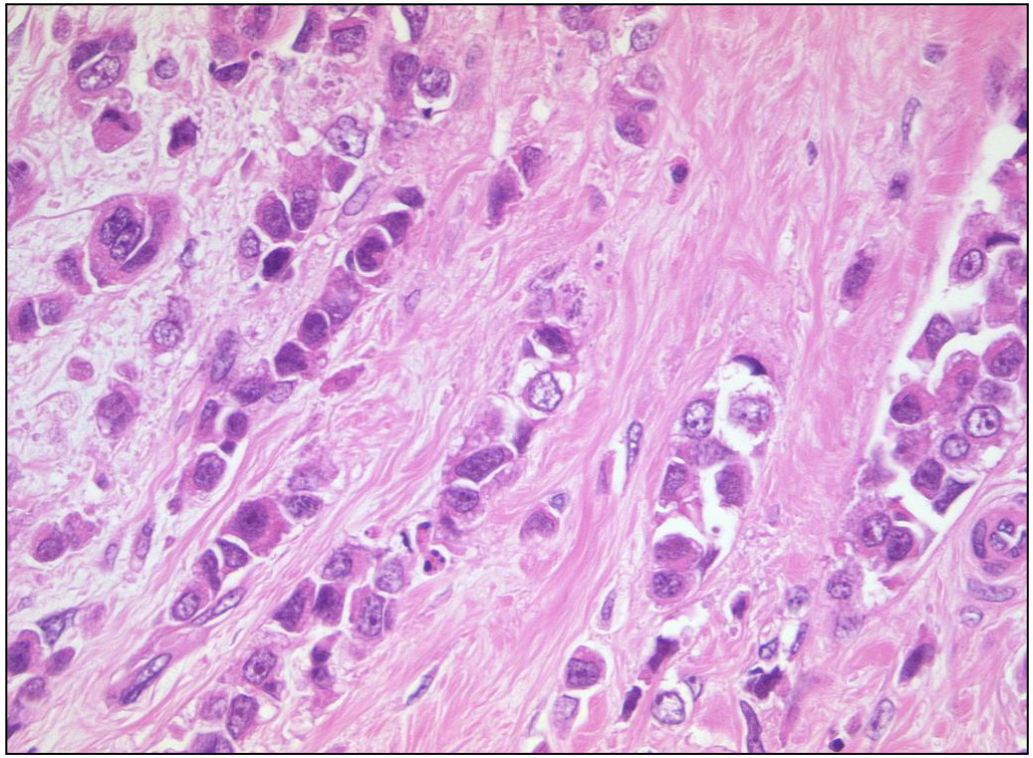
Techniques in pathology



Carsten Denkert
Translational Cancer Research Group
Institute of Pathology
Charité Universitätsmedizin Berlin

Berlin, Germany
7.5.14
IMPAKT Meeting, Brussels

Conflict of interest statement

- Research funding, honoraria, shareholder: Sividon Diagnostics
- Research funding: Siemens Medical Solutions

Outline – techniques in pathology

- Introduction
  - research strategies in pathology
  - strengths and weaknesses
- FFPE tissue
  - H&E
  - immunohistochemistry
  - digital imaging
  - RNA analysis
  - DNA analysis
  - NGS sequencing
- workflow options

Options for research in pathology

- Why are we doing the research project?
- What are the aims?

Options for pathologists:

  - „traditional approach“ - definition and description of new tumor entities
  - hypothesis-generating research
  - predictive biomarker-focussed research
  - practice changing research

Level of evidence for biomarker studies

Type of tumor marker study		Definition	Possible level of evidence	
A	Prospective	clinical trial designed to address tumormarker	1	validation preferred, but not required
B	Prospective using archived samples "prospective-retrospective"	prospective biomarker design, existing samples collected in clinical trial	1	two studies with identical results
			2	only one study
C	Prospective observational	prospective registry and sample collection, no standardized treatment and follow-up	2	two studies with identical results
			3	only one study
D	Retrospective observational	collection of samples from archive, no standardized treatment	4-5	hypothesis generating, no clinical utility

Simon, Paik, Hayes JNCI, 2009

Published Ahead of Print on October 15, 2012 as 10.1200/JCO.2012.42.6858
The latest version is at http://jco.ascopubs.org/cgi/doi/10.1200/JCO.2012.42.6858

Publication of Tumor Marker Research Results: The Necessity for Complete and Transparent Reporting

Lisa M. McShane and Daniel F. Hayes

Table 1. Requirements for a Marker-Based Test to Reach Level IB Evidence of Clinical Utility Based on Prospective-Retrospective Studies

1. Adequate amounts of archived specimen must be available from enough patients from a prospective trial (which for predictive factors should generally be a randomized design) for analyses to have adequate statistical power and for the patients included in the evaluation to be clearly representative of the patients in the trial.
2. The marker-based test should be analytically and preanalytically validated for use with archived specimens.
3. The plan for marker evaluation should be completely specified in writing before the performance of marker assays on archived specimens and should be focused on evaluation of a single completely defined marker-based test.
4. The results from archived specimens should be validated using specimens from one or more similar, but separate, studies.

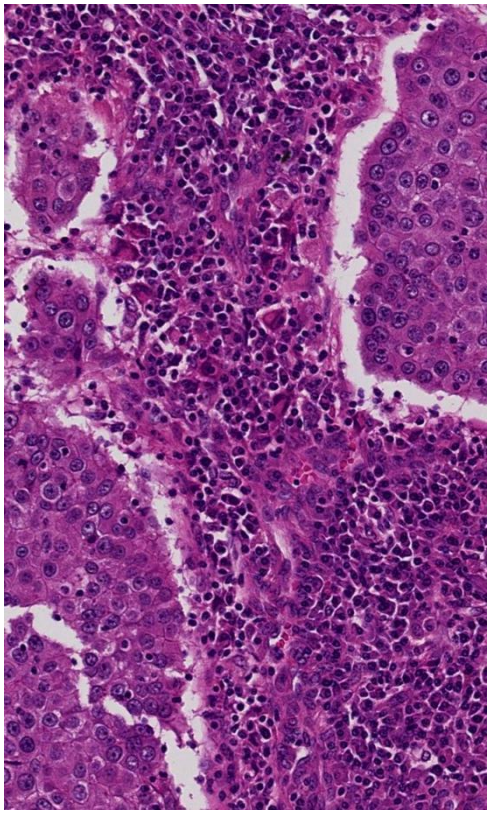
NOTE. Guidelines adapted.[22]

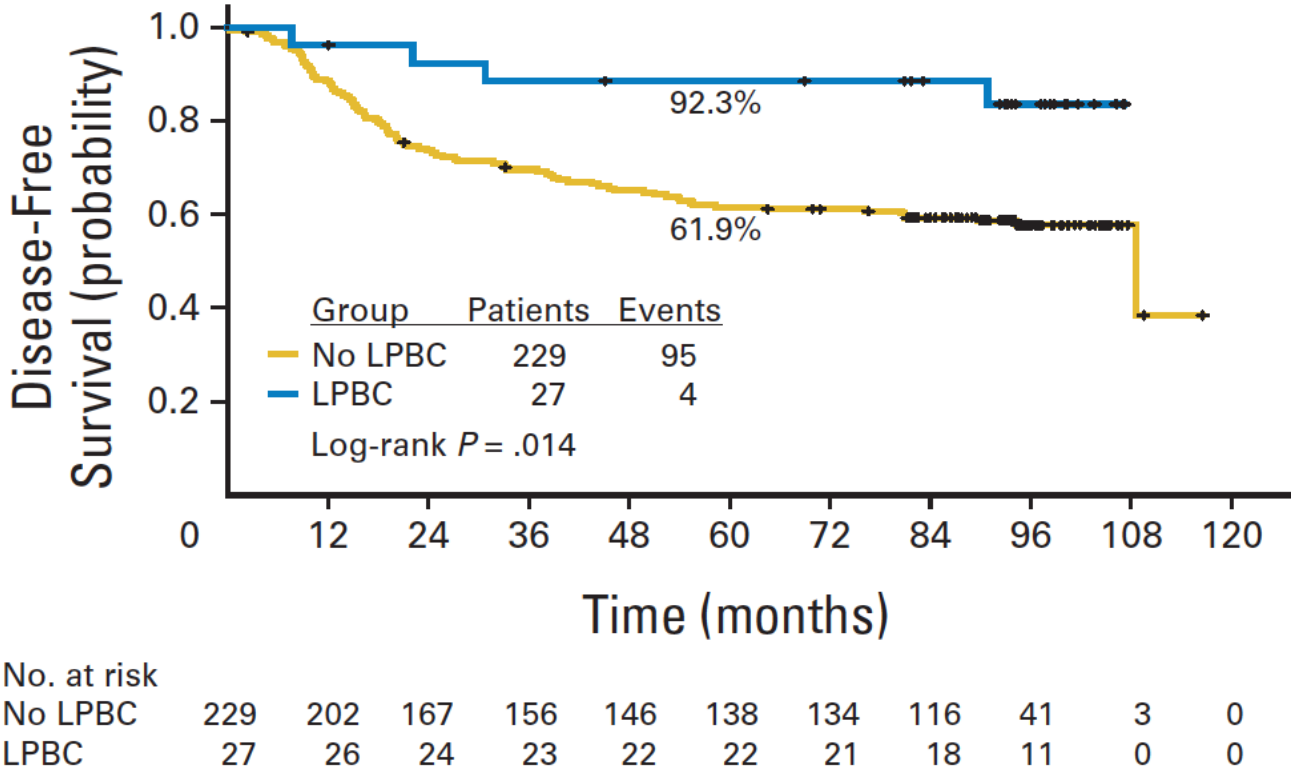
Requirements for pathology research 2014

