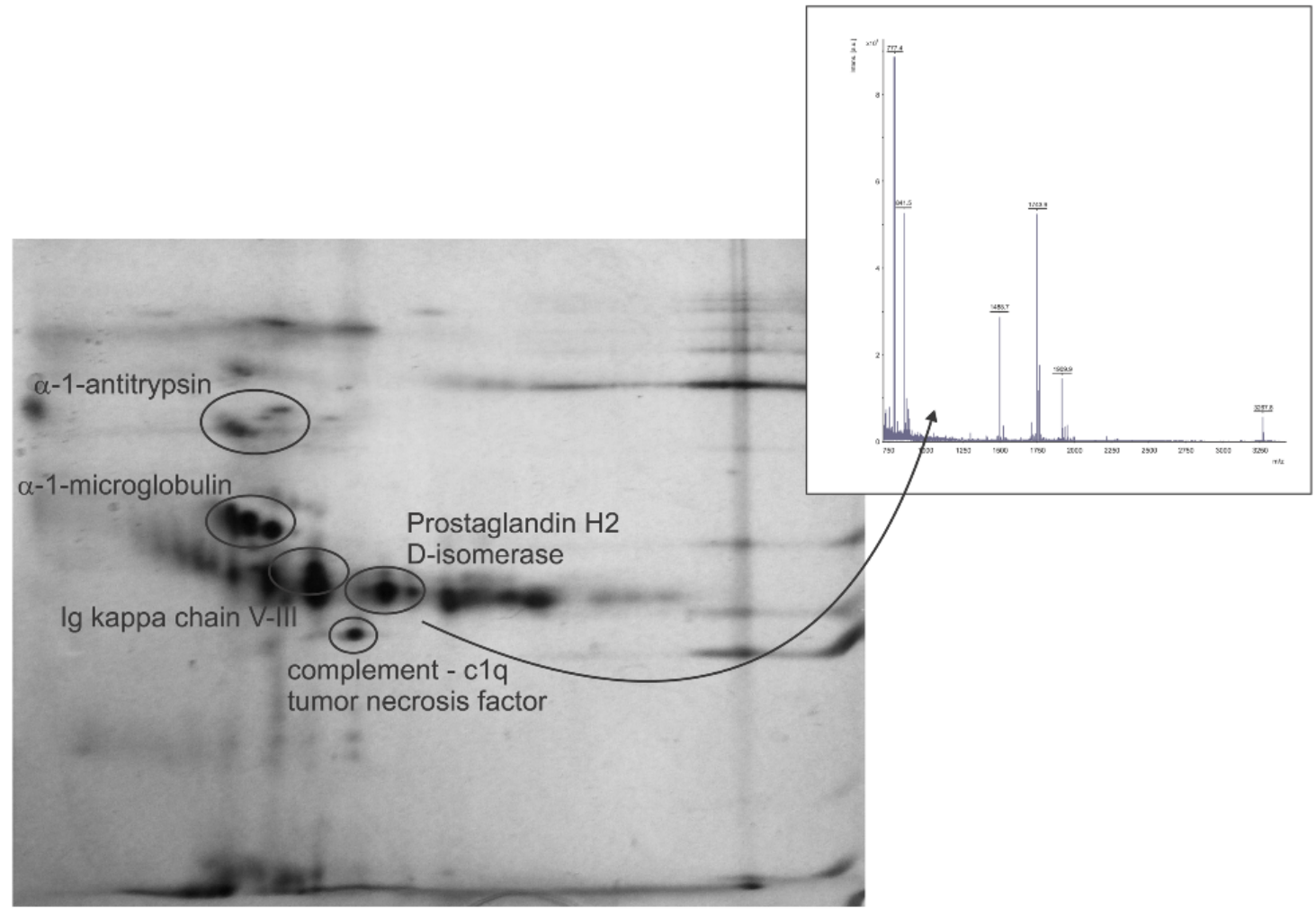
- ✓ Study of proteins' changes in terms of diagnosis, monitoring of medication and determination of disease prognosis using proteomic techniques
- ✓ 2D technique optimization for the analysis of the proteins
  - ✓ albumin's separation method
  - ✓ effect of proteases in the urine
- ✓ Identification of separated proteins using MALDI-TOF MS as a possible biomarkers of the disease

Methods

- **Disease groups:**
  - Idiopatic membranous nephropathy, Wegener´s granulomatosis, Amyloidoses AL, Lupus erythematodes, Focal segmental glomerulosclerosis, Anderson-Fabry disease
- **Group division:**
  - proteinuria: < 1 g/ 24h, 1 – 5 g/ 24h, > 5 g/ 24h
  - renal function: < 150 µmol/L, 150 – 300 µmol/L, > 300 µmol/L
- **Sample preparation:**
  - 24h urine collection
  - proteins were visualized by silver (Silver Bullit Kit, Amresco)
  - proteins´spectrum were analyzed using Phoretix 2D expression software


pI
Mr
3
10
50 kDa
20 kDa
IgA nephropathy
Lupus nephritis
albumin
alpha-1-antitrypsin precursor
transferin
inter-alpha-trypsin inhibitor
transthyretin precursor
Wegener's granulomatosis
Membranous nephropathy

2D electrophoreogram of Anderson-Fabry disease proteome



Changes of selected proteins

No	**Protein**	Fabry disease (average ± SD %)[a,]	Expected MW	Expected pI
1	prostaglandin H2 D-isomerase	398 ± 25*	28 kDa	6.1
2	complement – c1q tumor necrosis factor-related protein	289 ± 17*	23 kDa	5.8
3	Ig kappa chain	591 ± 7*	27 kDa	5.5

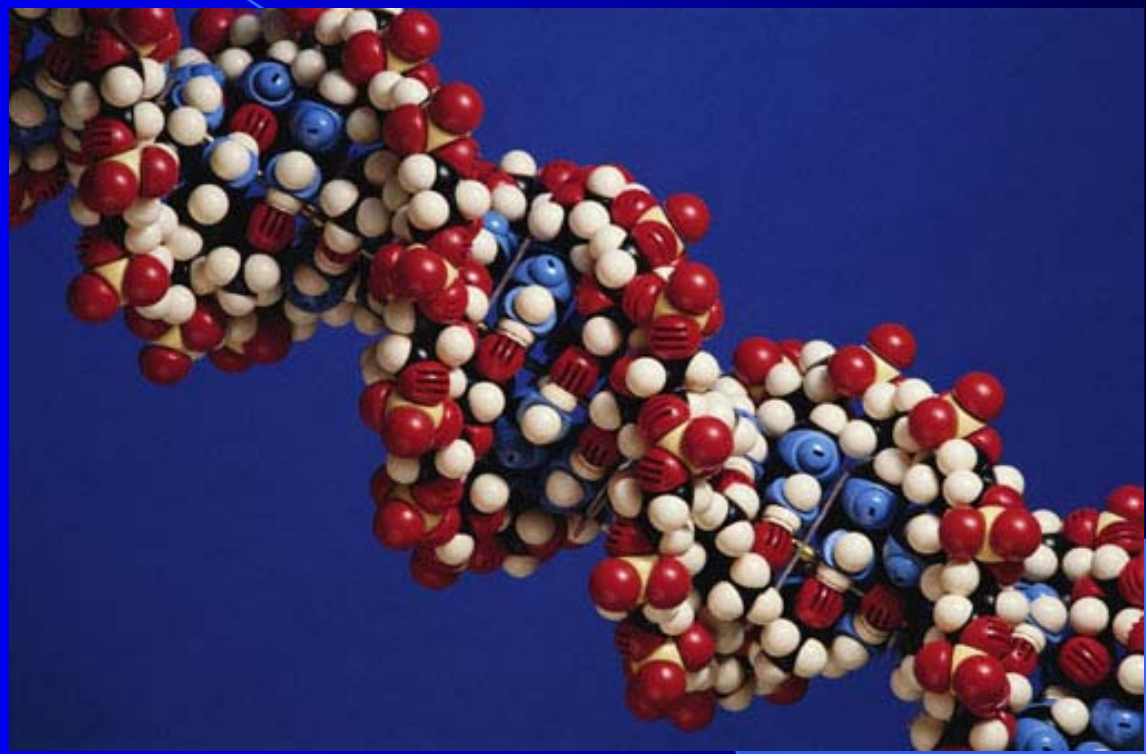
P < 0.05 vs. Healthy control

a) N = 20: [(amount of protein in sample AFD / amount of protein in sample healthy control) x 100]

Conclusion

- implementation and 2D technique optimization
- AFD patients revealed no significant differences in protein composition as compared to control individuals
- quantity of several proteins in AFD was substantially different
- modification of H2 D-isomerase

MOLECULAR DIAGNOSTICS



Main topics

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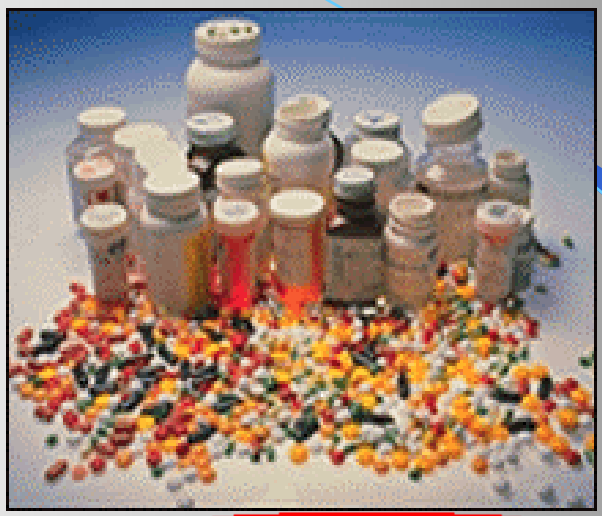
Traditional dosage approach: One size fits all

PHARMACOGENOMICS: THE LATEST!

- Pharmacogenetic tests can predict whether a drug will be effective or cause adverse, or even deadly side effects
- Tests are especially applied to psychiatric and cardiac drugs
- Approximately 70 % drugs have been identified that are catabolized by cytochrome P450 enzymes
  - More than 50 variations are known of the 2D6 gene that controls these enzymes
- 30% of persons of North African origin, 20% of persons of Middle East origin and 2% of Caucasians are born with 3 or more copies of the 2D6 gene causing extra rapid catabolism of certain drugs

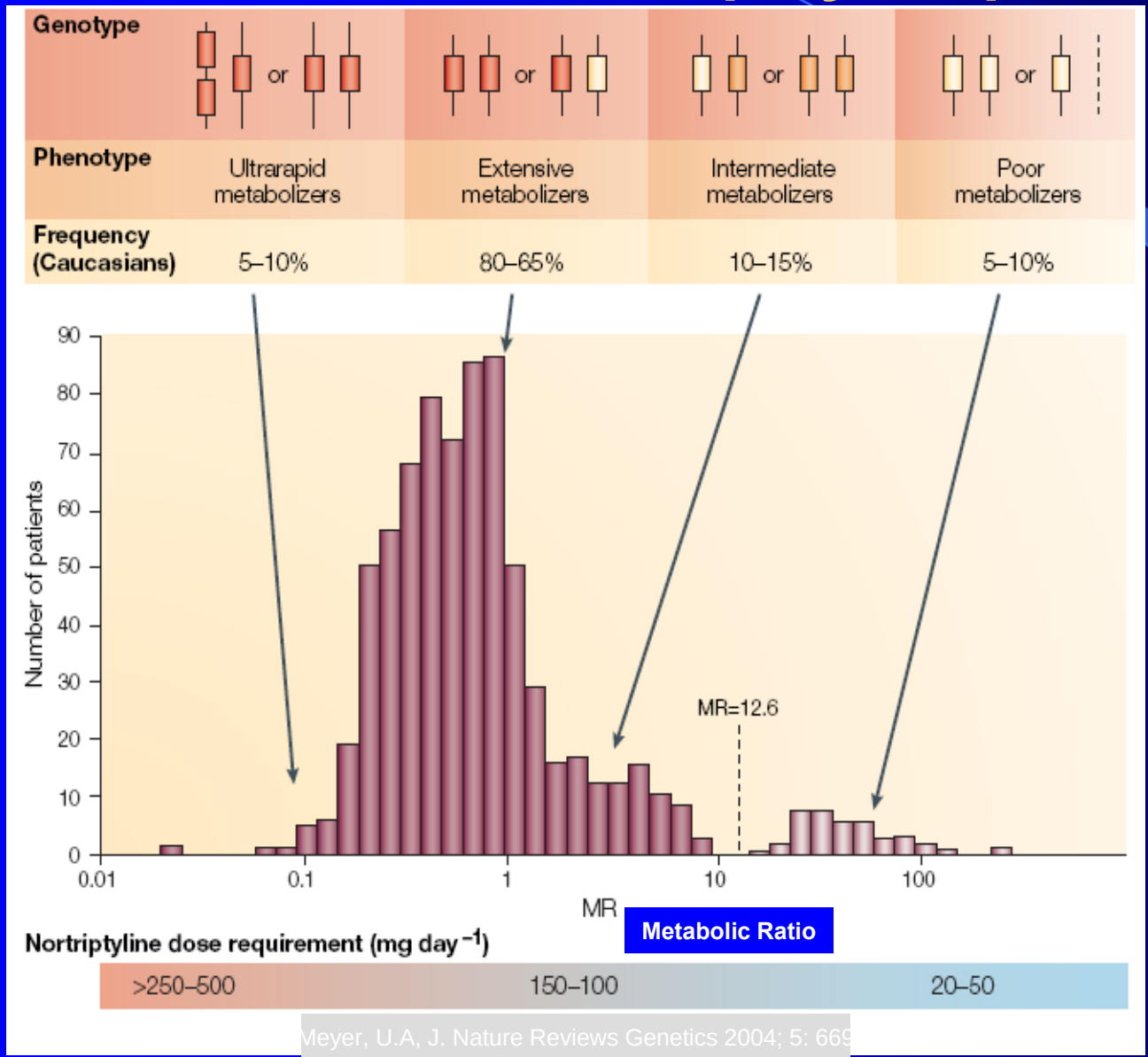
Cytochrome P-450 & Drug Metabolism



4 Types of Metabolizers (I)

- **Ultrarapid metabolizers (UM)**
  Carry multiple copies (3-13) of functional alleles and produce excess enzymatic activity
- **Extensive Metabolizers (EM)**
  Possess at least one normal functional allele
- **Intermediate metabolizers (IM)**
  Possess one reduced activity allele and one null allele
- **Poor metabolizers (PM)**
  Carry two mutant alleles which result in complete loss of enzyme activity