- large sample cohorts (aim: >1000 samples, but difficult to reach)
  - only possible for FFPE tissue
  - heterogenous cohorts vs tumor-type specific cohorts ?
    - prognostic markers may simply be markers of luminal differentiation
    - very difficult to find markers in TNBC cohorts
  - additional cohorts for validation
- prespecified analysis plan as a document
- methods validation

H&E based studies - Prognosis of TNBC – increased lymphocytic infiltrate defines a good prognosis group

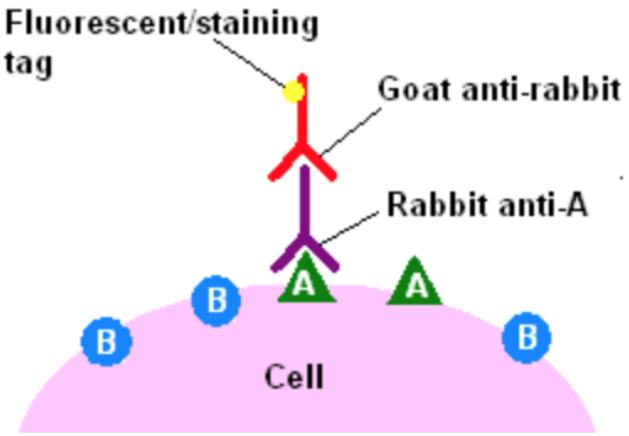
Loi et al, JCO 2013 BIG2-98 study (total n=2009, TNBC n=256)



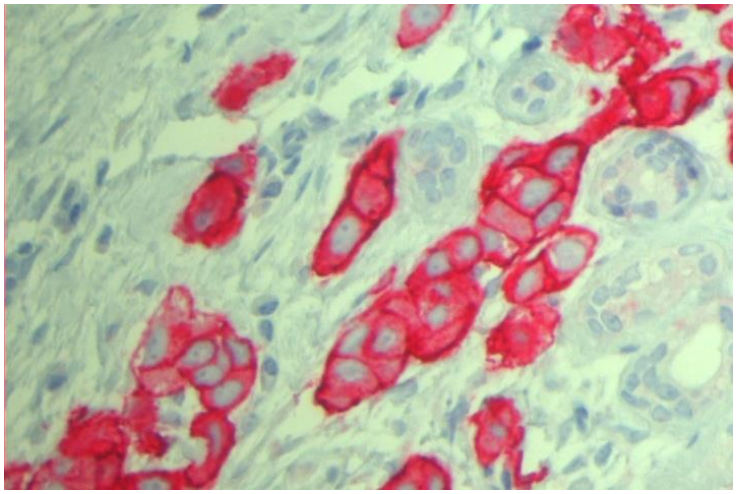


Immunohistochemistry = „in situ proteomics“

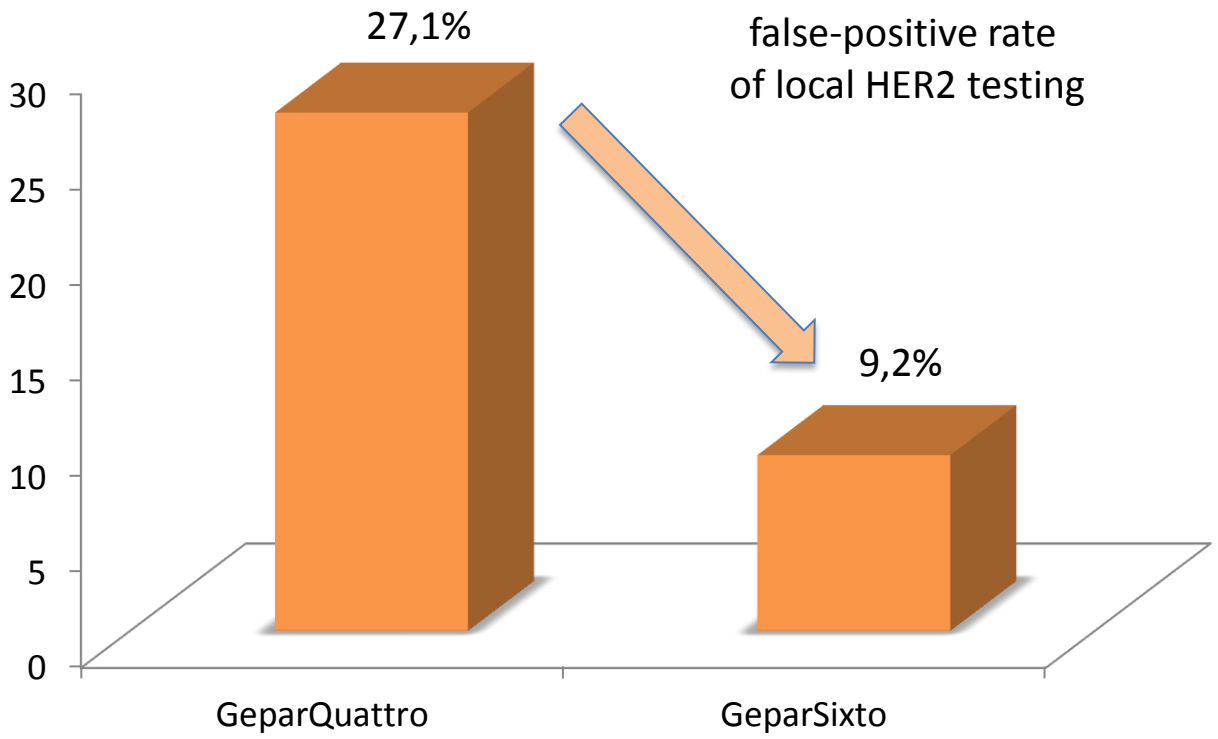
- antibody-based detection of molecular markers on tissue slides
- Advantages:
  - easy, useful on FFPE tissue
  - combined molecular and morphological information
  - type of cells, localisation in cells
- Disadvantages:
  - standardisation issues
  - staining intensity
  - percentage of of positive cells

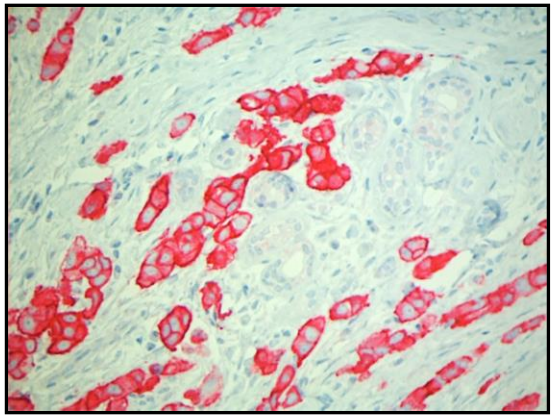


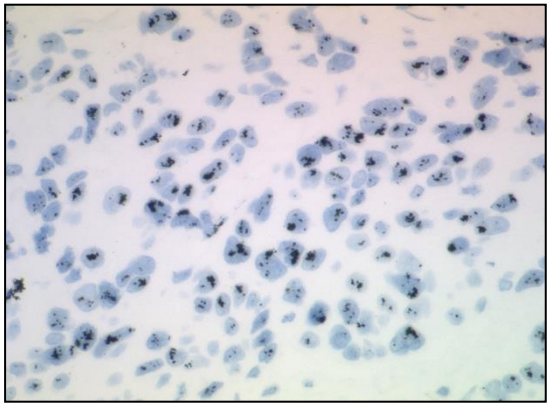
Wikipedia image File:Immunohistochemicalstaining2.PNG



Comparison of local and central HER2 in GBG trials







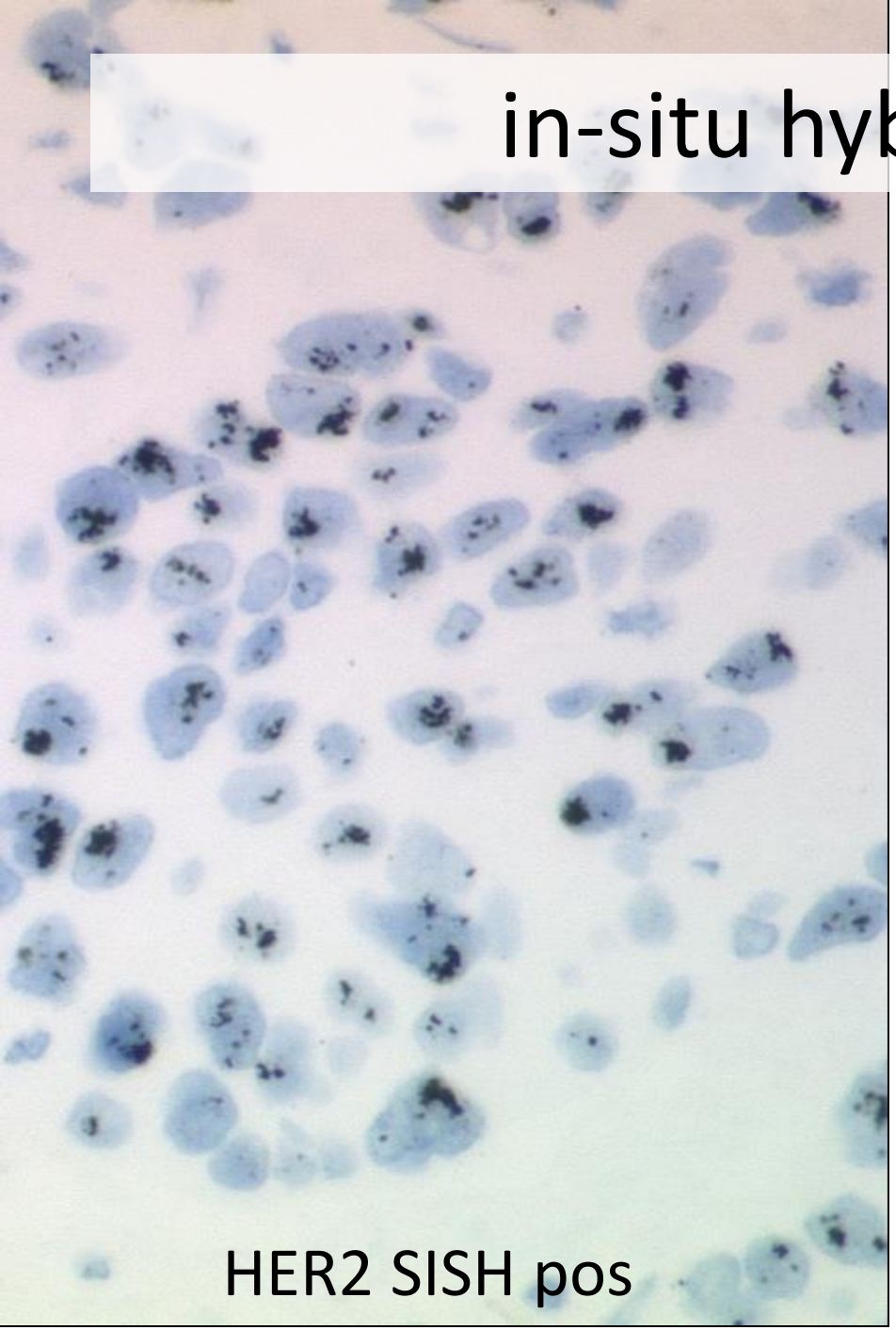
GeparQuattro
N=217 local HER2+
2005-2006