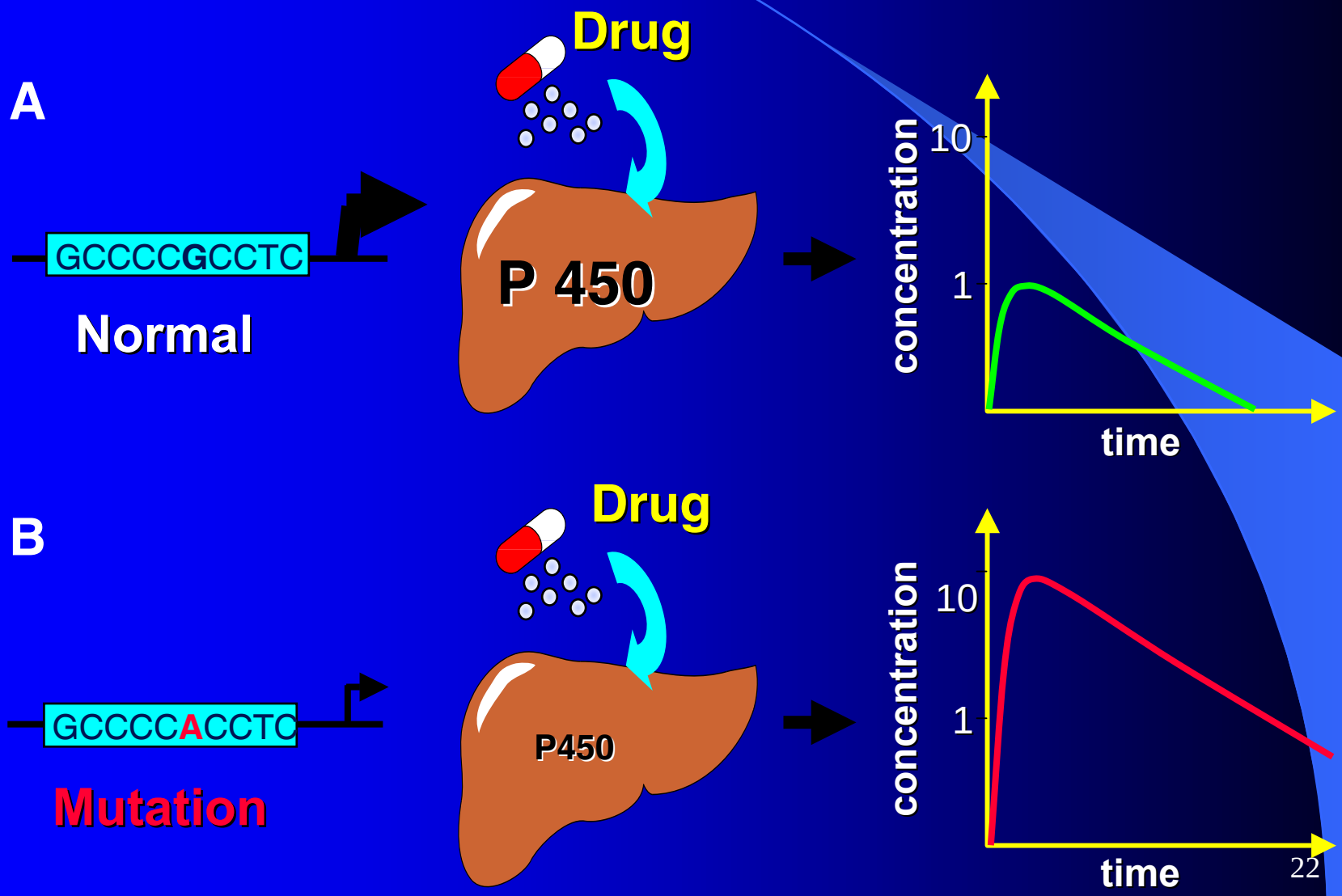
CYP2D6 – Genotypes – phenotype relationships of CYP2D6 polymorphisms



Meyer, U.A, J. Nature Reviews Genetics 2004; 5: 669

Genetically conditioned variability of drug metabolism

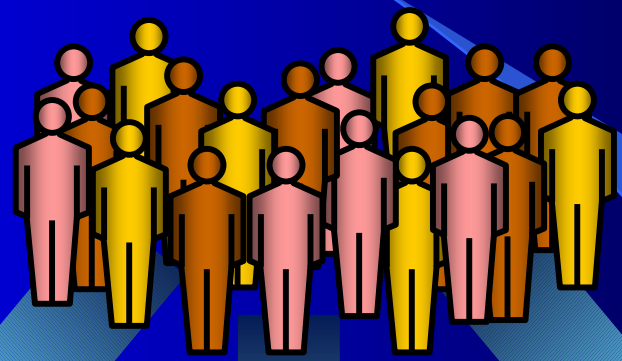
The same dose ➔ the different concentration in plasma



Optimizing Treatment …

Responder identification – better drug efficacy

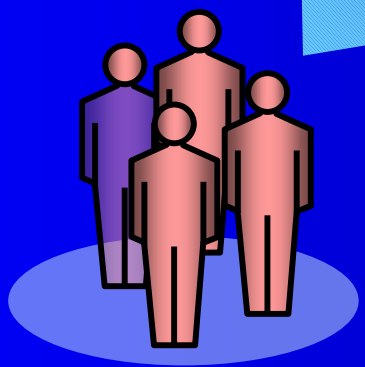
Optimal treatment



Not treated

No efficacy, consider alternatives,

save $



No response

Responders

Not treated

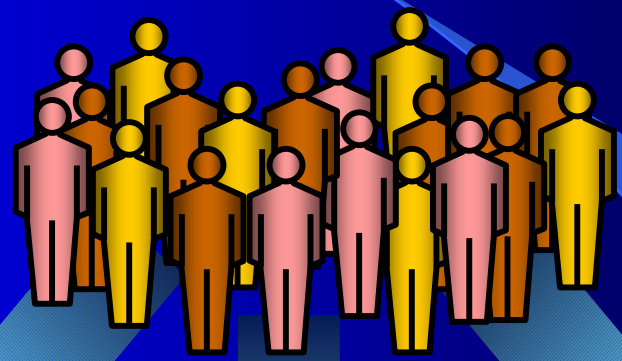
Avoid / manage ADR, save $

Adverse Drug Events

Optimizing Treatment …

Dosage alteration for better patient outcomes

Optimal treatment



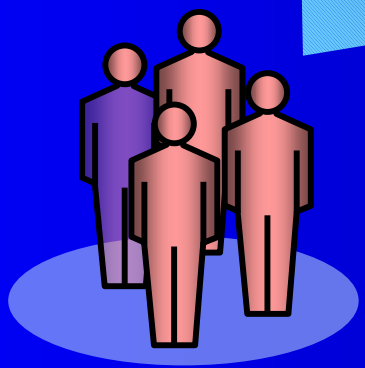
Treated with lower dose

Avoid ADR, possible cure, save $

Not treated

No efficacy, consider alternatives,

save $



No response

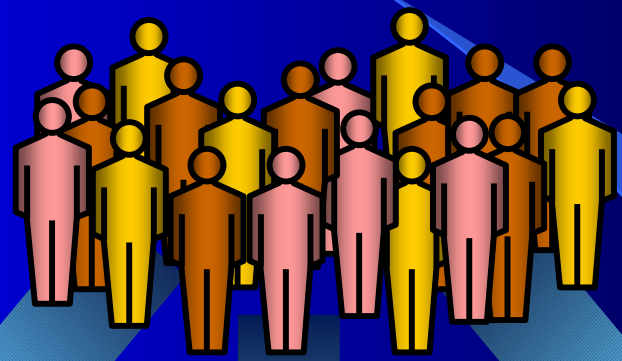
Responders

Adverse Drug Events

Optimizing Treatment …

Dosage alteration for better patient outcomes

Optimal treatment



Treated with higher dosage taking into consideration the increased drug metabolism

Possible cure

No response

Responders

Treated with lower dose

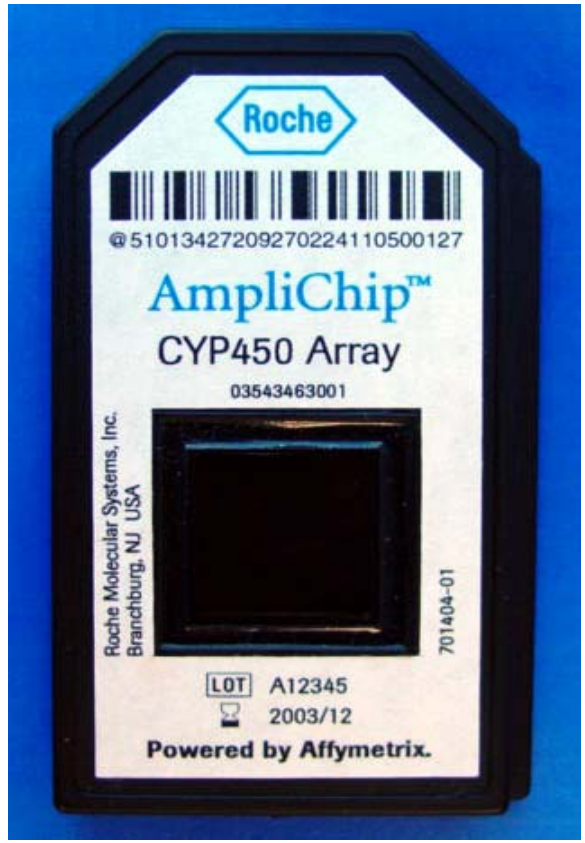
Avoid ADR, possible cure, save £

Adverse Drug Events

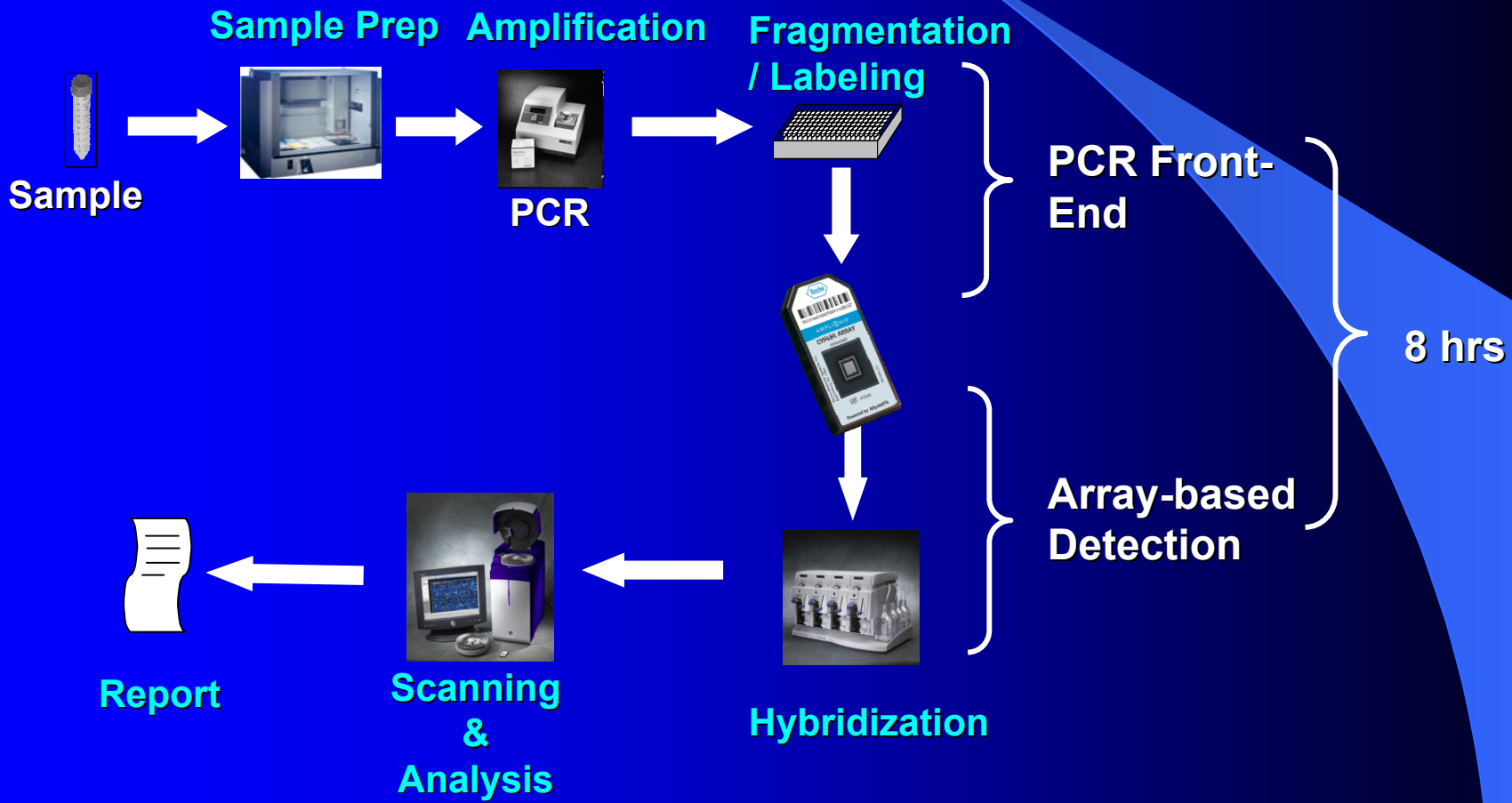
Example of pharmacogenomics

AmpliChip CYP450 Array
is a combination of PCR and microarray technologies

- **To address the relevant genetic variations, each array contains over 10,000 different probes complementary to sense and anti-sense p450 genomic DNA**
  - 2004 (October) - AmpliChip® CYP 450 CE IVD certified launched
  - 2005 (January) - AmpliChip® CYP 450 FDA approved



AmpliChip CYP450 Test Work Flow



Routine PGx testing

- **Methodology**
  - **Genotype**
    - Microarray – Amplichip (CYP2D6, CYP2C19)
    - FRET-PCR (Förster Resonance Energy Transfer)
    - Multiplex PCR- hybridization
    - PCR-RFLP

PGx testing

- **TPMT - azathioprine**
  - Currently 350 pts. with IBD enrolled
  - Phenotype-genotype correlation
  - Phenotype-based individualized dosing
  - Finalization expected in 2009-2010
  - Preliminary results: serious myelotoxicity of azathioprine due to TPMT deficiency

Slanar O et al. Nucleosides Nucleotides Nucleic Acids. 2008 Jun;27(6):835-8.
Slanar O et al. Nucleosides Nucleotides Nucleic Acids. 2008 Jun;27(6):661-5.
Frohman EM et al. Mult Scler. 2006 Feb;12(1):108-11.

PGx testing

- **CYP2D6 – tramadol**
  - **patients**
    - Currently 250 pts after knee laparoscopy
    - Endpoint: analgesic efficacy vs. CYP2D6 genotype
    - Finalization expected in 2010-2011
  - **healthy volunteers**
    - PK + PD (drug-induced miosis), PK/PD
    - Results: CYP2D6 genotype-specific PK and urinary excretion of tramadol and O-demethyltramadol

Slanar O et al. Eur J Clin Pharmacol. 2007 Apr;63(4):419-21.
Slanar O et al. Physiol Res. 2007;56(1):129-36.
Slanar O et al. Eur J Clin Pharmacol. 2006 Jan;62(1):75-6

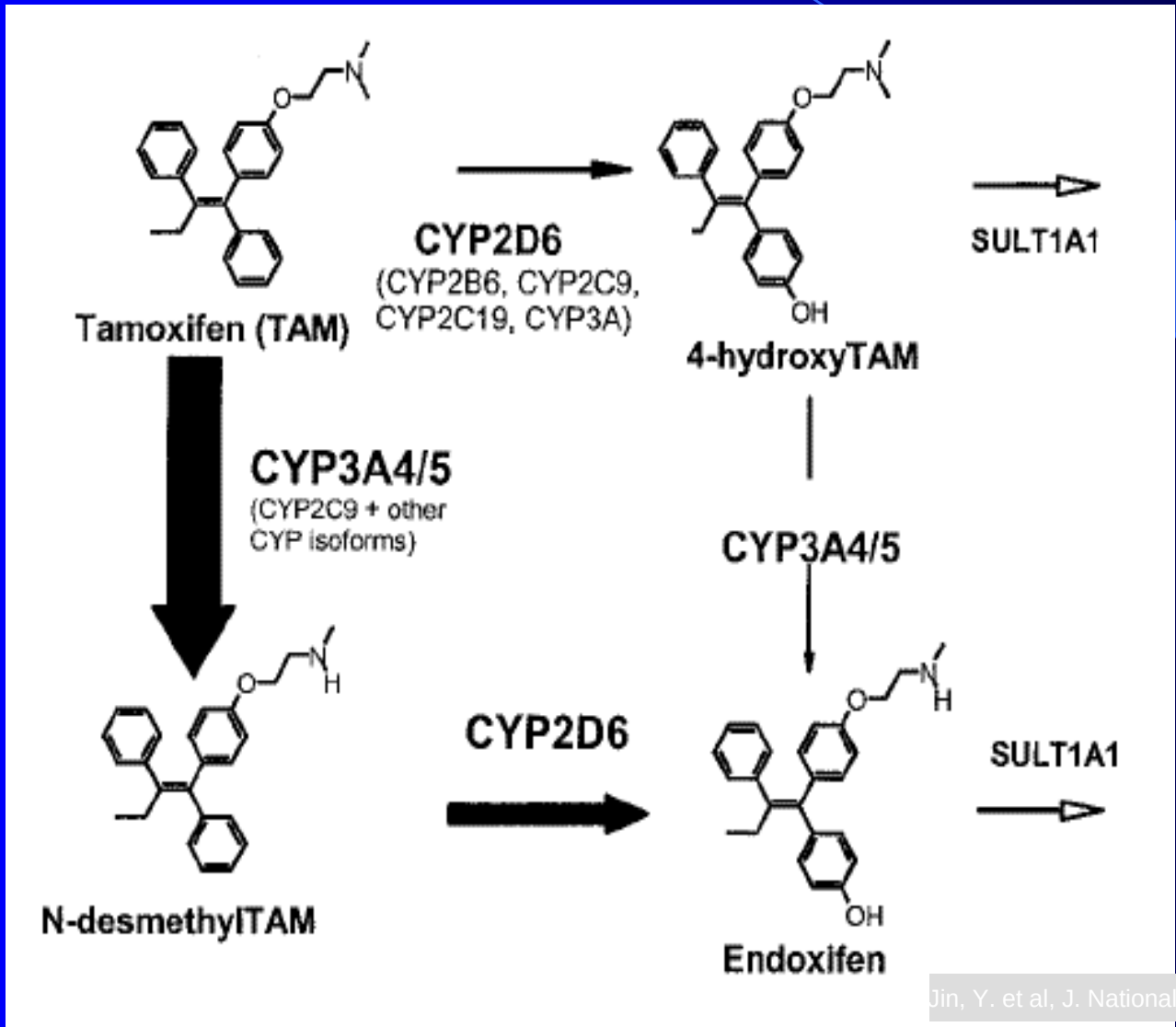
PGx testing

- **MDR1 (P-glycoprotein) – opiates**
  - Genotype + expression
  - Transplacental drug permeation in vivo
  - PK in mother + penetration to the infant's circulation, PD effects
  - Study population: 200 patients
  - Finalization expected in 2010

Breast Cancer treatment

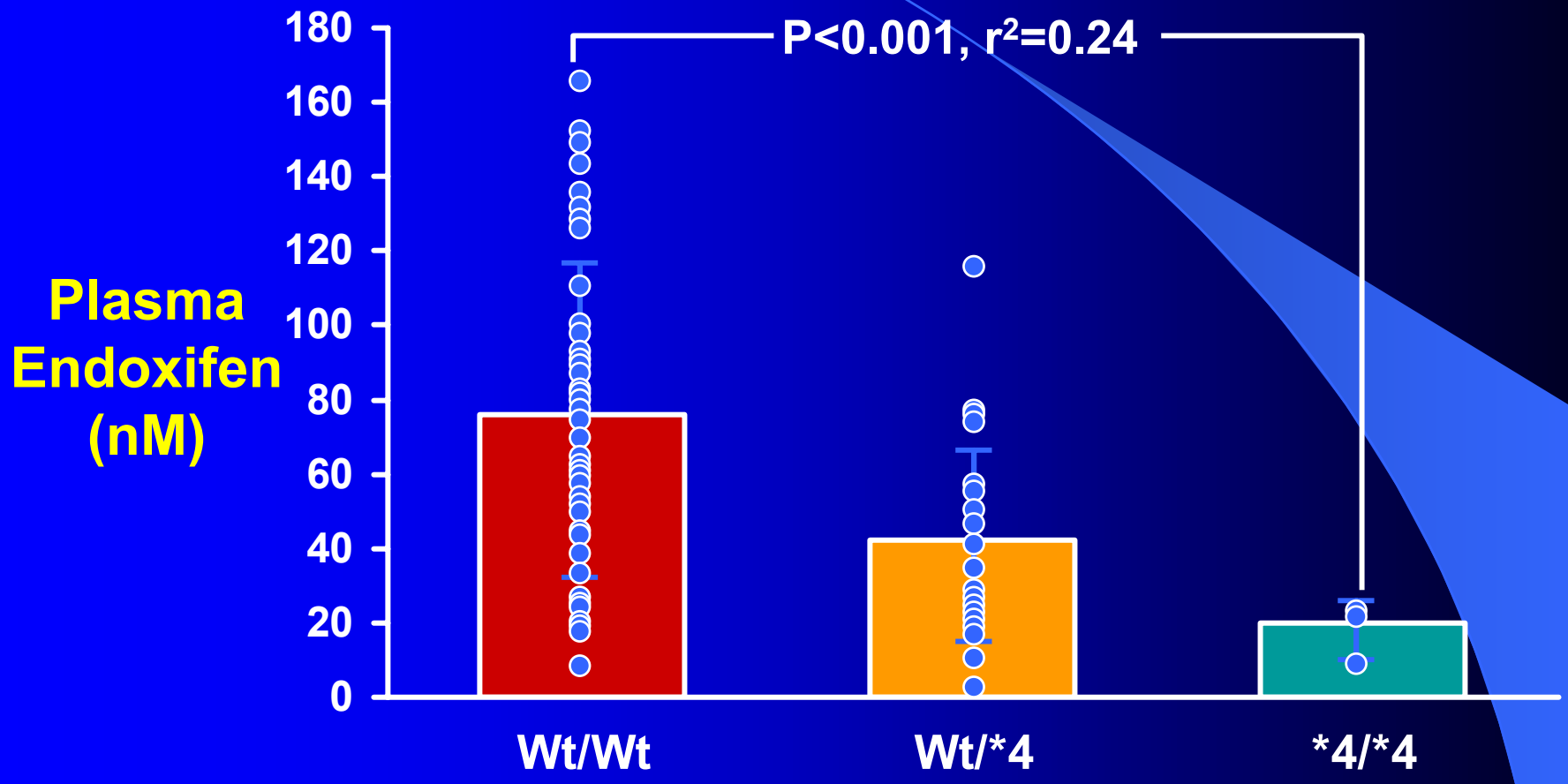
- **Tamoxifen in pre- and post-menopausal women**
- Blocks estrogen receptors and controls tumor growth
- (Activating enzymes: **CYP 2D6**, CYP3A5, CYP2C9, CYP2C19, SULT1A)

Tamoxifen – Biotransformation of tamoxifen and its metabolites



Jin, Y. et al, J. National Cancer Institute 2005; 97: 30-39

CYP2D6 Genotype and Endoxifen Plasma Levels



CYP2D6*4 (most common genetic variant associated with the CYP2D6 poor metabolizer state)

Jin Y et al., Journal of National Cancer Institute **97**:30, 2005

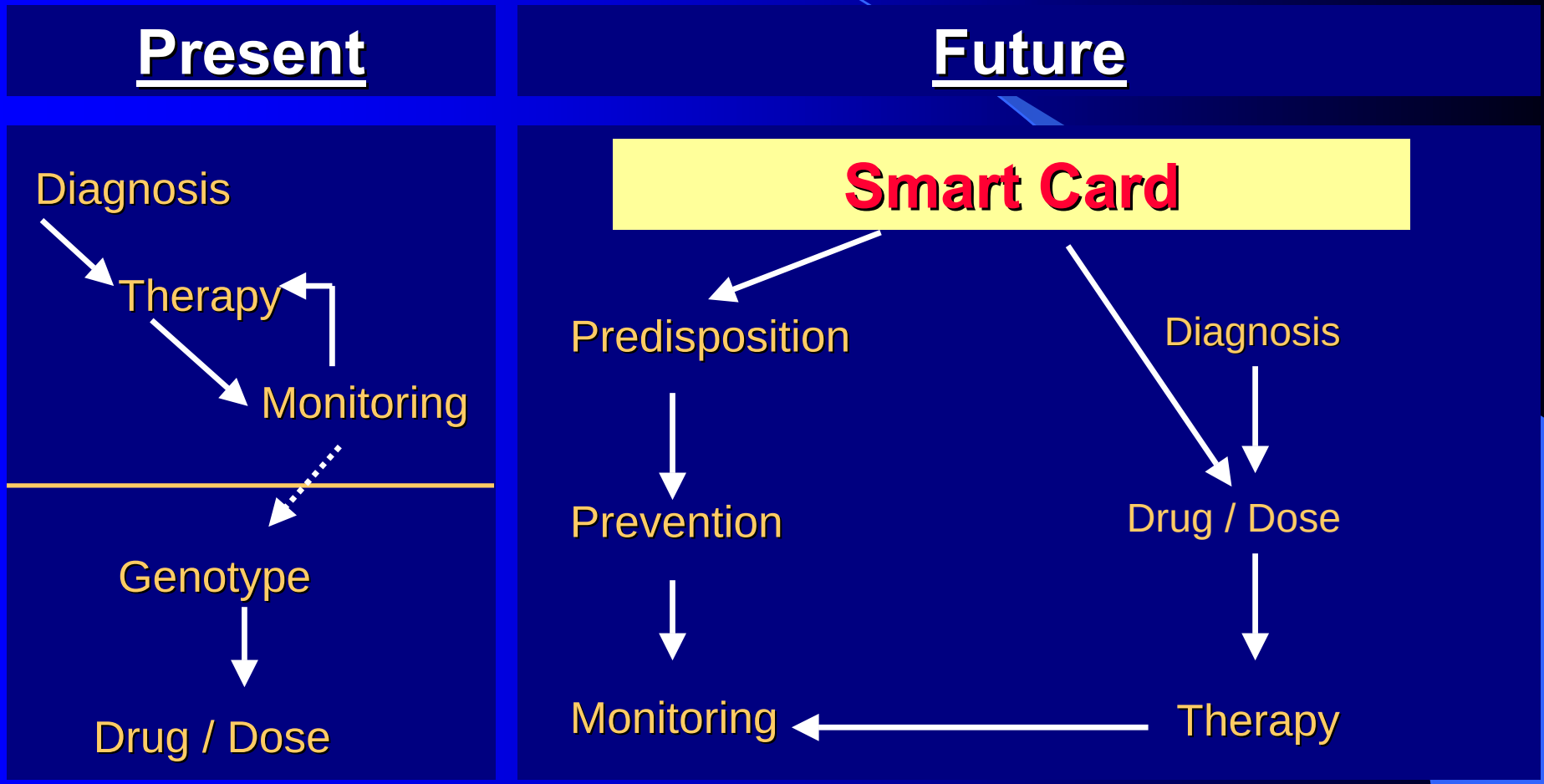
PGx testing

- **CYP2D6 – tamoxifen**
  - 160 pts. with non-metastatic breast cancer
  - Follow up 5 years
  - Primary endpoint disease progression
  - Finalization expected in 2010
  - Preliminary results (n=84)
    - CYP2D6 wild-type genotype – progression rate: 18%
    - CYP2D6 – intermediate or poor metabolzers - - progression rate: 30%

Conclusions

- Group relatively small up to now – however there is a trend towards statistical significance
- **Deficiency of CYP 2D6 activity probably increases risk of relaps of breast cancer during Tamoxifen therapy**
- **Diagnostics of CYP 2D6 genotype before breast cancer treatment could improve the decision making process between Tamoxifen and Aromatase inhibitors**

Pharmacogenomics



according to diagnosis

according to the patient predisposition

Far future?

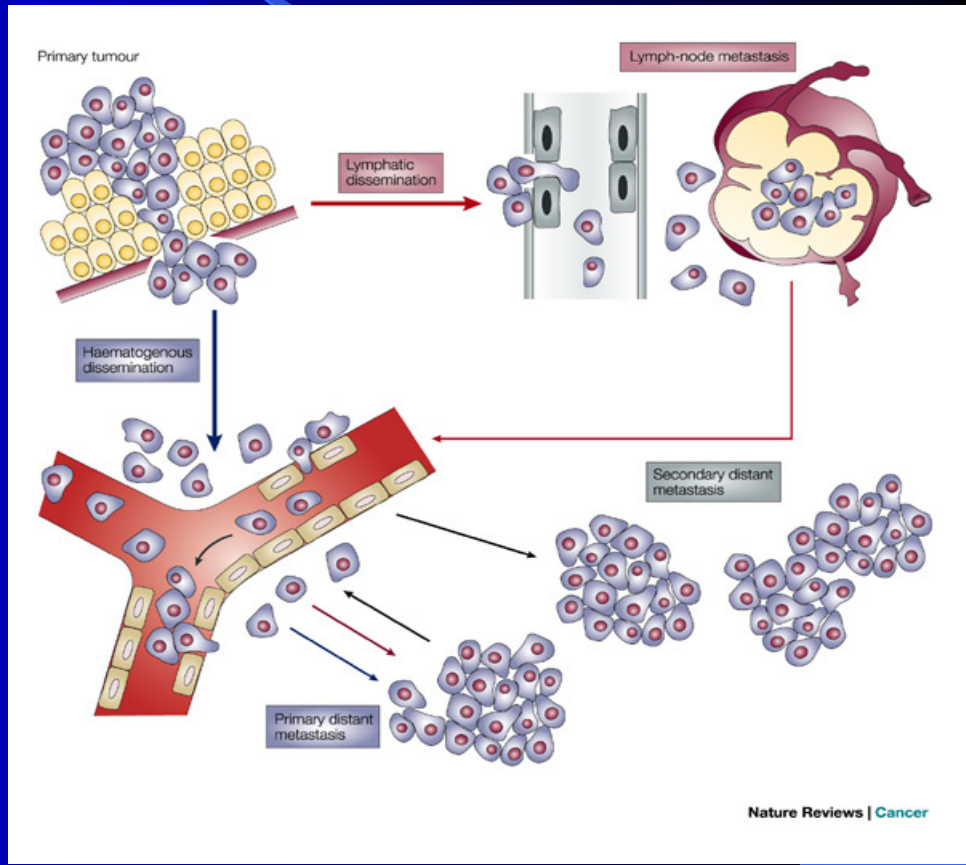
“Here is my Sequence”

Main topics

- Proteomics
- Pharmacogenomics
- Circulating tumour cells
- Molecular cytogenetics
- Future trends, technologies and labs