GeparSixto*
N=158 local HER2+
2011-2012

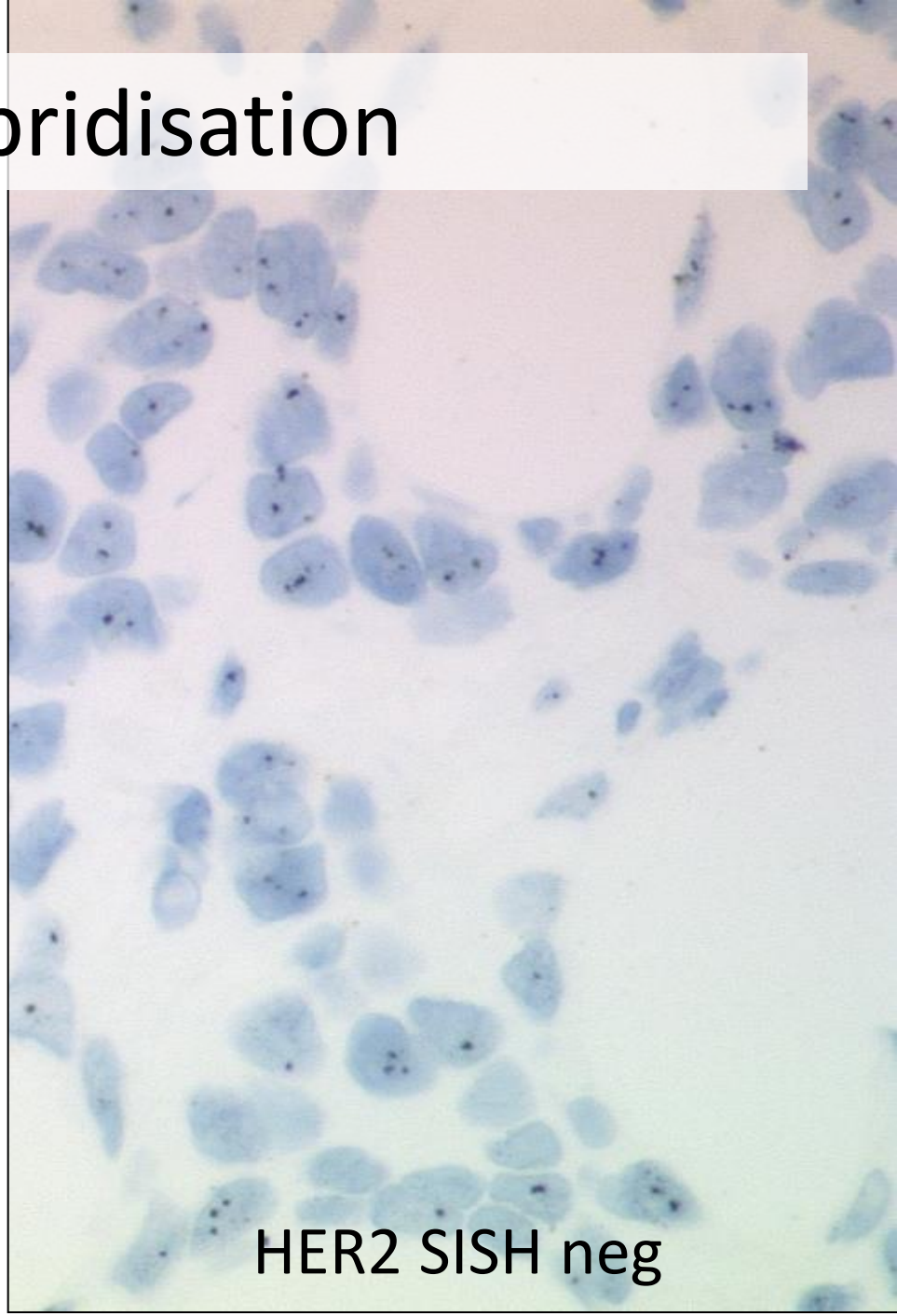
Preliminary 2.5.12

GBG
GERMAN
BREAST
GROUP

in-situ hybridisation



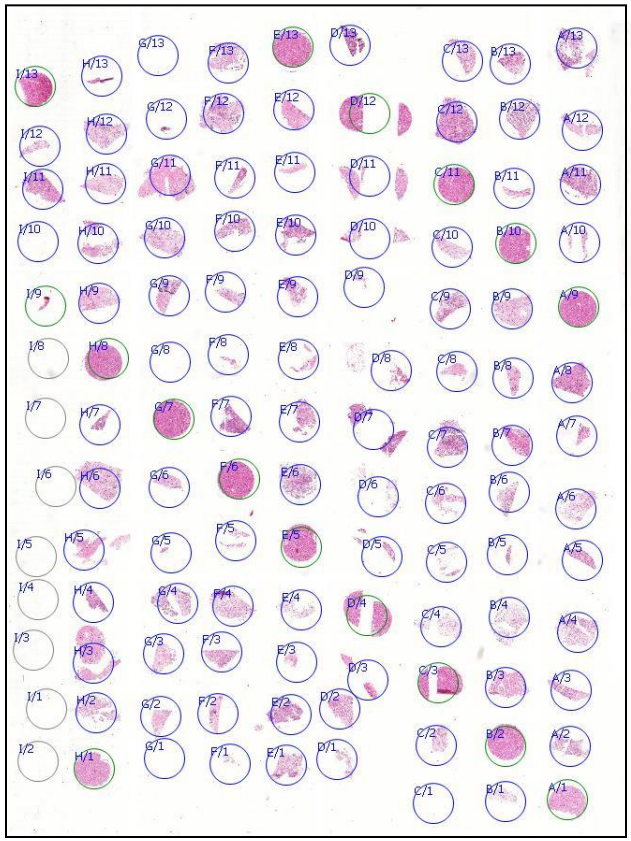
HER2 SISH pos



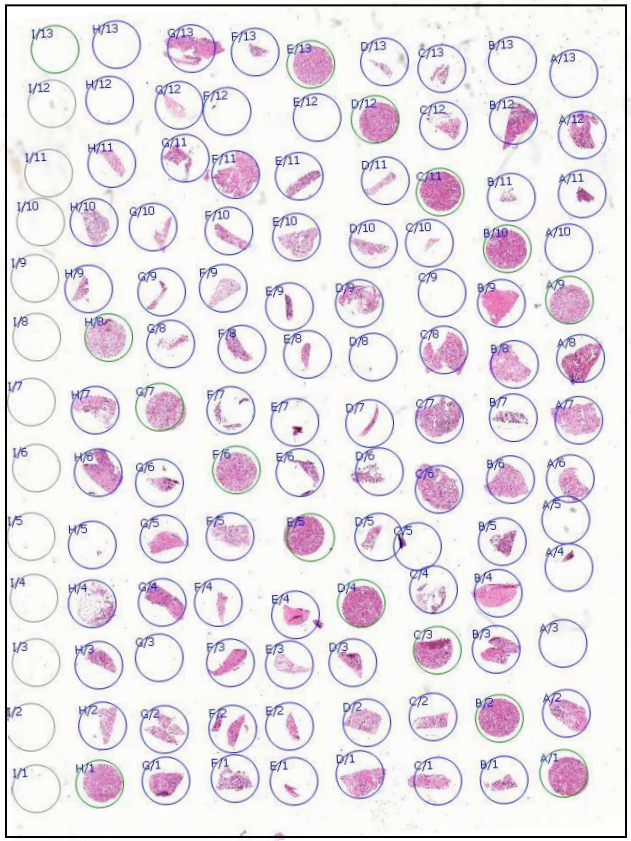
HER2 SISH neg

TMA – tissue microarray

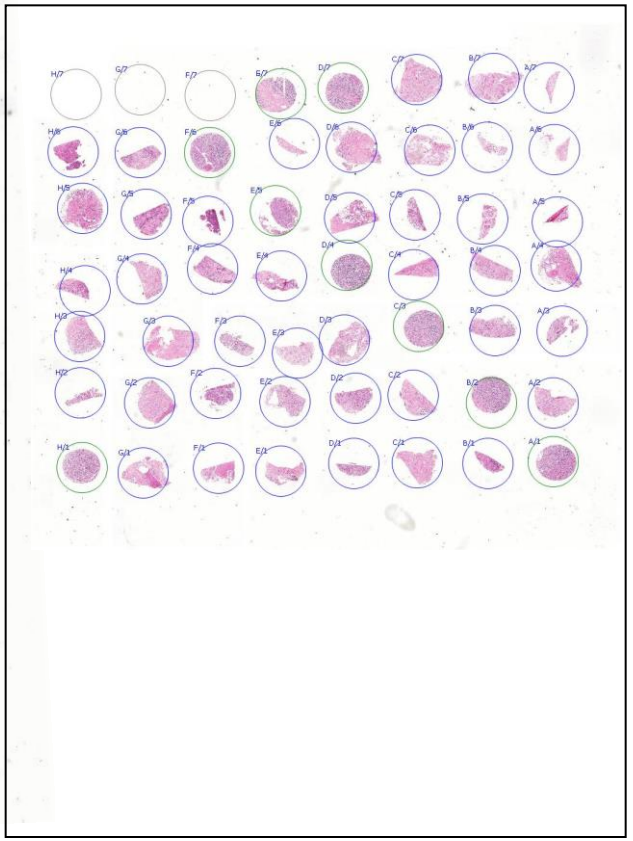
TMA from 227 pretherapeutic core biopsies from HER2-positive Tumors from the GeparQuattro-trial



TMA 1

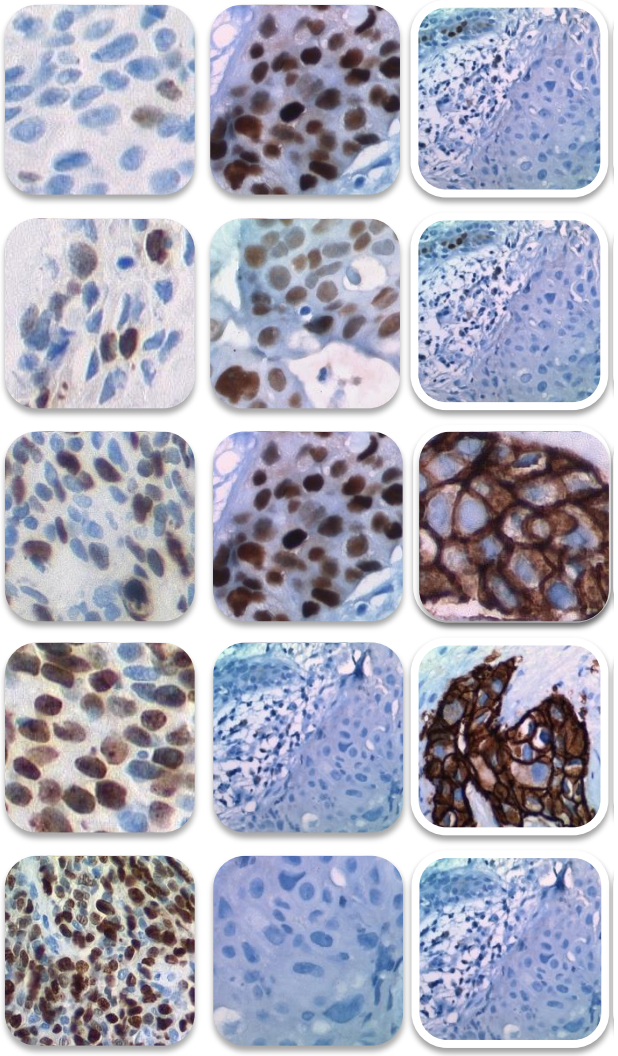


TMA 2



TMA 3

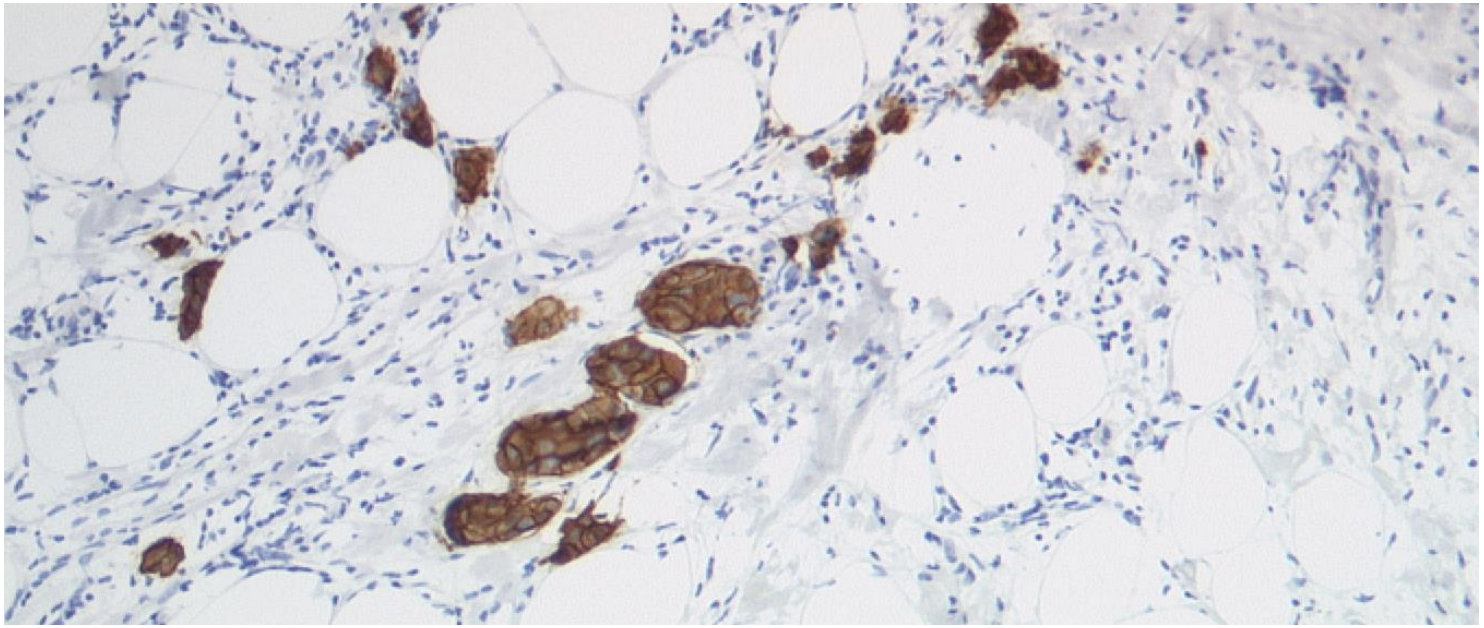
Limitations of immunohistochemistry

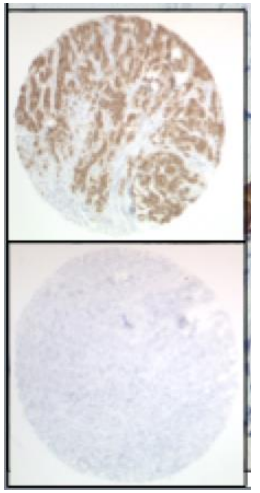


- Technical issues
  - Quantification of markers and cutoffs, e.g. Ki67
  - Interobserver variability
  - Assay standardisation
- More complex questions:
  - Endocrine Tx vs. Chemo-endocrine Tx
  - Different types of anti-HER2 therapy
  - Response to anti-angiogenic Tx

Immunohistochemistry - standardisation

- use of Autostainers
- internal / external controls
- quantitative markers – image analysis

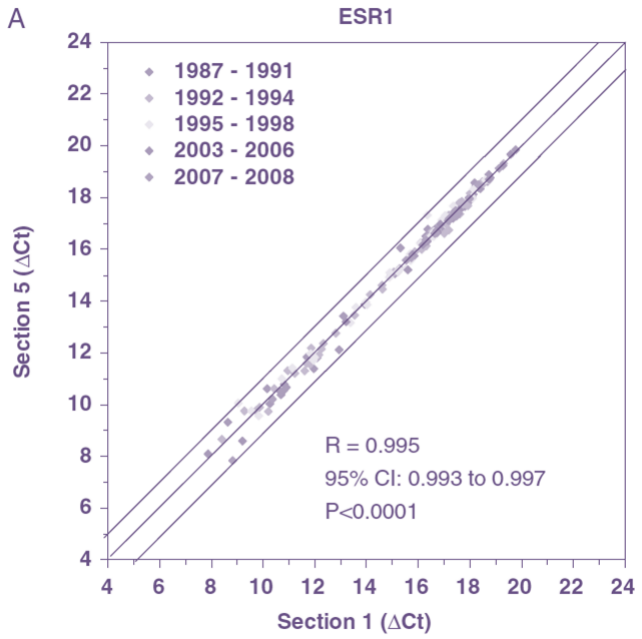


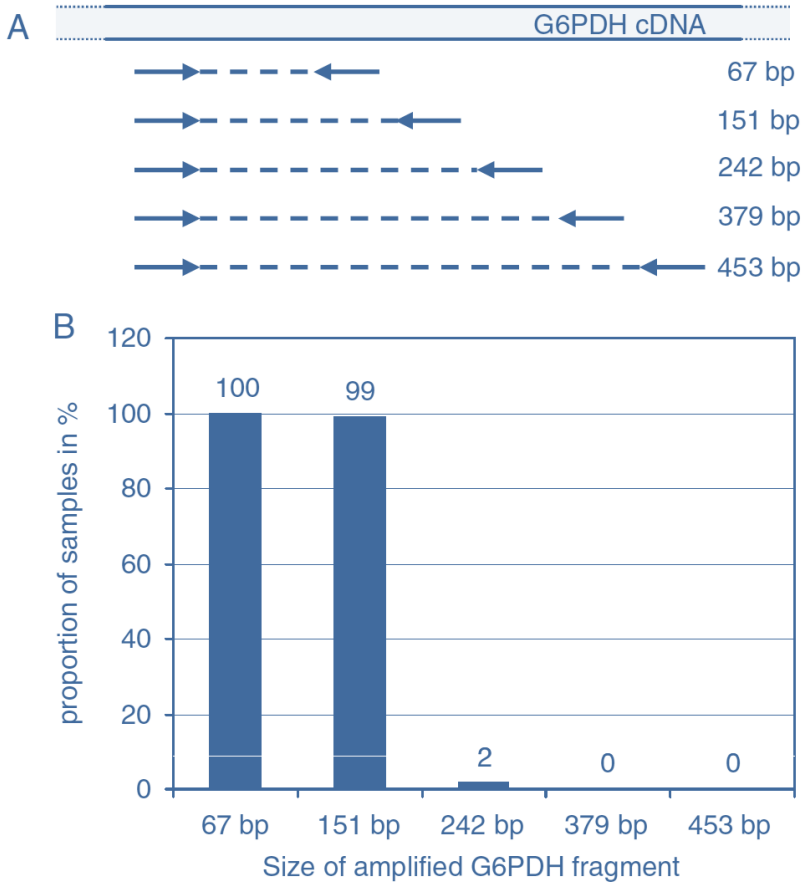


mRNA biomarkers in FFPE tissue

1. mRNA isolation is feasible from FFPE tissue („Formalin is an RNA-protective substance“)
2. mRNA analysis can be used to assess breast cancer biomarkers
3. EndoPredict / Oncotype Dx – routine mRNA expression analysis in breast cancer