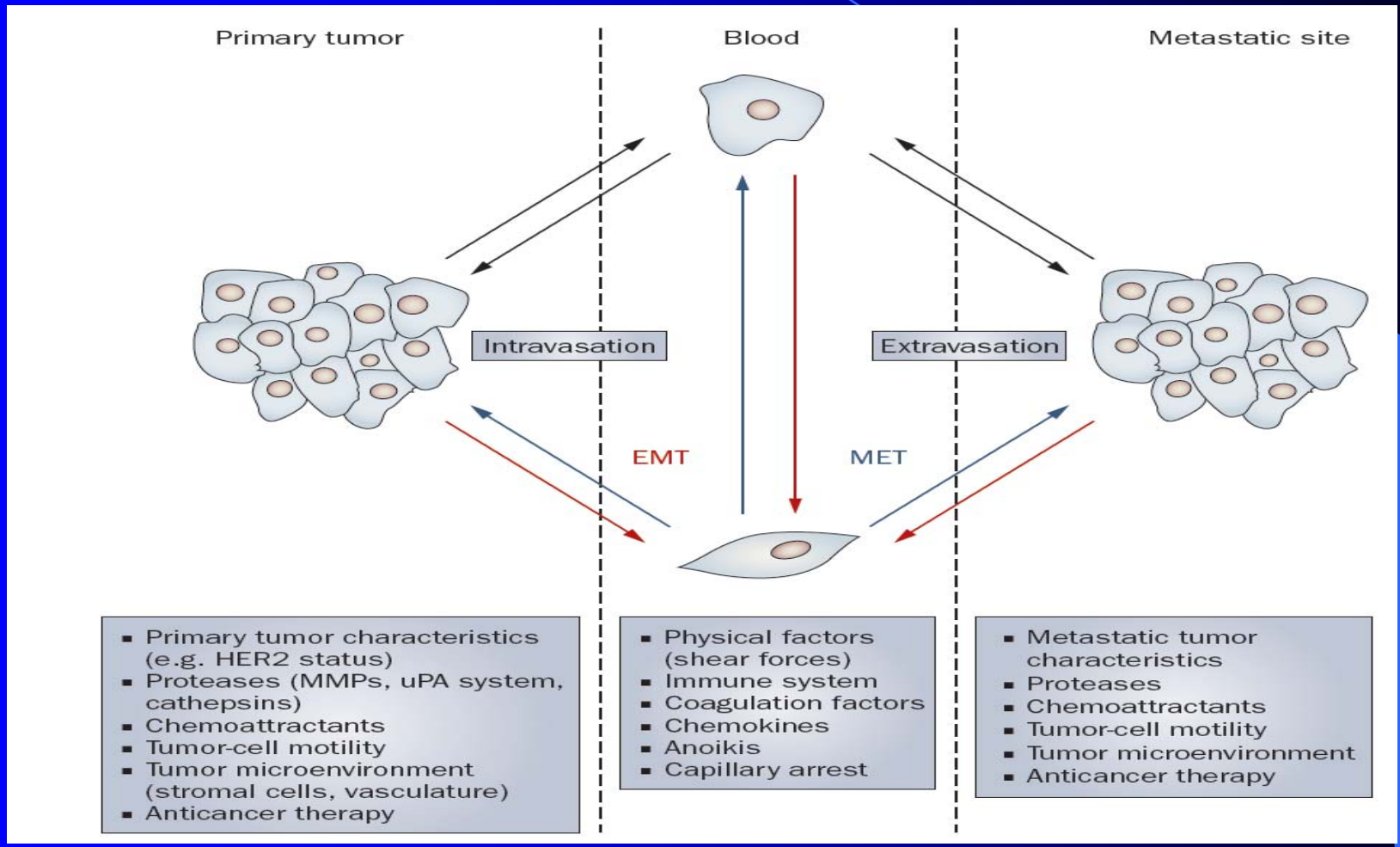
Crucial role of CTCs

- **in the metastatic cascade**
  - tumor cells must invade the basement membrane and surrounding tissue and enter the bloodstream or lymphatics
- **tumor dissemination**
  - changes in cell-to-cell adhesion and extracellular matrix (ECM) adhesion
    - switch in cadherin expression (E-cadherin, N-cadherin)
  - degradation of the ECM
    - MMPs, uPA system (poor prognosis)
- **tumor progression**
  - epithelial-mesenchymal transition (EMT)
  - process of „self-seeding“



Klaus Pantel & Ruud H. Brakenhoff
Nature Reviews Cancer 4, 448-456 (June 2004)

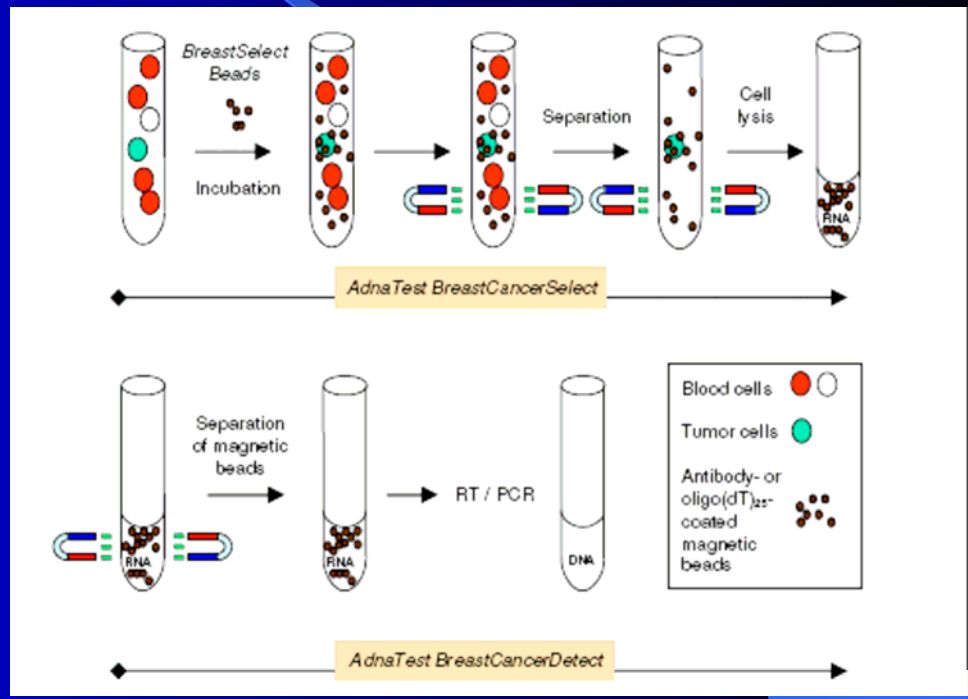
Factors affecting CTCs count



Mego M:*Nature Reviews Clinical Oncology* **7**, 693-701 (December 2010).

AdnaGen BreastCancer system (Langenhagen, Germany)

- BreastCancer Select test
  - enables the immunomagnetic enrichment of tumor cells via epithelial and tumor associated antigens (EpCAM, MUC1)
    - antibodies against epithelial and tumor associated antigens are conjugated to magnetic beads (Dynabeads) for the labeling of tumor cells in peripheral blood
    - the labeled cells are extracted by a magnetic particle concentrator and are subsequently lysed



AdnaGen BreastCancer system (Langenhagen, Germany)

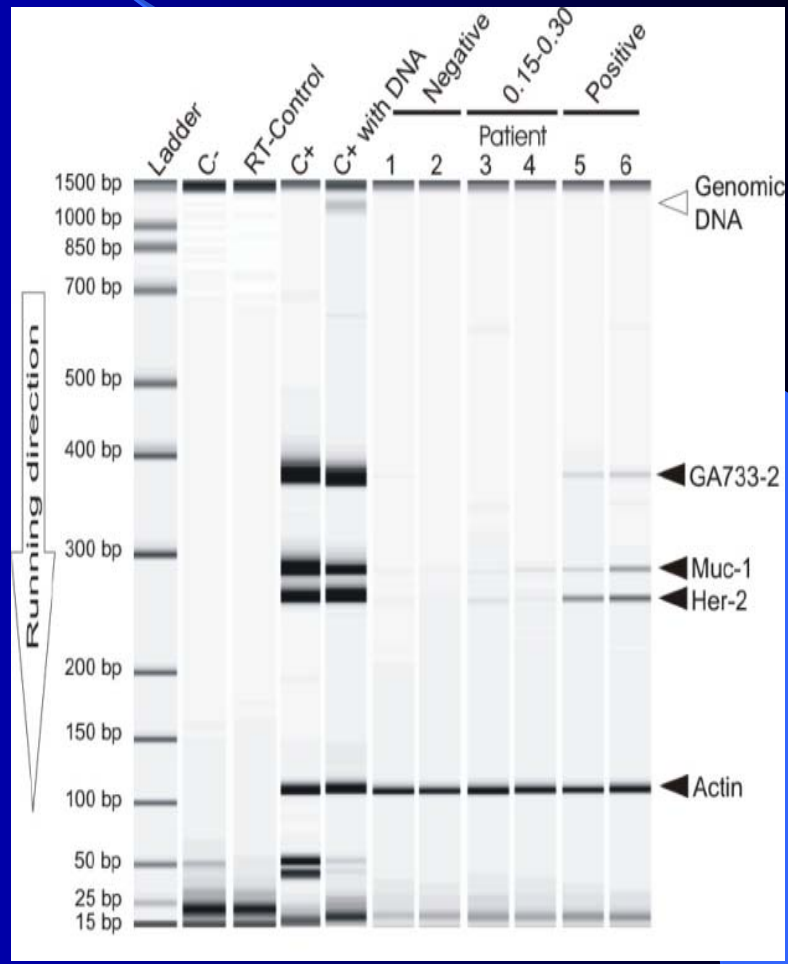
- BreastCancer Detect test
  - contains oligo (dT)25-coated beads for the isolation of mRNA from the lysate of pre-enriched tumor cells.
  - reverse transcription results in cDNA, which is the template for tumor cell detection and characterization by multiplex-PCR.
  - with the *PrimerMix BreastDetect* three tumor associated antigens and one control gene are amplified. The primers generate fragments of the following sizes:

    GA733-2 : 395 bp

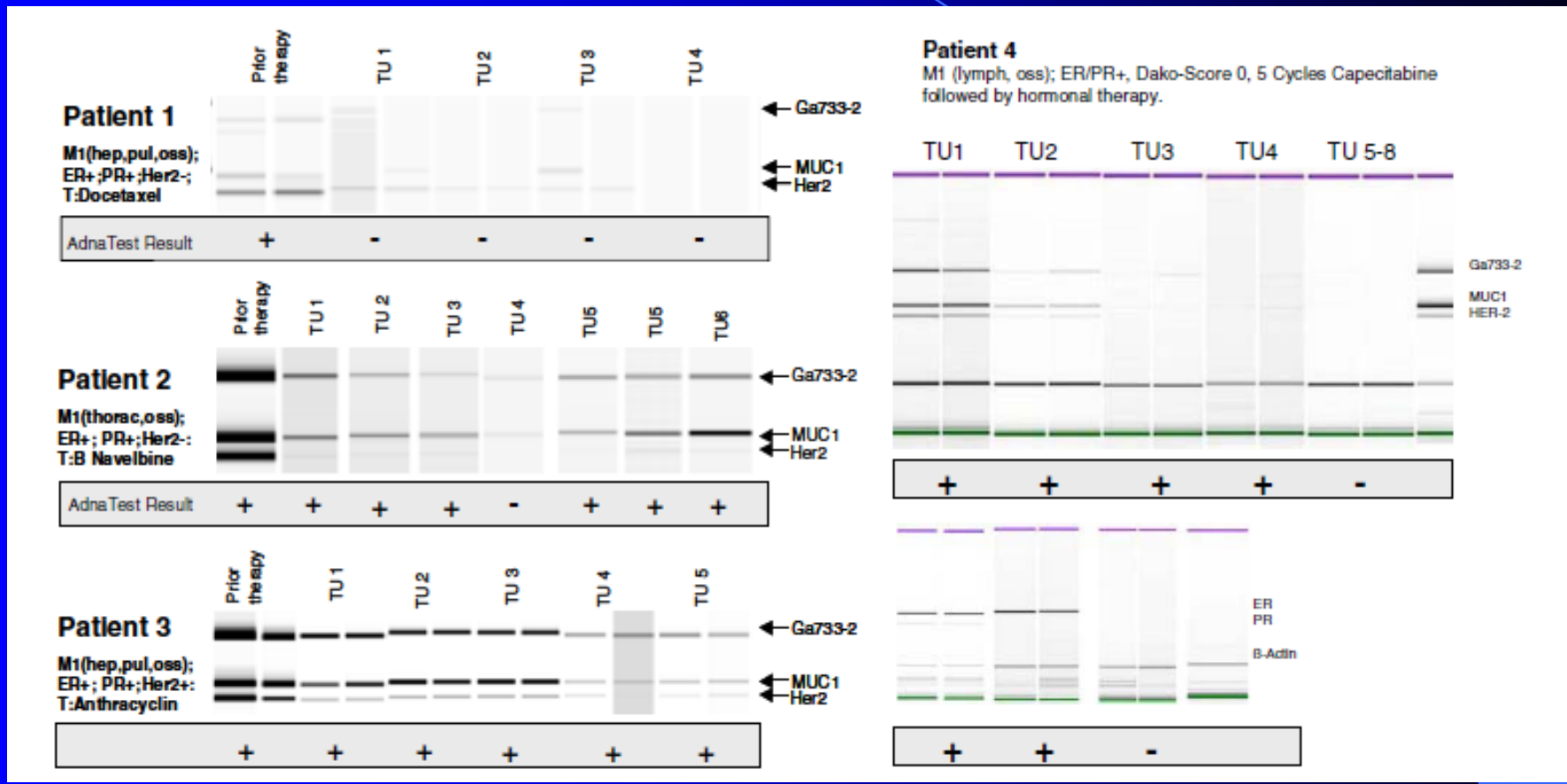
    Muc-1 : 293 bp

    Her-2 : 270 bp

    Actin : 114 bp (internal PCR control).



Monitoring of palliative therapy of breast cancer patients using Adnagen platform. Before each therapy unit (TU) blood samples were analyzer in duplicates.



Breast Cancer Res Treat (2009) 115:581–590

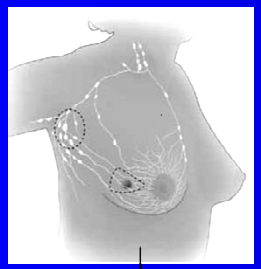
CTC positivity rate

The CTC positivity has been described in 33 % in patients with an early breast cancer (M0) undergoing adjuvant chemotherapy.

In the metastatic patients the CTCs have been described in 43% of patients at least in one sampling. After treatment (CHT, RT) the positivity rate decreased to the 12%.

In the group of neoadjuvant patients 35% samples have been positive before therapy, after 2 CHT- cycles only 5% remained positive.