RNA Isolation from FFPE tissue is feasible

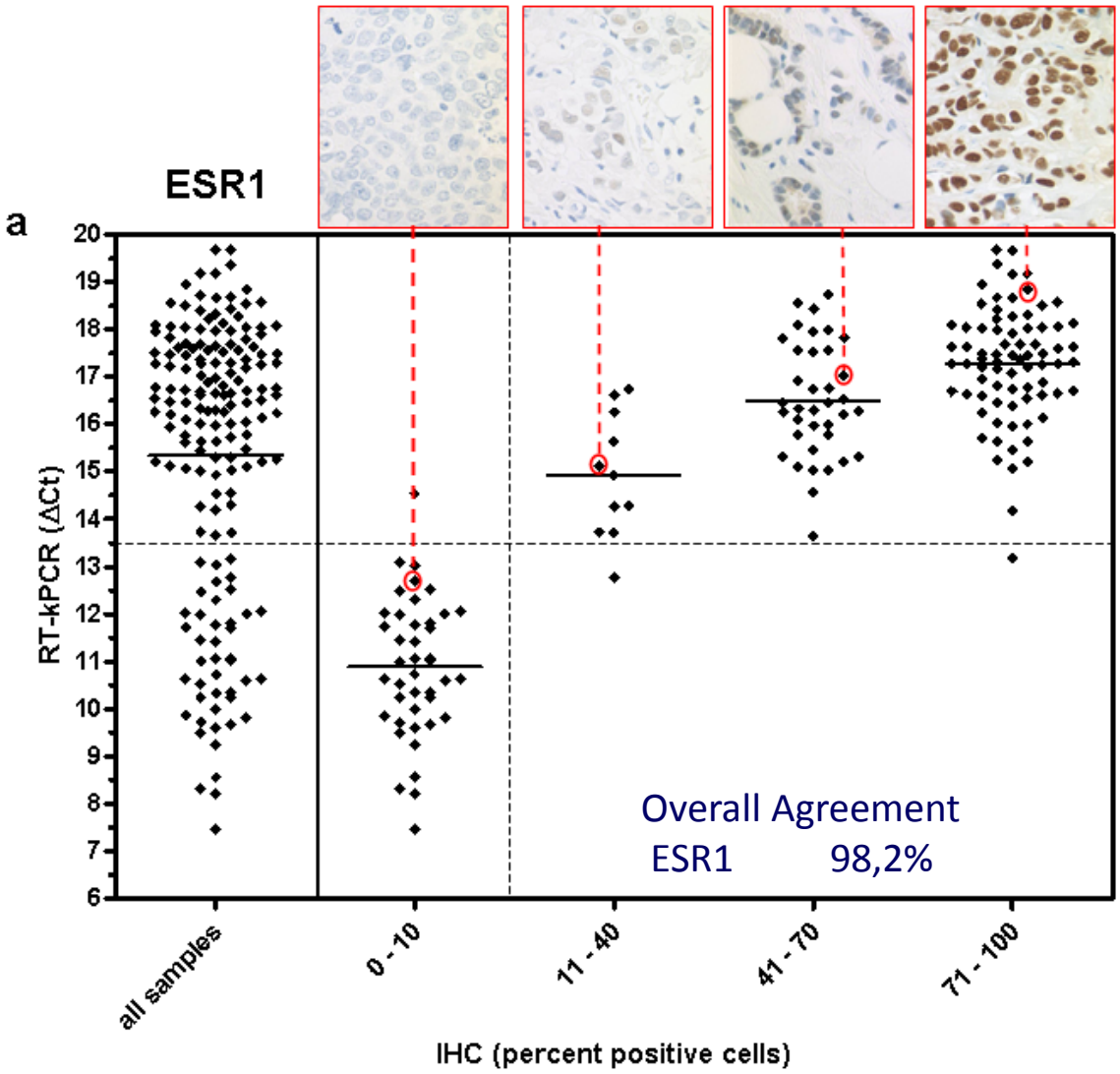


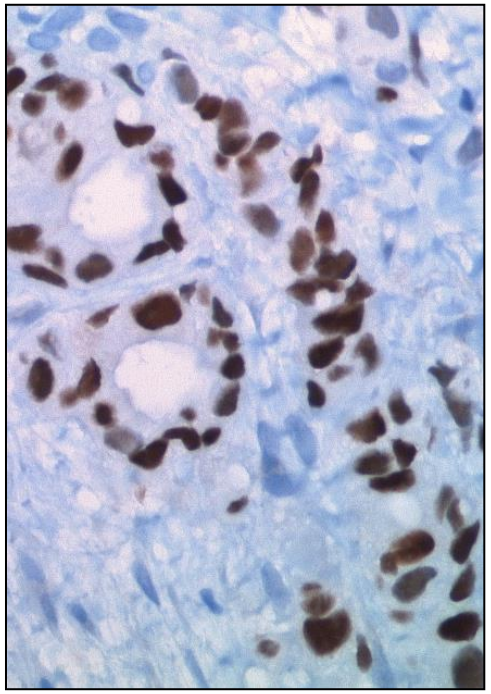


- 167 FFPE samples, age up to 21 years
- 501 RNA isolations
- Fragment length: ca 150bp
- RNA: ca. 1ug/10um section
  - = ca. 100 PCR reactions
- High concordance of consecutive sections

Müller et al., 2011

Concordance for ESR1 measured by RNA analysis and immunohistochemistry





Müller et al., 2011

Recurrence Score - Oncotype DX

16 genes and 5 control genes

Proliferation
Ki-67
STK15
Survivin
Cyclin B1
MYBL2

Estrogen
ER
PR
Bcl2
SCUBE2

GSTM1

BAG1

Invasion
Stromelysin 3
Cathepsin L2

CD68

HER-2
GRB7
HER-2

Reference
Beta-actin
GAPDH
RPLPO
GUS
TFRC

$$RS = +0.47 \times \text{HER-2 group score} - 0.34 \times \text{ER group score} + 1.04 \times \text{proliferation group} + 0.10 \times \text{invasion group score} + 0.05 \times \text{CD68} - 0.08 \times \text{GSTM1} - 0.07 \times \text{BAG1}$$

Category	RS (0 – 100)
Low risk	RS < 18
Intermediate risk	RS ≥ 18 and < 31
High risk	RS ≥ 31

Sparano JCO 2007

Recurrence Score - Oncotype DX

16 genes and 5 control genes

Proliferation
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BAG1

Invasion
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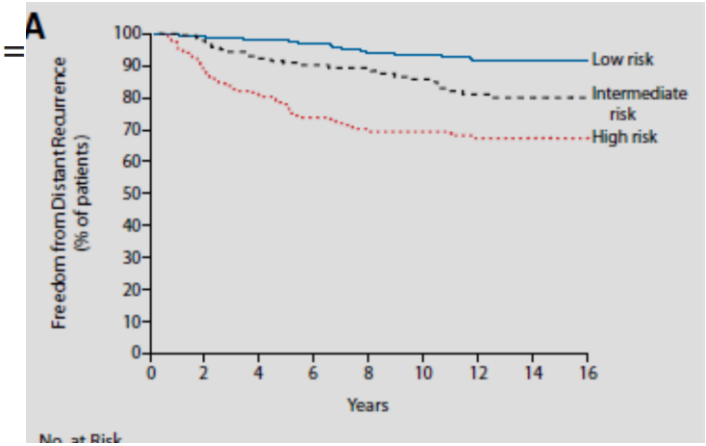
CD68

HER-2
GRB7
HER-2

Reference
Beta-actin
GAPDH
RPLPO
GUS
TFRC

RS =



Category	RS (0 – 100)
Low risk	RS < 18
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High risk	RS ≥ 31

Sparano JCO 2007

Development of the Endopredict assay

Training

Multicenter

Tam Monotherapy
(n=964)

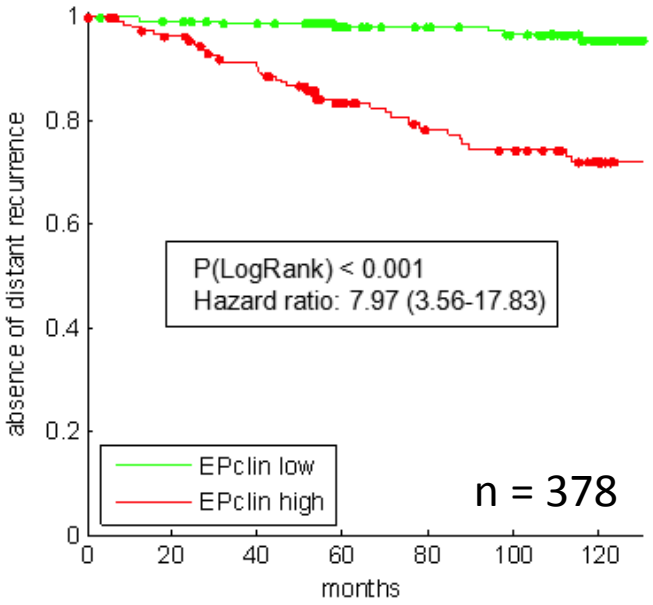
Validation I

ABCSG 6
TAM vs.
1st gen. AI
(n=378)

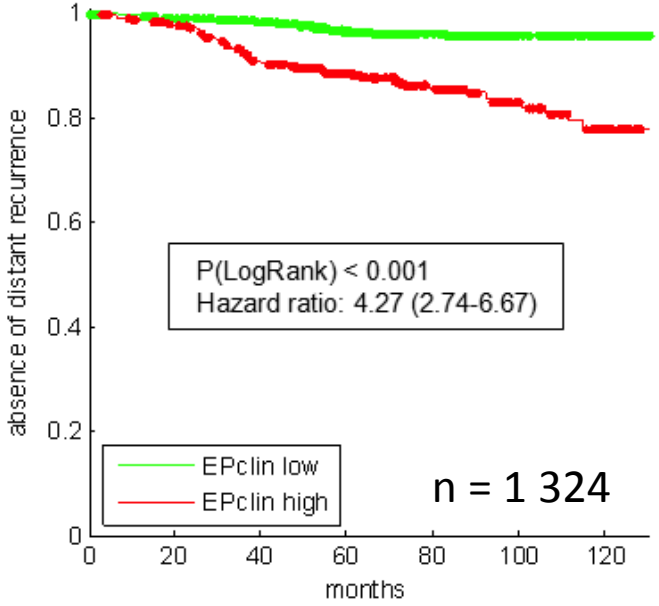
Validation II

ABCSG 8
TAM vs.
TAM/Anastrozol
(n=1.324)

ABCSG-6

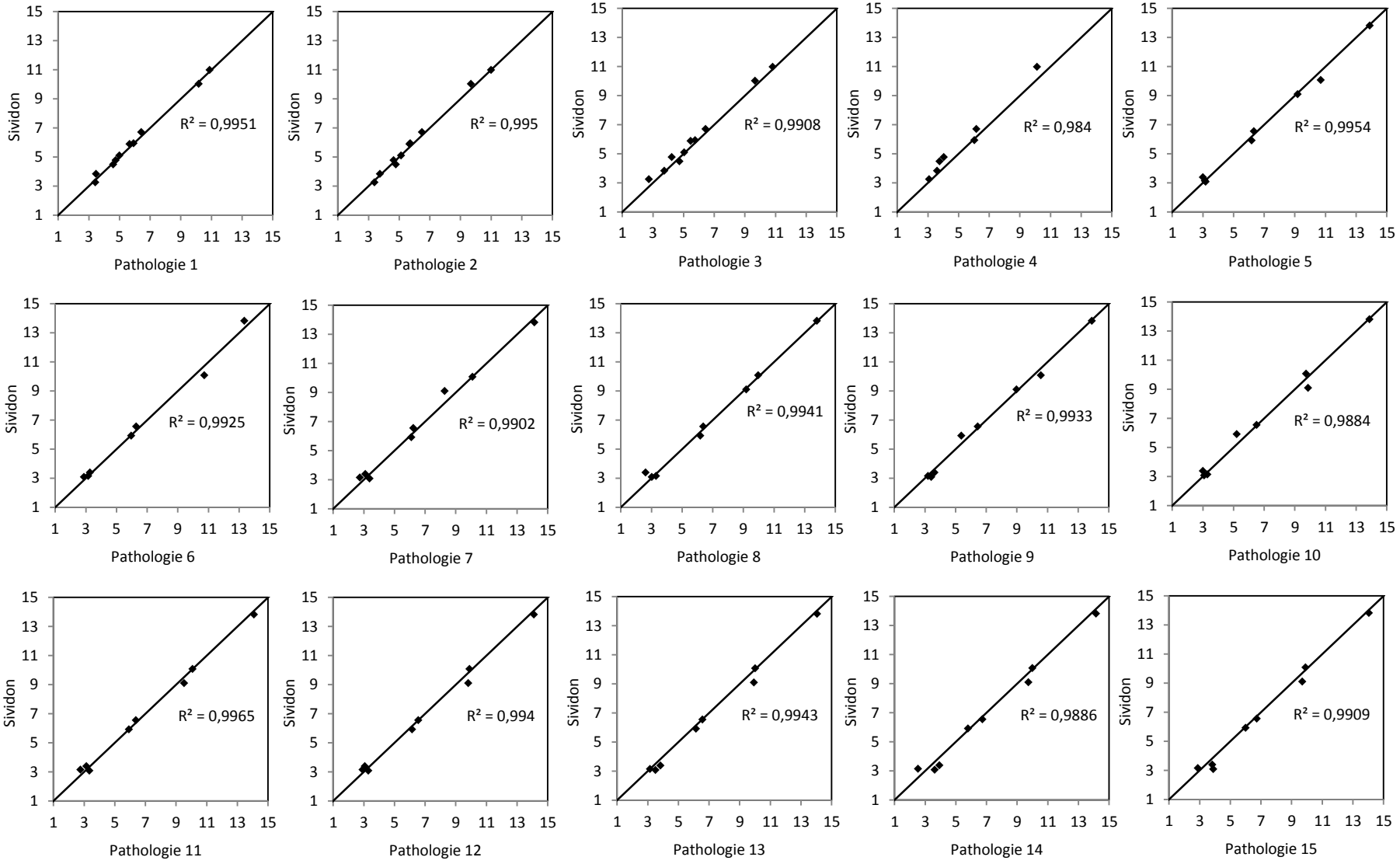


ABCSG-8



Filipits et al. Clinical Cancer Res. 2011

Endopredict Test – 15 different laboratories



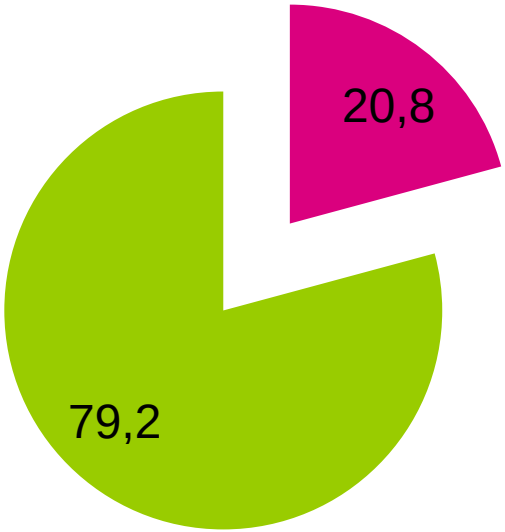
DNA markers and mutation analysis

- DNA is more stable than mRNA
- can be isolated from FFPE tissue
- fragmentation occurs – focus on analysis of small fragments

- classical Sanger sequencing
- NGS sequencing

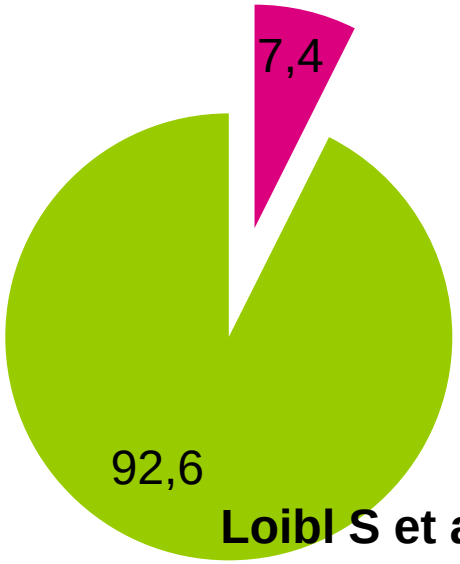
PIK3CA Mutation analysis (Exon 9 & 20) in FFPE samples of the GeparQuinto and GeparSixto study