Circulating tumor cells testing



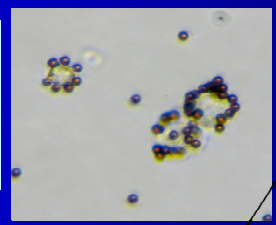
BLOOD COLLECTION

CTC ISOLATION
Immunomagnetic separation

RNA ISOLATION

BONE MARROW SAMPLING

CTC – and DTC visualization

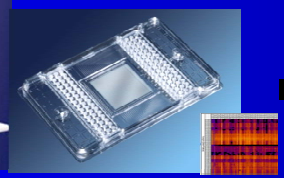


MULTIPLEX PCR

AGILENT BIOANALYZER 2100

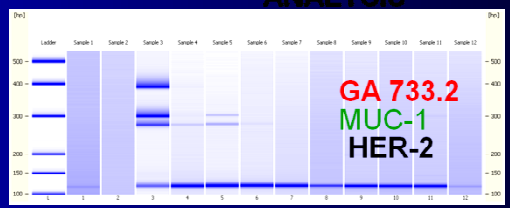
HIGH- THROUGHPUT gene expression profiling

BIOMARK (Fluidigm, U.S.)
48 samples x 48 geneš = 2034 rxn



Pre-Amp Highthroughput PCR

SEMI- QUANTITATIVE ANALYSIS



Areas where CTCs evaluation could impact clinical care

1. Stratification of patients with early disease
2. Subdivision of patients with advanced cancer into different prognostic groups
3. As an intermediate endpoint („surrogate“) of survival for therapeutic efficacy
4. Molecular subclassification of advanced cancer patients

Main topics

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- Circulating tumour cells
- **Molecular cytogenetics**
- Future trends, technologies and labs

Molecular cytogenetic analysis in oncology

→ **specification of diagnosis and prognosis of malignant diseases**

Specification of the tumor

Identification of prognosis

Monitoring of treatment → **determination of remission**
→ **early identification of relaps**

Monitoring of bone marrow transplantation
(opposite sex of donor and recipient, markers, chromosomal polymorphism)

Instability of tumor cell genome

➡ mutations and genetic or chromosomal aberrations

➡ one of the most important events in the genesis of malignant process

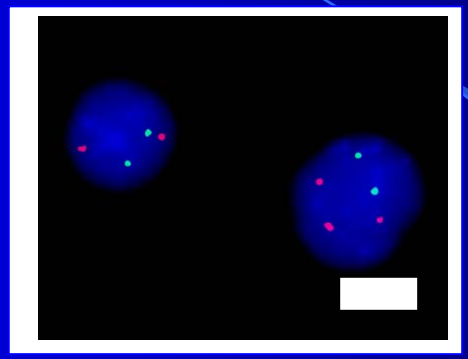
Hematological malignancies: prognostic values of specific chromosomal changes have been determined

Solid tumors: little is known about the molecular genetic pathway of genesis and progression

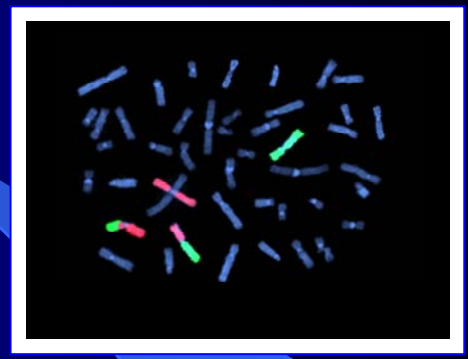
Molecular cytogenetic methods enable the detection of numerical and structural chromosomal aberrations in non-dividing nuclei of interphase tumor cells.

Molecular cytogenetic methods

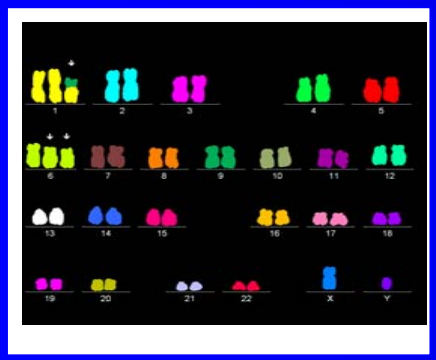
Conventional Cytogenetics (CC)



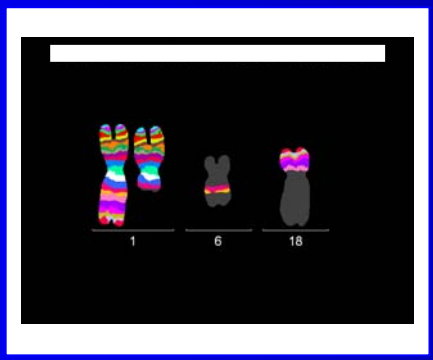
I-FISH



WCP-FISH

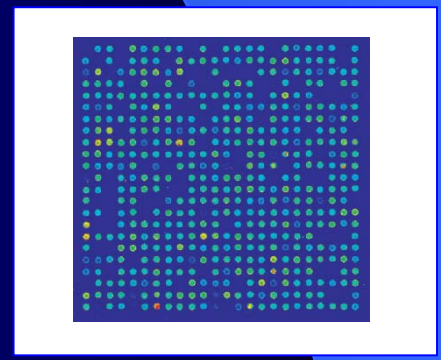


mFISH



mBAND

CGH



arrays

FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH)

Sensitive molecular-cytogenetic method

- allows to analyse karyotype and to detect numerical and/or structural chromosomal aberrations in mitoses as well as in non-dividing intrephase cell nuclei

Molecular cytogenetic diagnostics of hematological malignancies

- Karyotypic investigation of hematologic malignancies - important in the clinical managment of patients with different diagnoses.
- Knowledge about genetic abnormalities - is necesseary for classification of leukemias according to the WHO classification.
- Many aberrations provide important prognostic information → classification of patients into appropriate treatment protocols.
- An increasing number of specific treatment approarches target geneticaly defined leukemia subtypes → cytogenetic analyses of hematological malignancies is mandatory for the proper treatment stratification.

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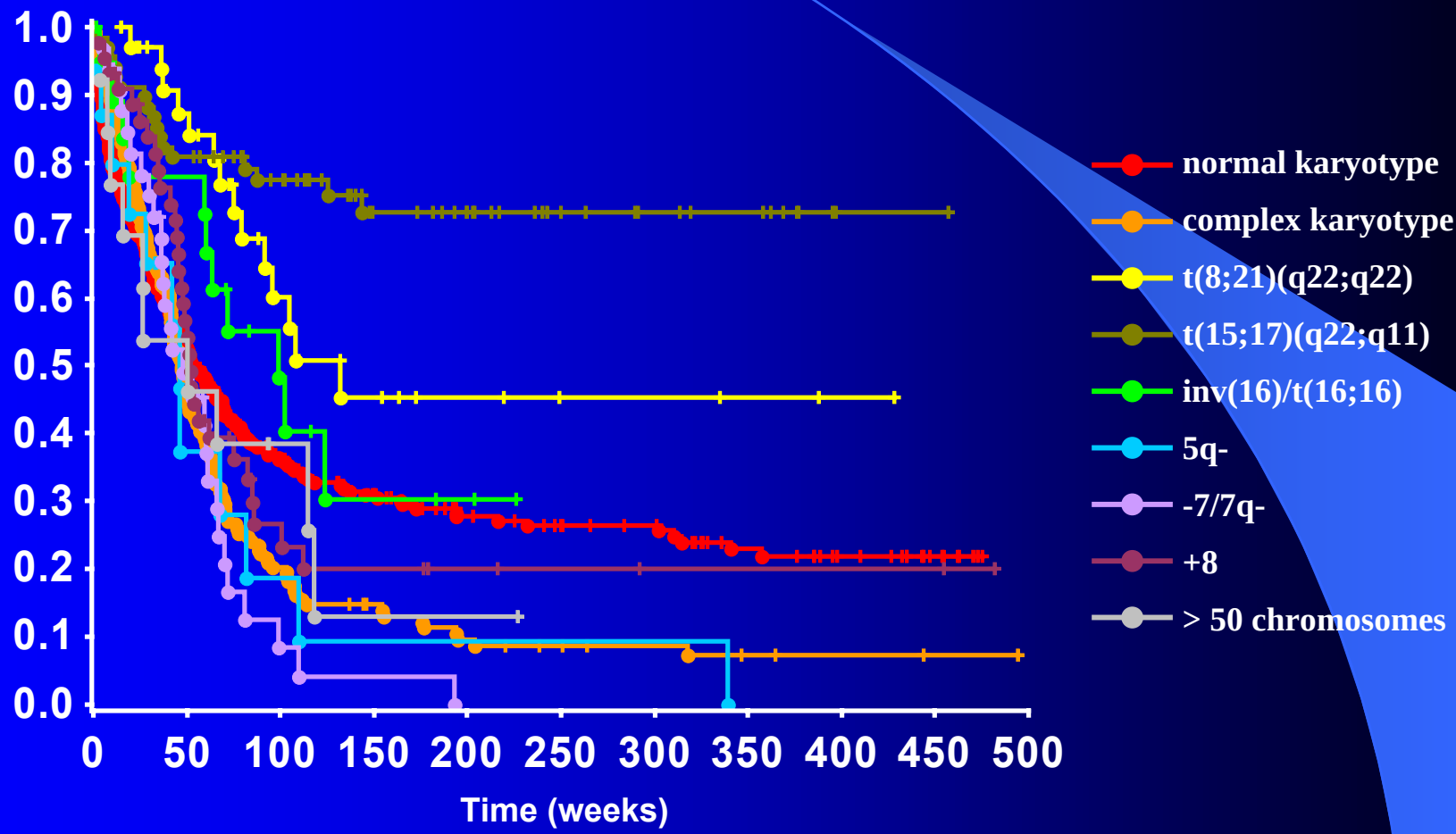
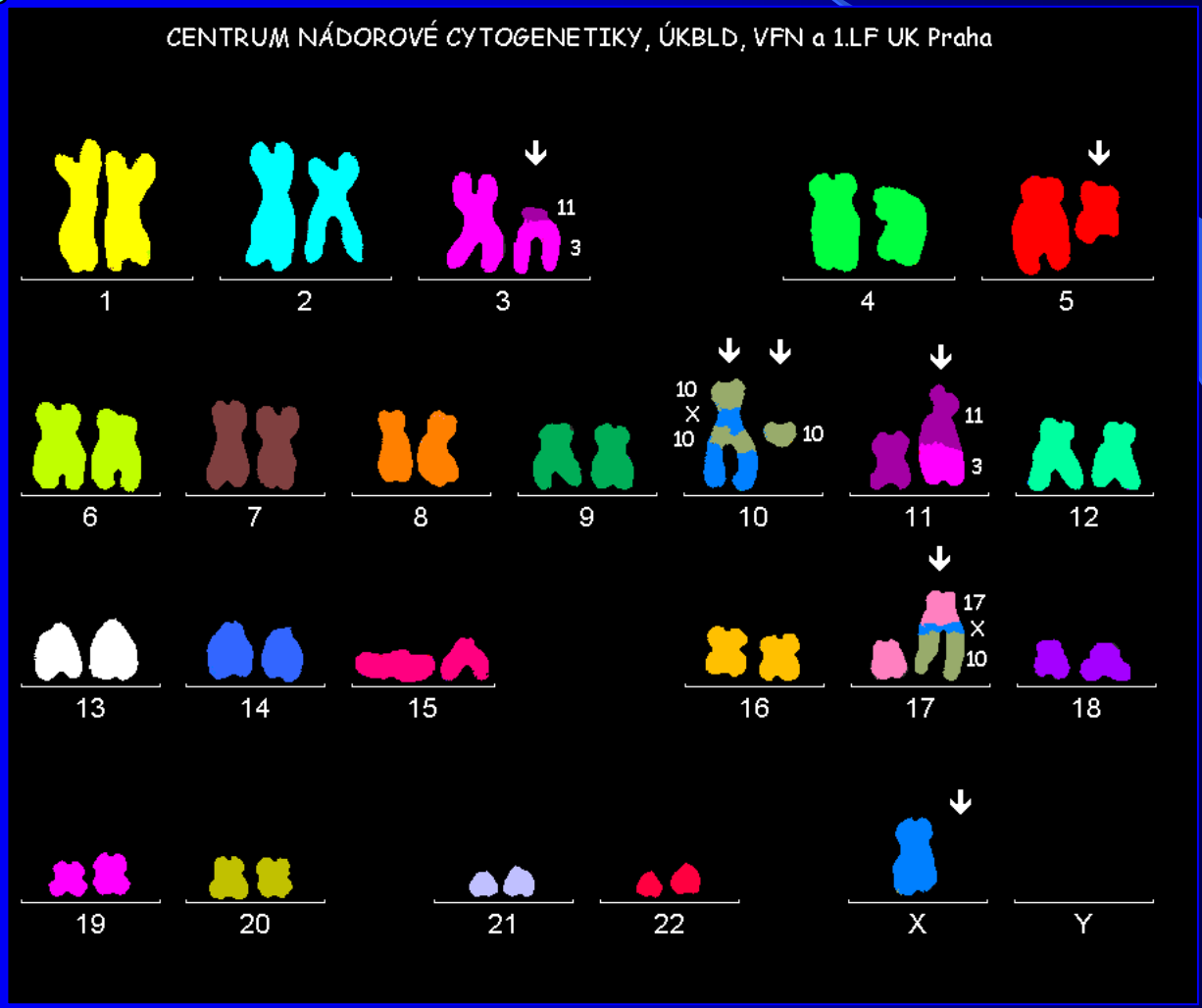
Prognostic impact of specific chromosomal aberrations in acute myeloid leukemia (AML)



Alert 2006

Multicolor FISH - mFISH

- analyses of complex chromosomal rearrangements in bone marrow cells of patients with hematological malignancies will bring us detailed informations about involvement of specific chromosomes or their regions into rearrangements



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Multicolor banding with high resolution - mBAND

- enables determination of exact breakpoints of chromosomal aberrations with much higher resolution than classical banding

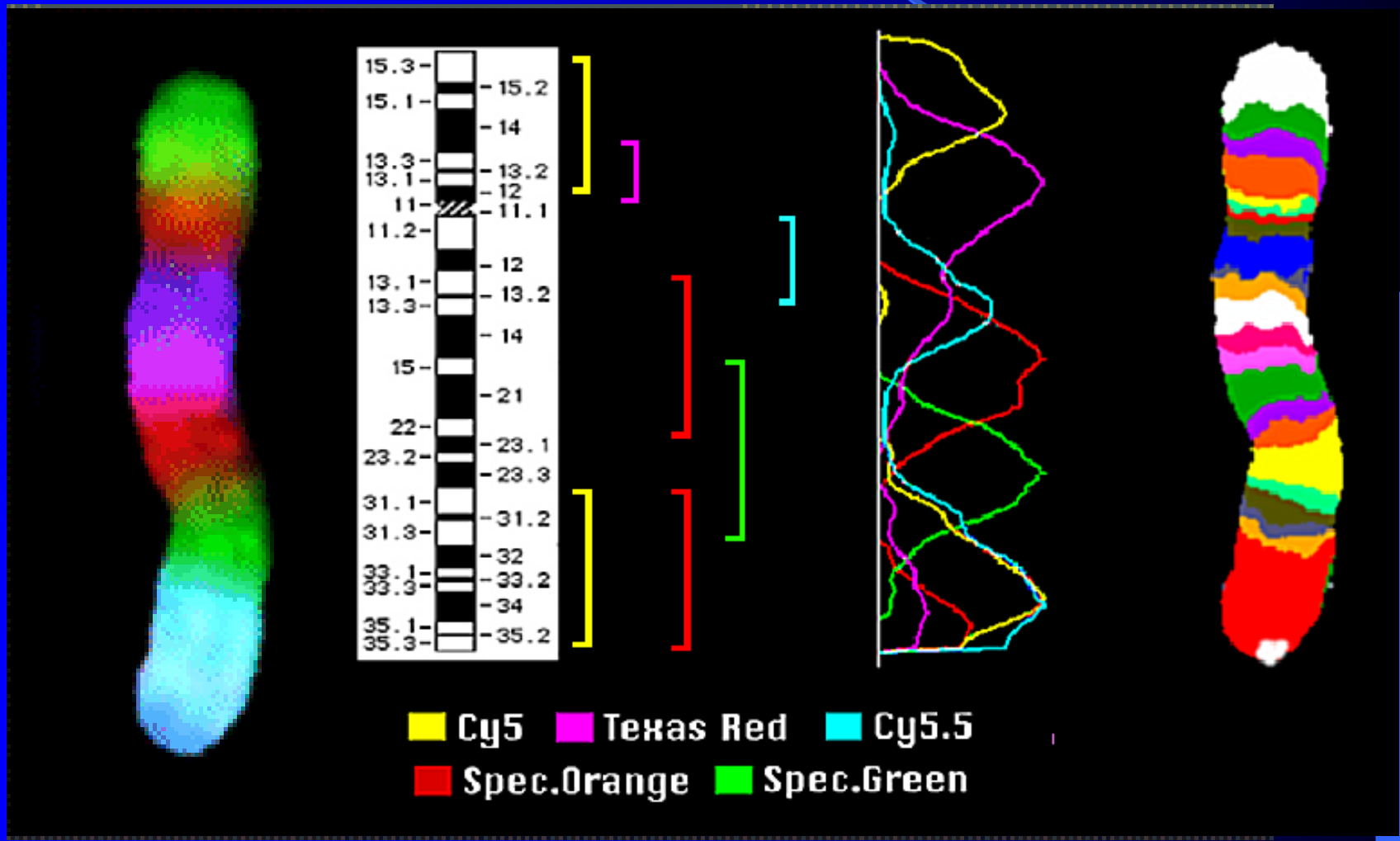
mBAND 1

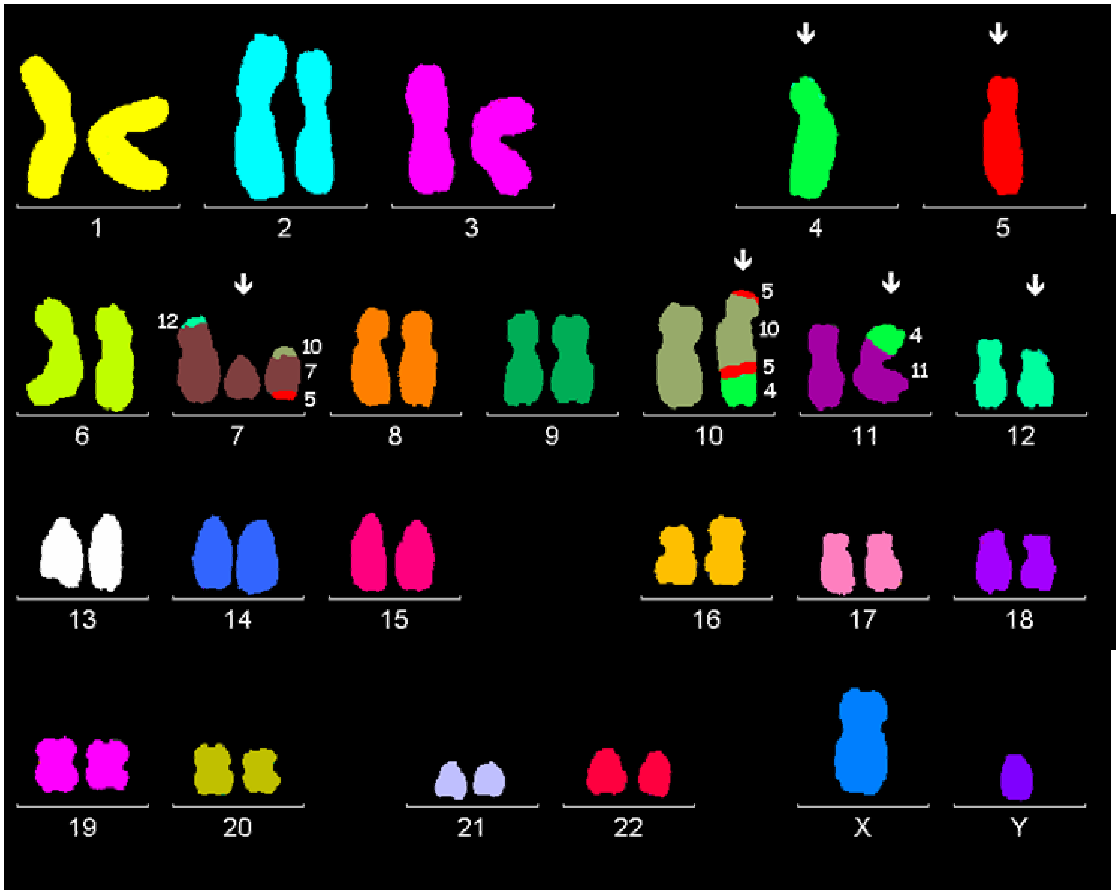
der(1)(1;11)(pter->q31::q22.2q23.3)

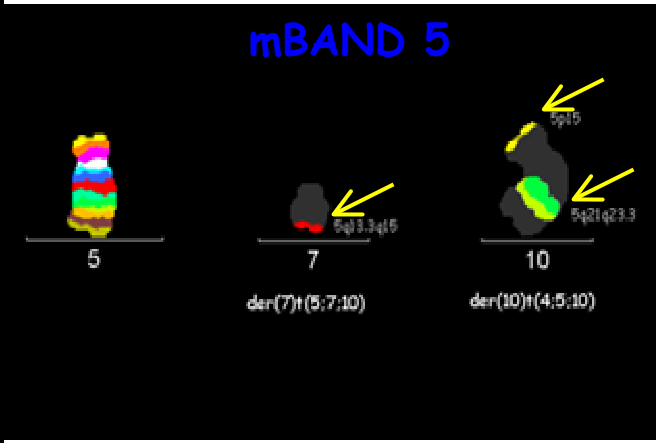
mBAND 11

der(1)(1;11)(pter->q31::q22.2q23.3)

Multicolor banding
- mBAND







45,XY,-4,-5,der(7)t(7;12)(p21;p13),del(7)(p11.1),der(7)t(5;7;10)(q13.3q15;?;?),
der(10)t(4;5;10)(q?;p15q21;p?q?),der(11)t(4;11)(?;p14),del(12)(p13)

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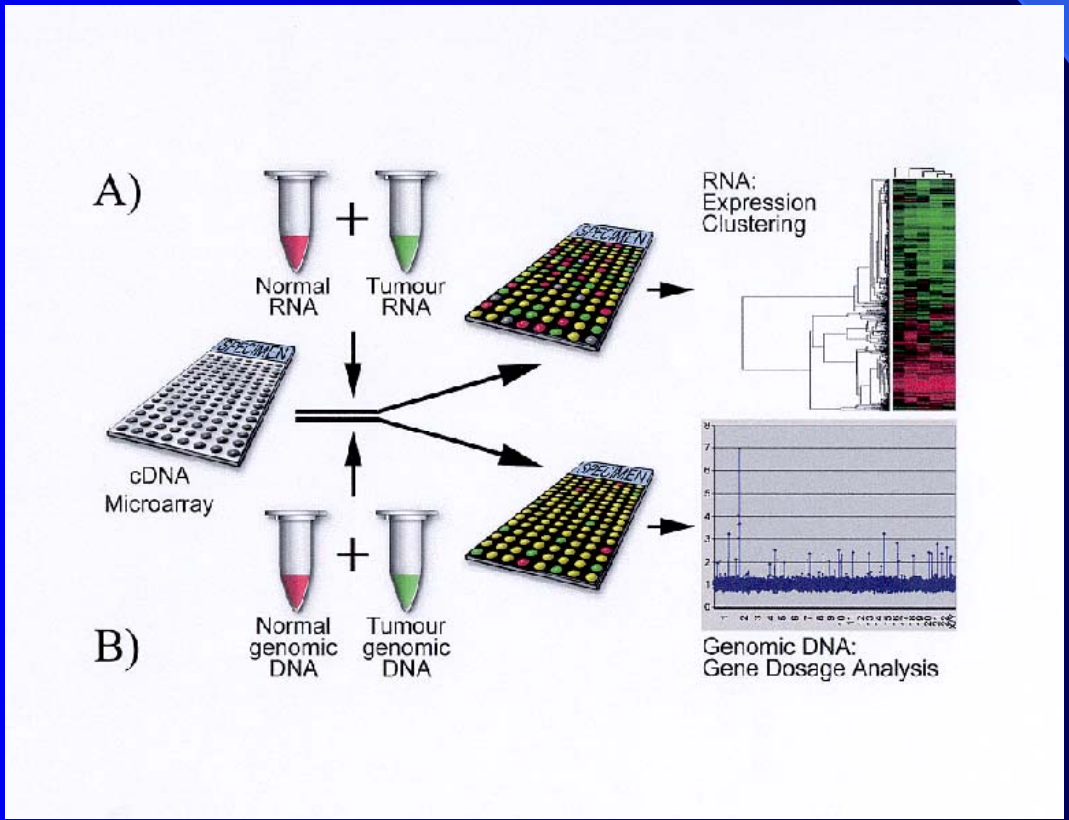
Array-based comparative genomic hybridization (aCGH)

- new tool to search for recurrent gains or loss of chromosomal regions throughout the genome according to detection with very high resolution of copy number changes at DNA level
- only recently is aCGH successfully utilised in analyses of malignant cells and the results revealed a large spectrum of genomic imbalancies, including novel recurrent deletions and amplifications



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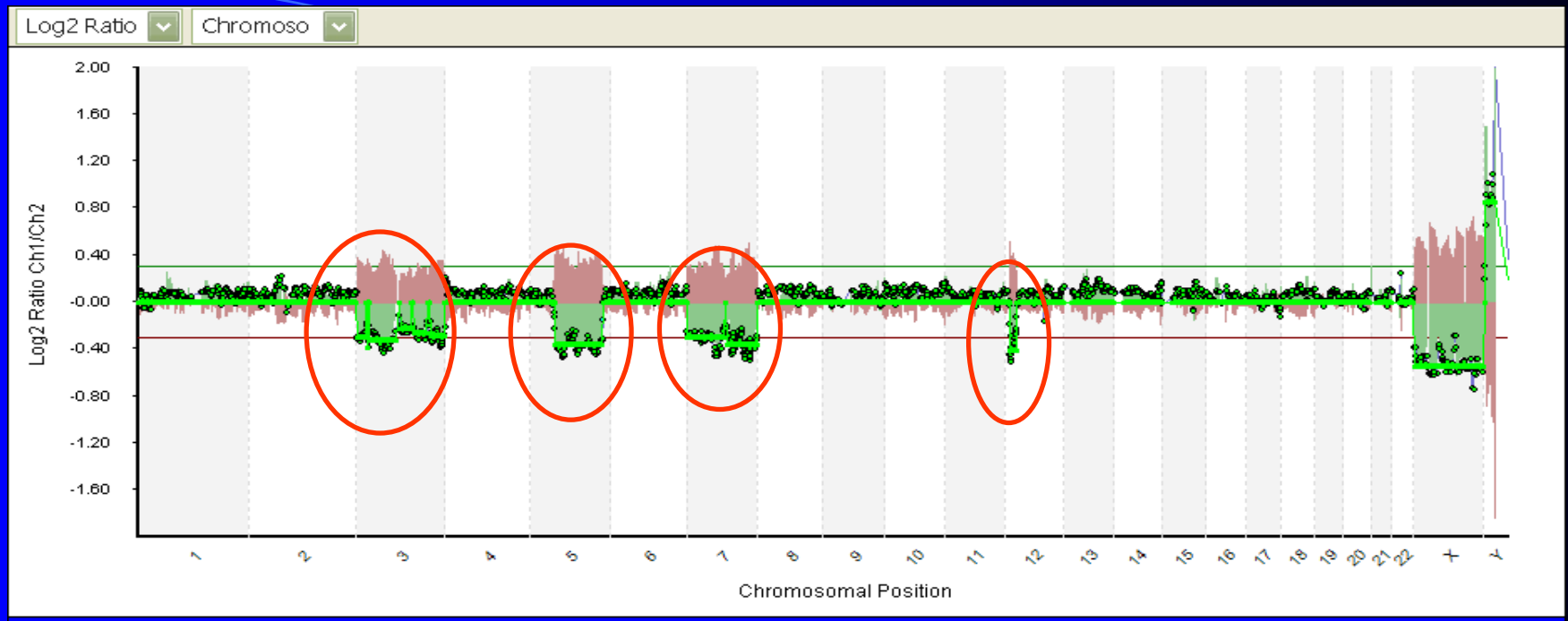

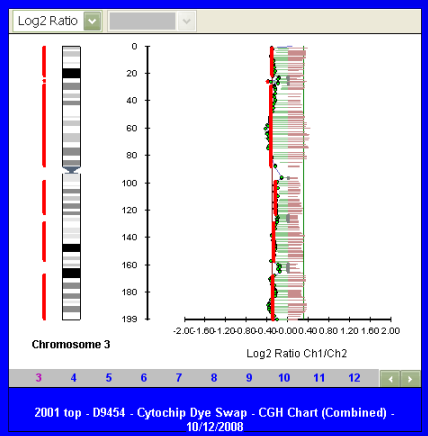

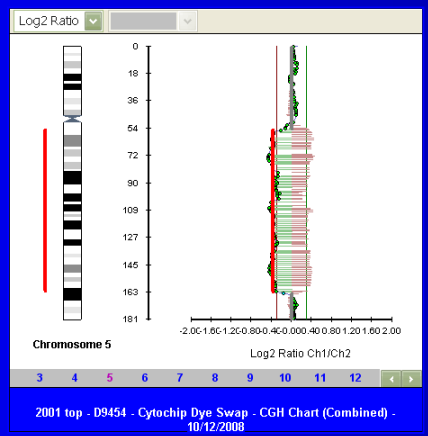

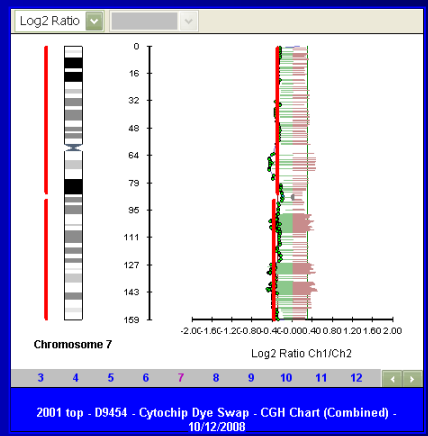

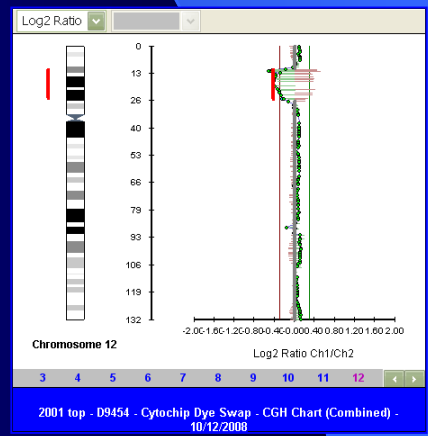

44,XY,-3,del(5)(q12),-7,del(12)(p12)