HER2+ tumours n=360

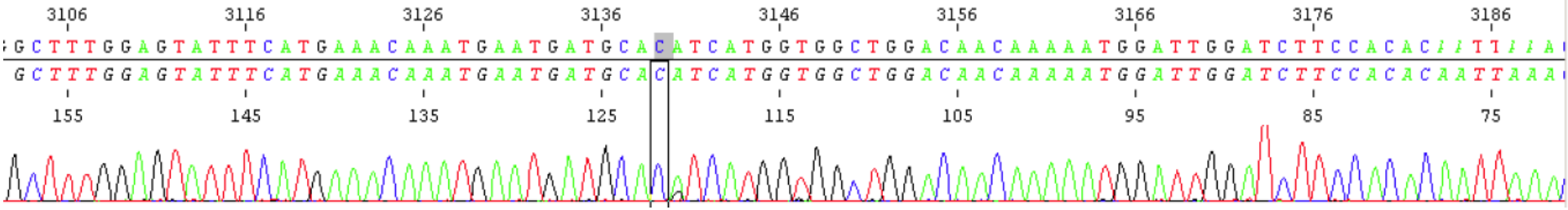


PIK3CA mt
PIK3CA wt

triple-negative tumours n=285

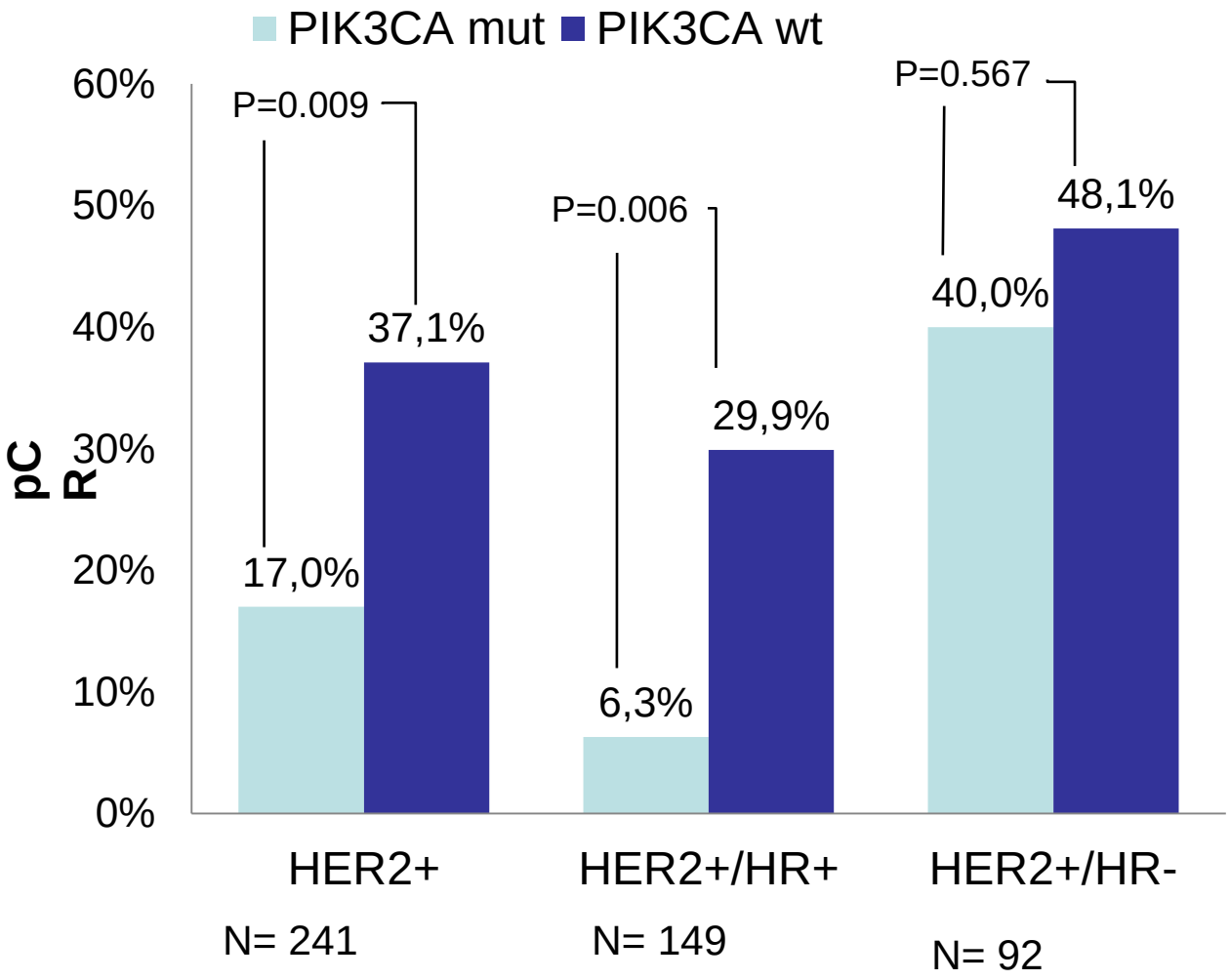


Loibl S et al. ASCO 2013



RESPONSIFY

pCR rate according to *PIK3CA* mutation status in GeparSixto study



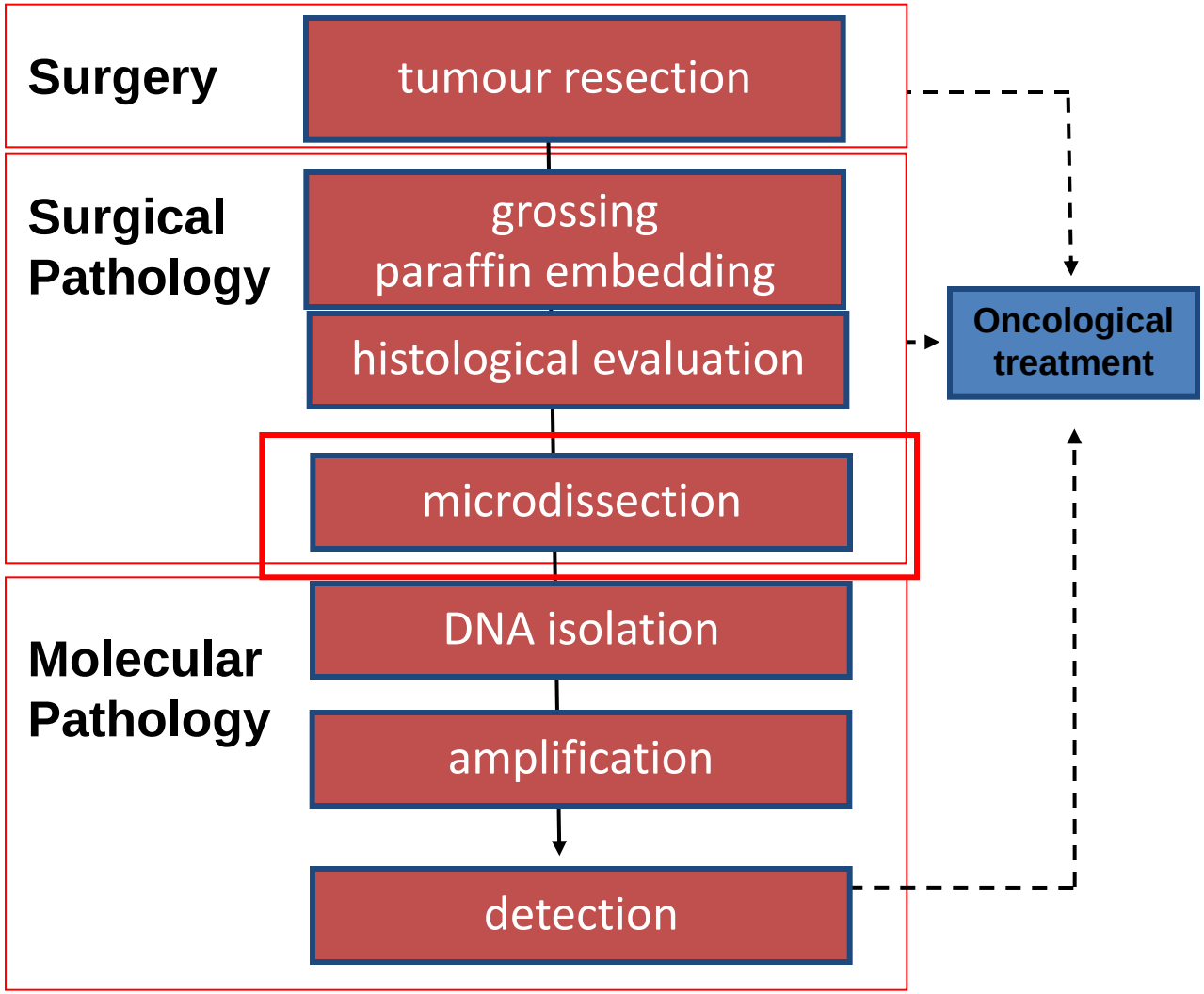
Multivariate

		Odds ratio	P-value
HR status	neg	1.00	0.006
	pos	0.44 (0.24-0.79)	
PIK3CA	wt	1.00	0.007
	mut	0.29 (0.12-0.71)	

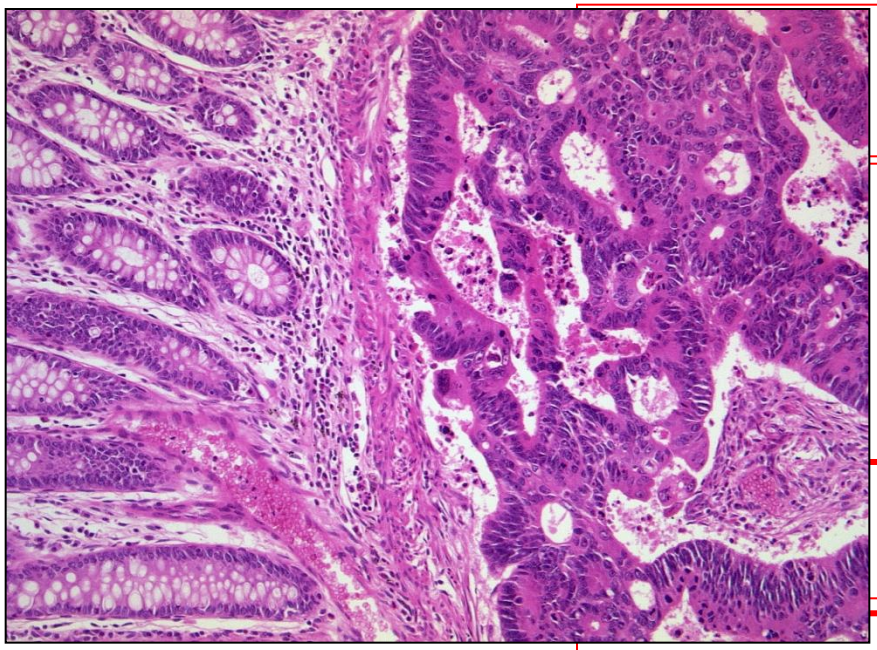
adjusted for therapy, age, tumour and nodal status, histotype and grading