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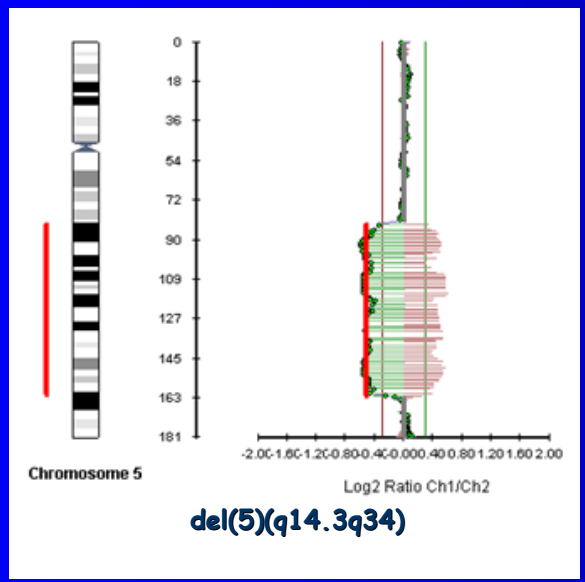
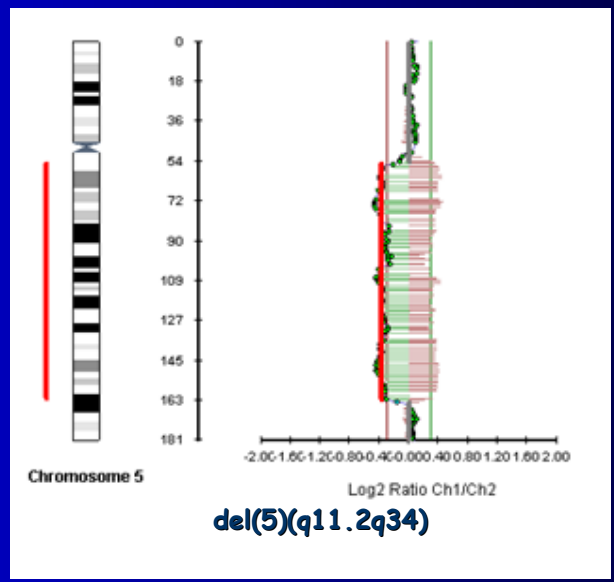
Extent of deletions 5q analysed by mBAND and aCGH:





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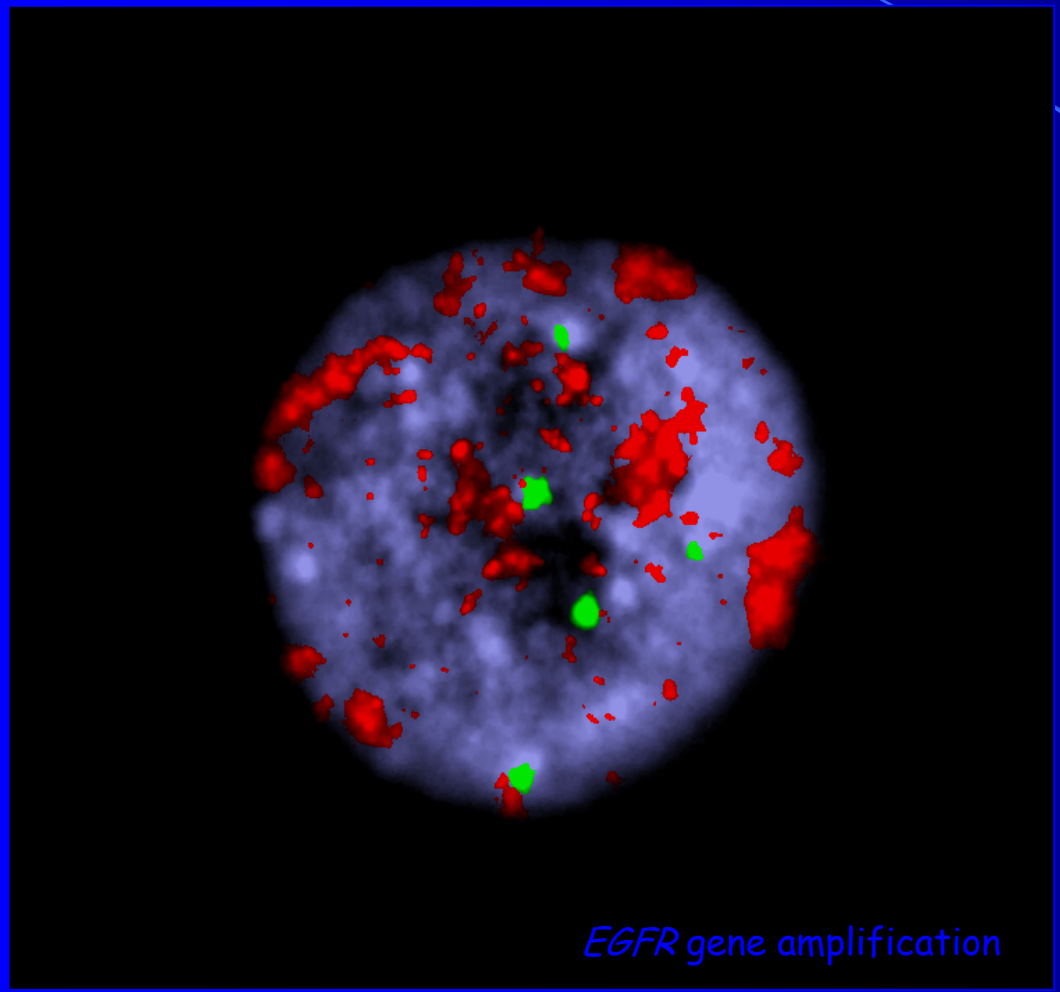
Molecular cytogenetic analyses of brain tumor cells

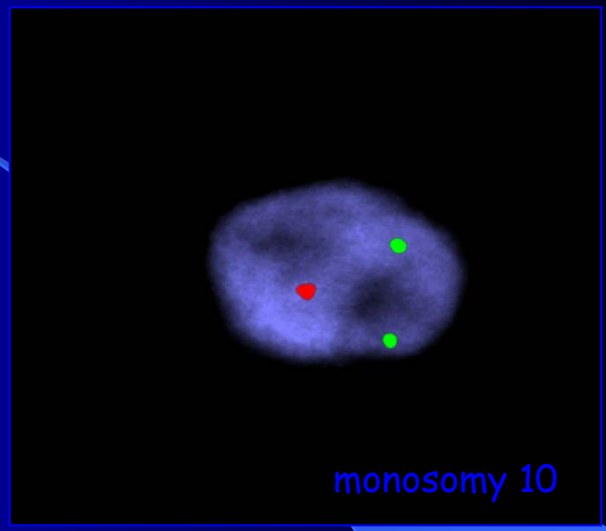
Diffuse gliomas:

- Most frequent tumors of the central nervous system
- Heterogeneous group of primary tumours (~25% of all brain tumors in adults) - various histological subtypes
- Differ in their response to treatment and in the prognosis of the disease
- New diagnostic and prognostic indicators must be sought to enable stratification of treatment
- One possibility is subclassification of patients according to specific chromosomal aberrations in tumour cells

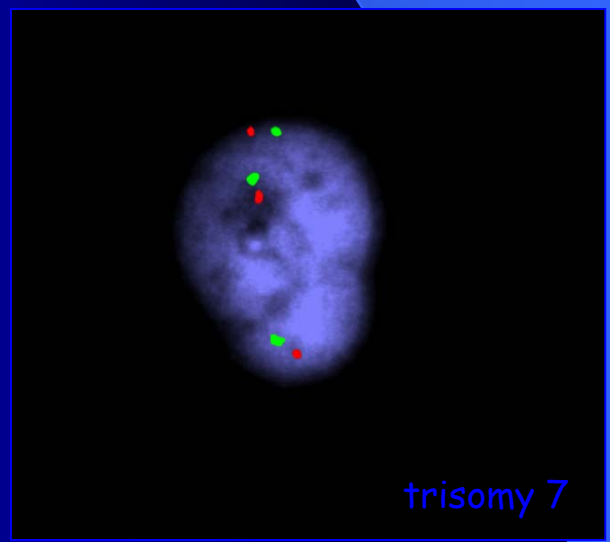
glioblastoma multiforme (WHO grade IV)



EGFR gene amplification



monosomy 10



trisomy 7

Contribution of molecular cytogenetic analyses to diagnosis and treatment of malignant brain tumors.

Molecular cytogenetic analyses are suitable diagnostic methods to detect chromosomal aberrations in brain tumor cells:

- ✓ In patients with astrocytoma confirms histological diagnosis and contributes to more accurate prognosis
- ✓ In patients with oligodendroglioma is essential part of diagnostics and considerably influences treatment and prognosis.

A systematic molecular cytogenetic analyses by means of I-FISH showed in our cohort advancement of diagnosis, grading and classification of brain tumors.

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Molecular cytogenetic analyses of bladder cancer cells

- Urothelial carcinoma is heterogenous malignancy with many different chromosomal aberration described.
- Identification of recurrent chromosomal aberrations might contribute to prediction of further course of the disease and response to the therapy.
- Interphase fluorescence *in situ* hybridization (I-FISH) offers the combination of cytology and detection of genetic alteration in non-dividing nuclei.

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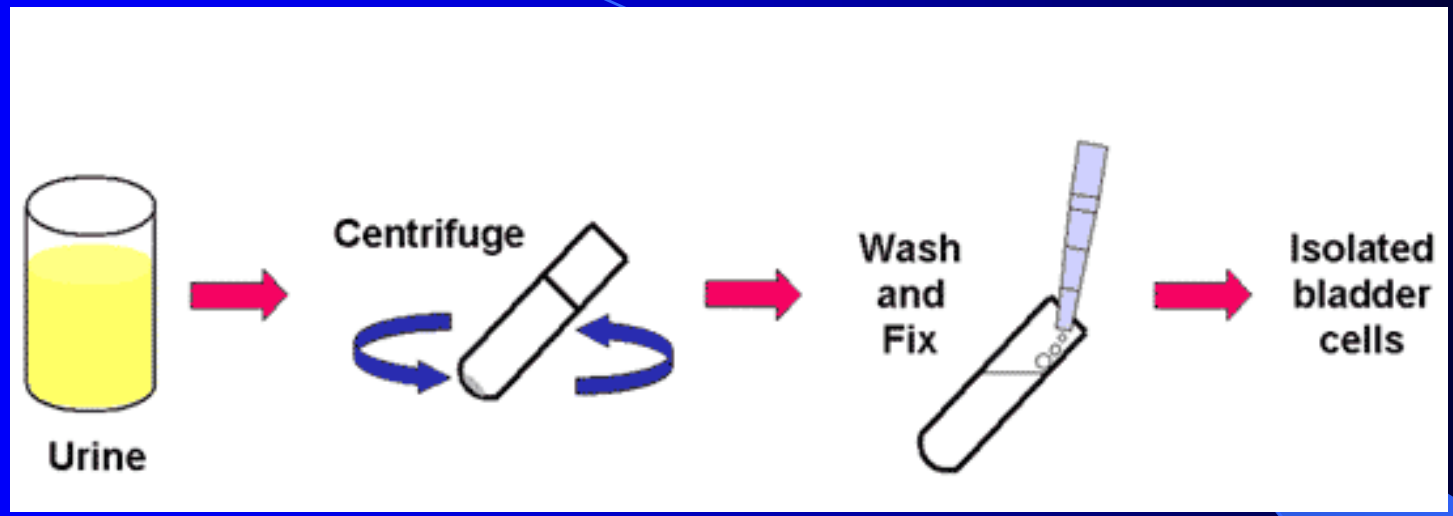
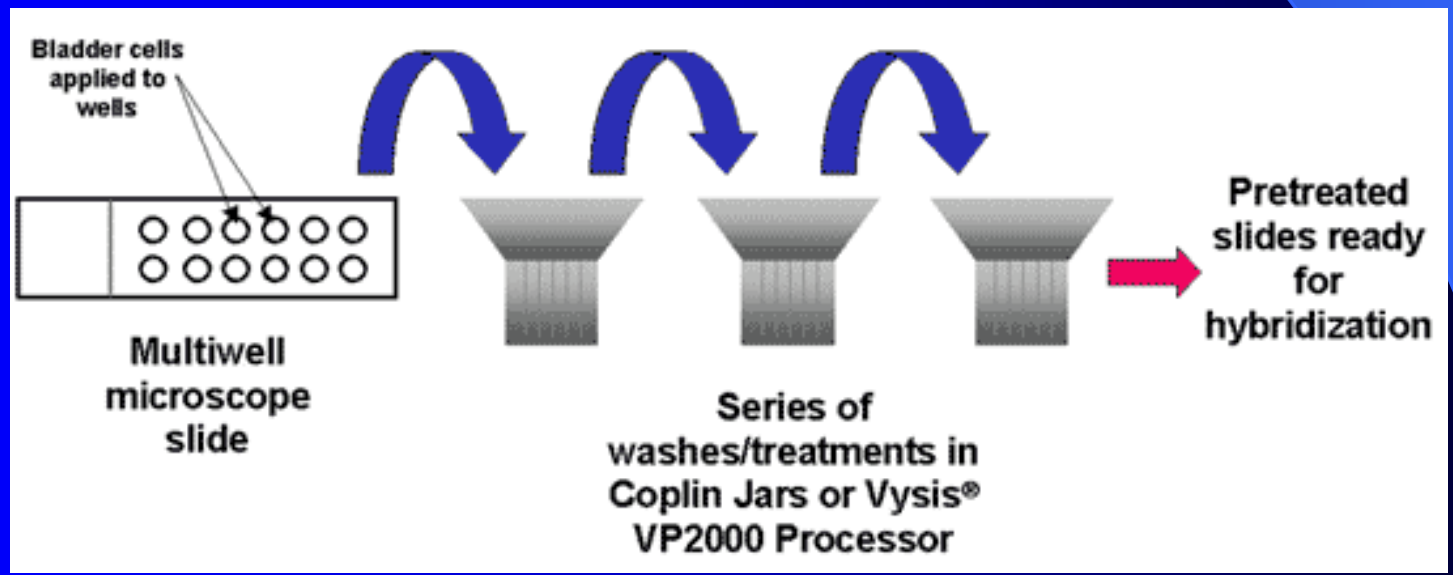
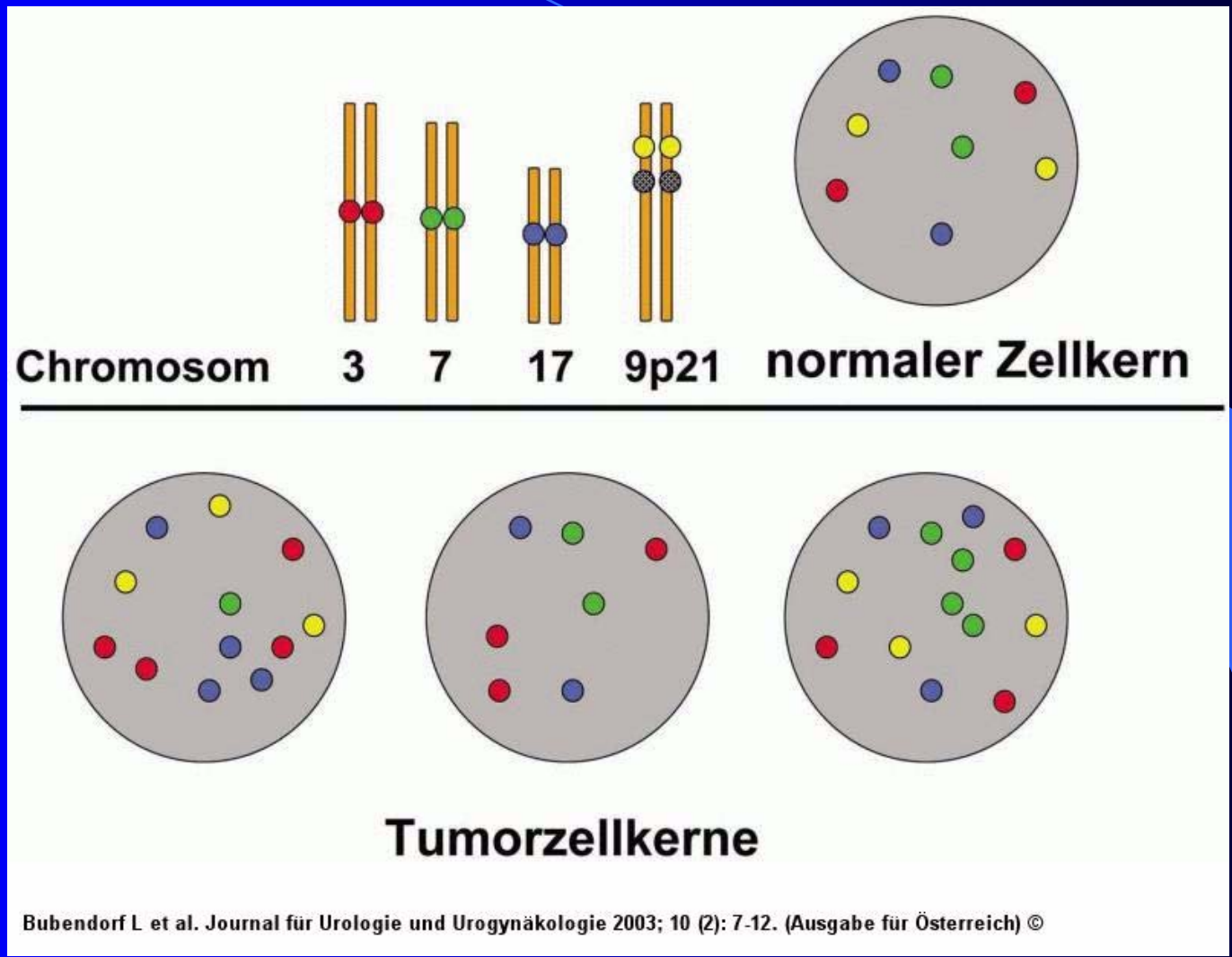
I-FISH in tumors of urinary bladder:



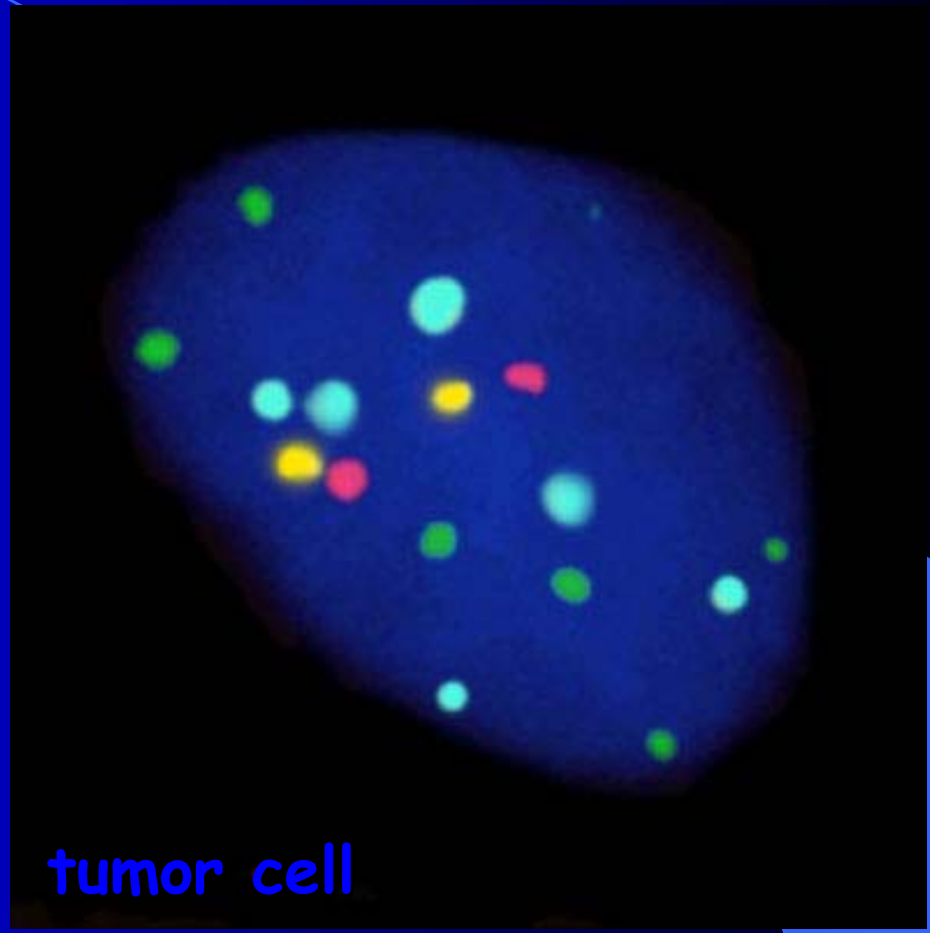


I-FISH in tumors of urinary bladder:



Bubendorf L et al. Journal für Urologie und Urogynäkologie 2003; 10 (2): 7-12. (Ausgabe für Österreich) ©

I-FISH in tumors of urinary bladder:



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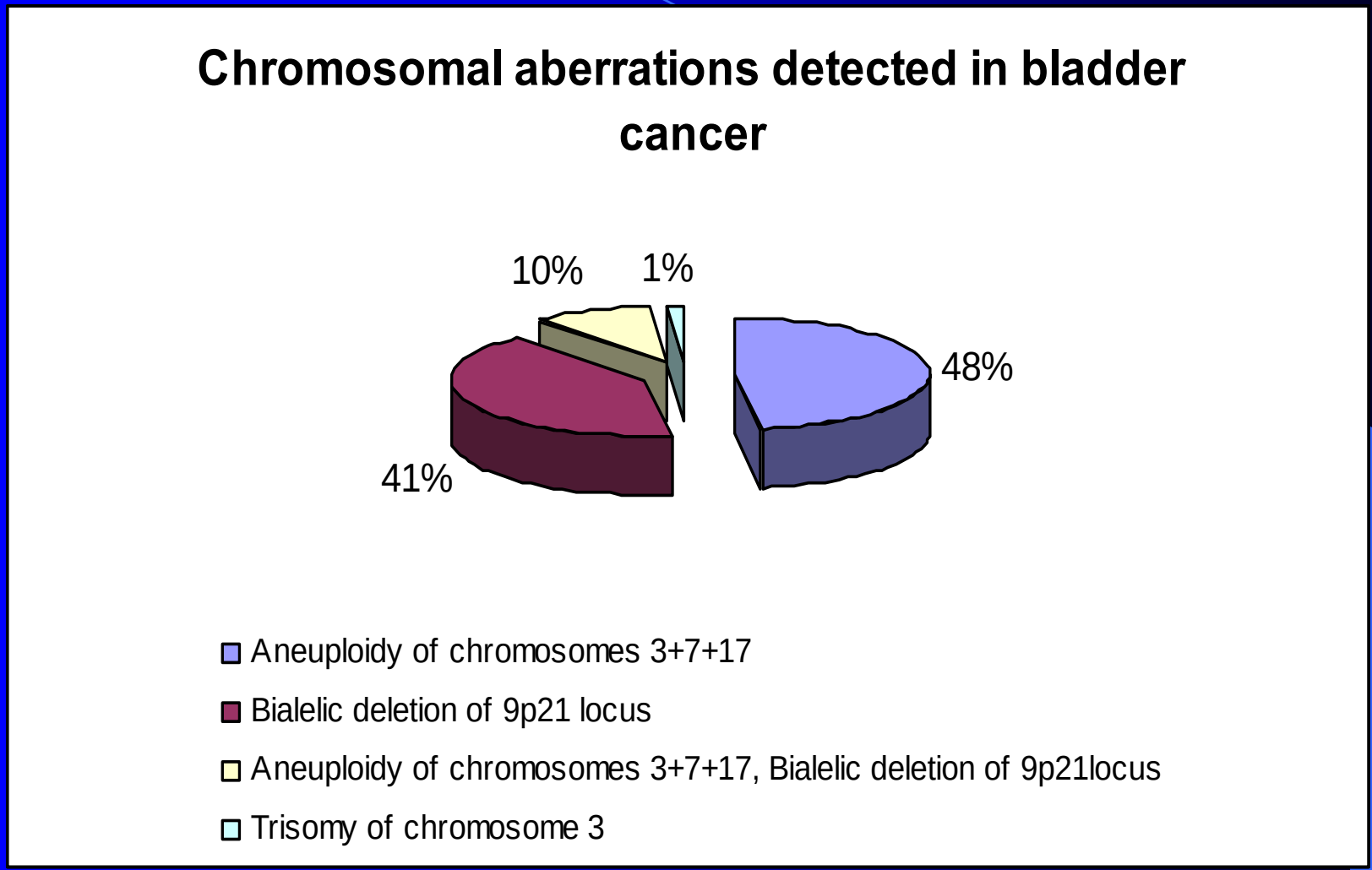
I-FISH in tumors of urinary bladder:



I-FISH in tumors of urinary bladder:

✓Early stages of bladder cancer are characterized mainly by bialelic deletion of 9p21 locus, advanced stage is represented by aneuploidy of different chromosomes.

✓The overall sensitivity of the method was 73,2 %, the specificity of the method was 87 %.

Our results confirm the usefulness of the UroVysion probes as a non-invasive screening tool to select patients for cystoscopy.

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CONCLUSIONS

- Conventional and molecular cytogenetic analyses of cancer cells have led to the definition of prognostic risk groups and the development of subtype-specific or risk-adapted therapy strategies in different types of malignant diseases.

- Results of examinations of malignant cells by molecular cytogenetic methods brought a new informations which are important for the understanding of pathogenetic mechanisms of origin and progression of leukemias and some selected solid tumors.

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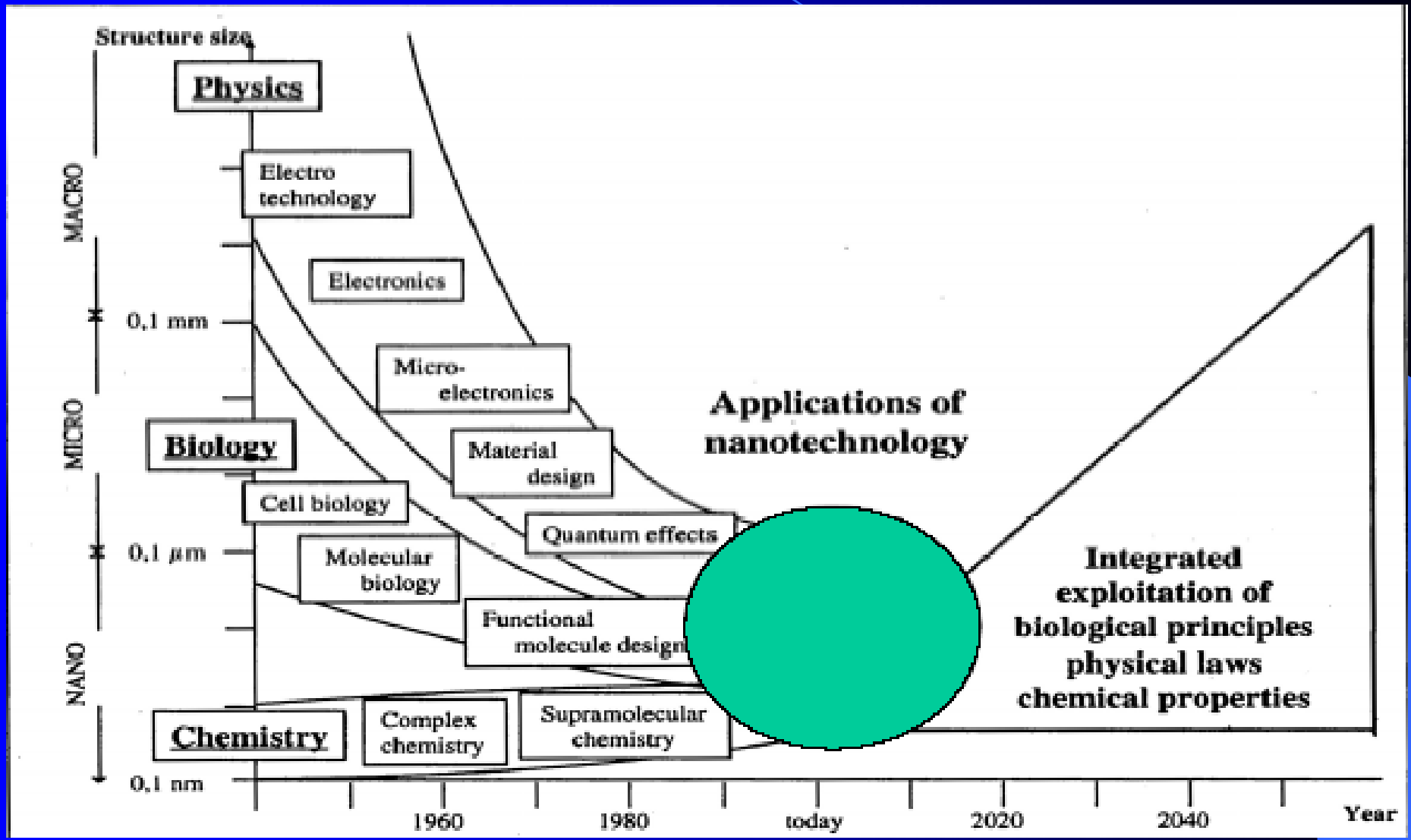
Main topics

- Proteomics
- Pharmacogenomics
- Circulating tumour cells
- Molecular cytogenetics
- Future trends, technologies and labs

Future trends – nanotechnologies



Future lab

- Short staffing
- Platform consolidation, integration and automation
- Dramatic increase in POCT and home testing
- Non invasive testing
- Niche market exploitation
  - e.g. women's health, geriatric health)
- Increased use of tandem mass spectrometry
- Use of molecular diagnostics (Chips and SNPs), single cell analyses
- Use of robotics
- Working from home: telecommuting
- Web-based communications

Ευχαριστούμε