RESPONSIFY

Workflow in molecular pathology



Workflow in molecular pathology



Molecular Pathology

tumour resection

grossing
paraffin embedding

histological evaluation

microdissection

DNA isolation

amplification

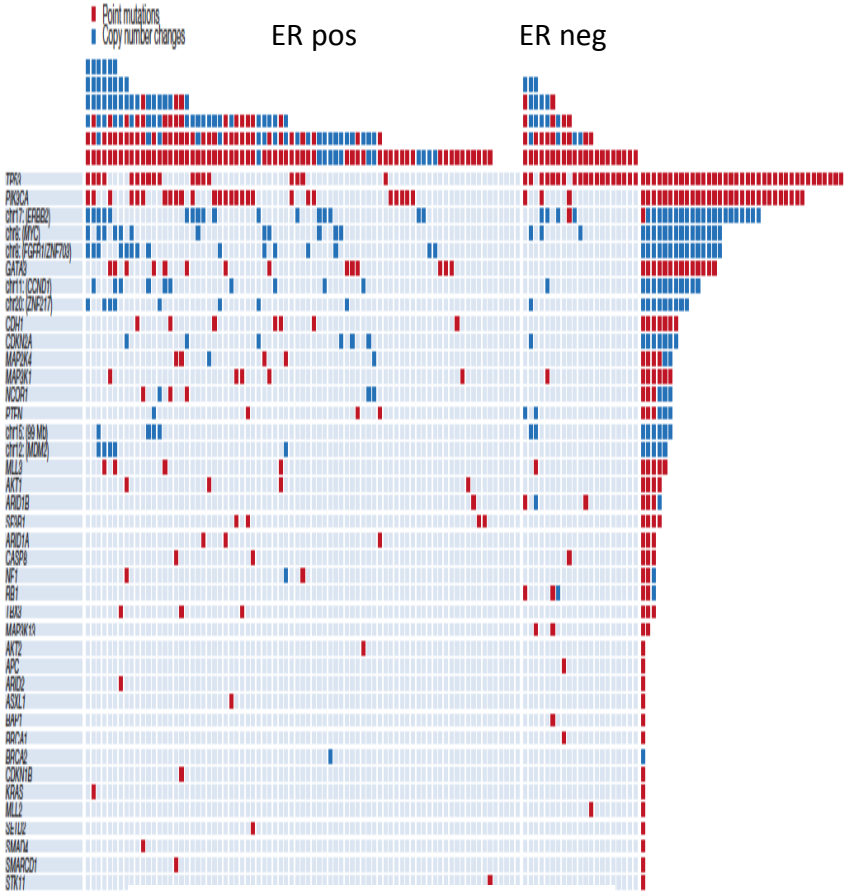
detection

Oncological treatment

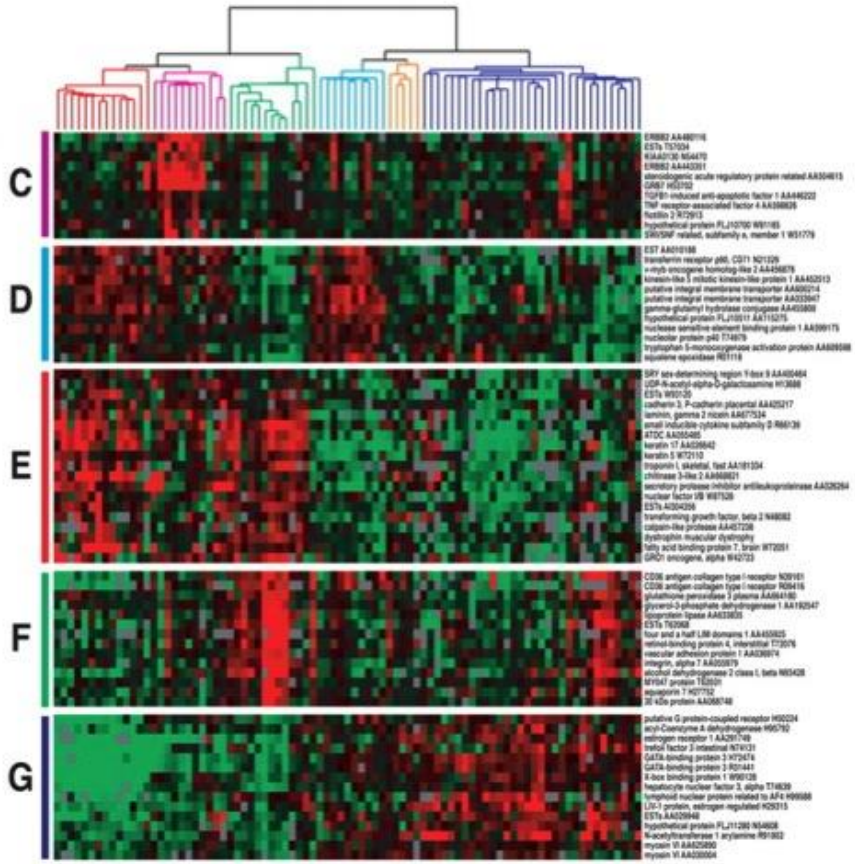
Next generation sequencing

- NGS: all genes / transcripts in a tumor are measured
- overview on all genetic alterations in a tumor
  - Mutations
  - Copy number variations
  - Amplifikations
  - Deletions
- applicable to routine pathology: targeted exome sequencing

High throughput technologies - mRNA and DNA alterations in breast cancer

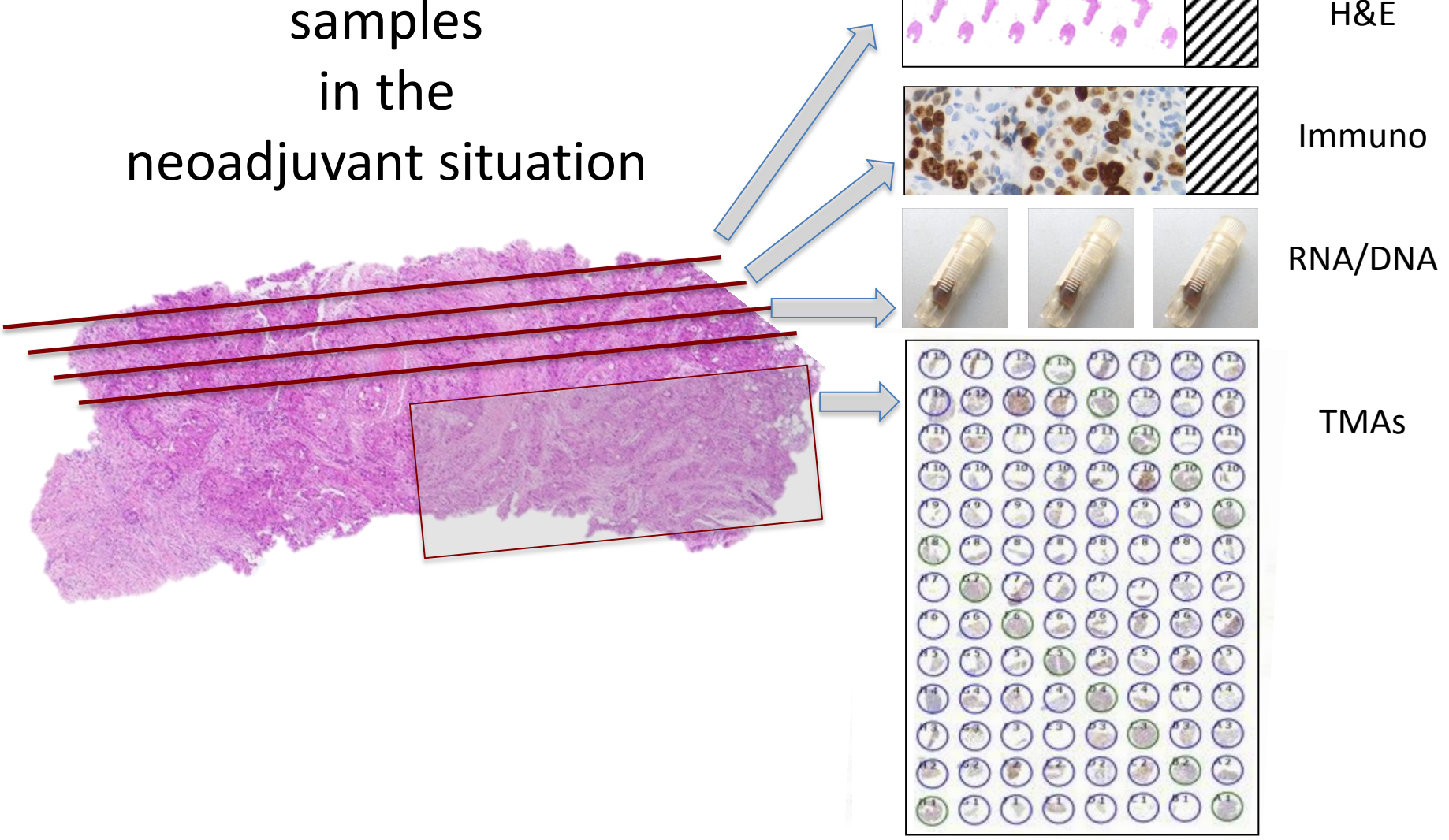


Stephens, Nature June 2012



Sorlie, et al. (2001)
Proc. Natl. Acad. Sci. USA 98, 10869-10874

Efficient workflow: Tumor samples in the neoadjuvant situation



GBG
GERMAN BREAST GROUP

Conclusion – methods in pathology

- FFPE tissue is suitable for analysis of protein, mRNA and DNA markers
- targeted exome sequencing as an upcoming method in routine pathology
- critical parameters are:
  - standardisation and quantification for immunohistochemistry
  - selection of primers for mRNA / DNA analysis
  - selection of tissue area for mRNA / DNA analysis
- requirements for practice changing research in pathology: high level of evidence, „prospective-retrospetive“ studies, large sample collections