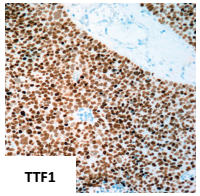
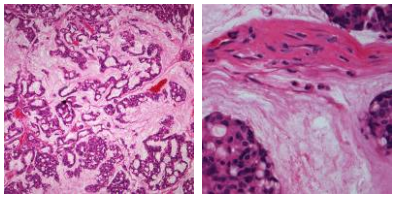
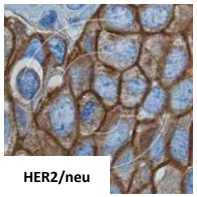
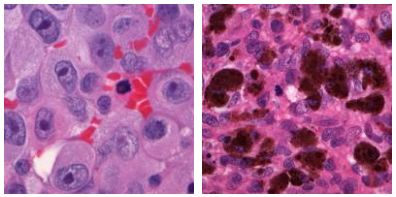
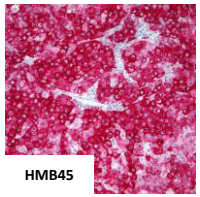
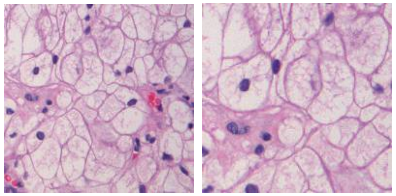
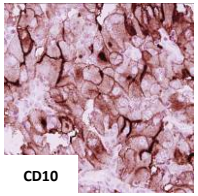
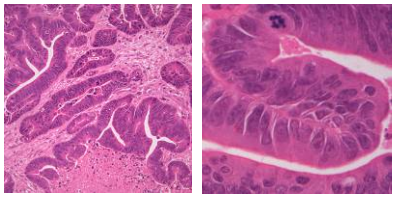
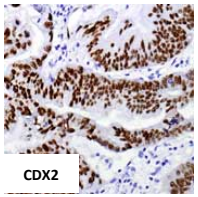
UniversitätsKlinikum Heidelberg

Micro-RNA-based identification of the primary tumor tissue

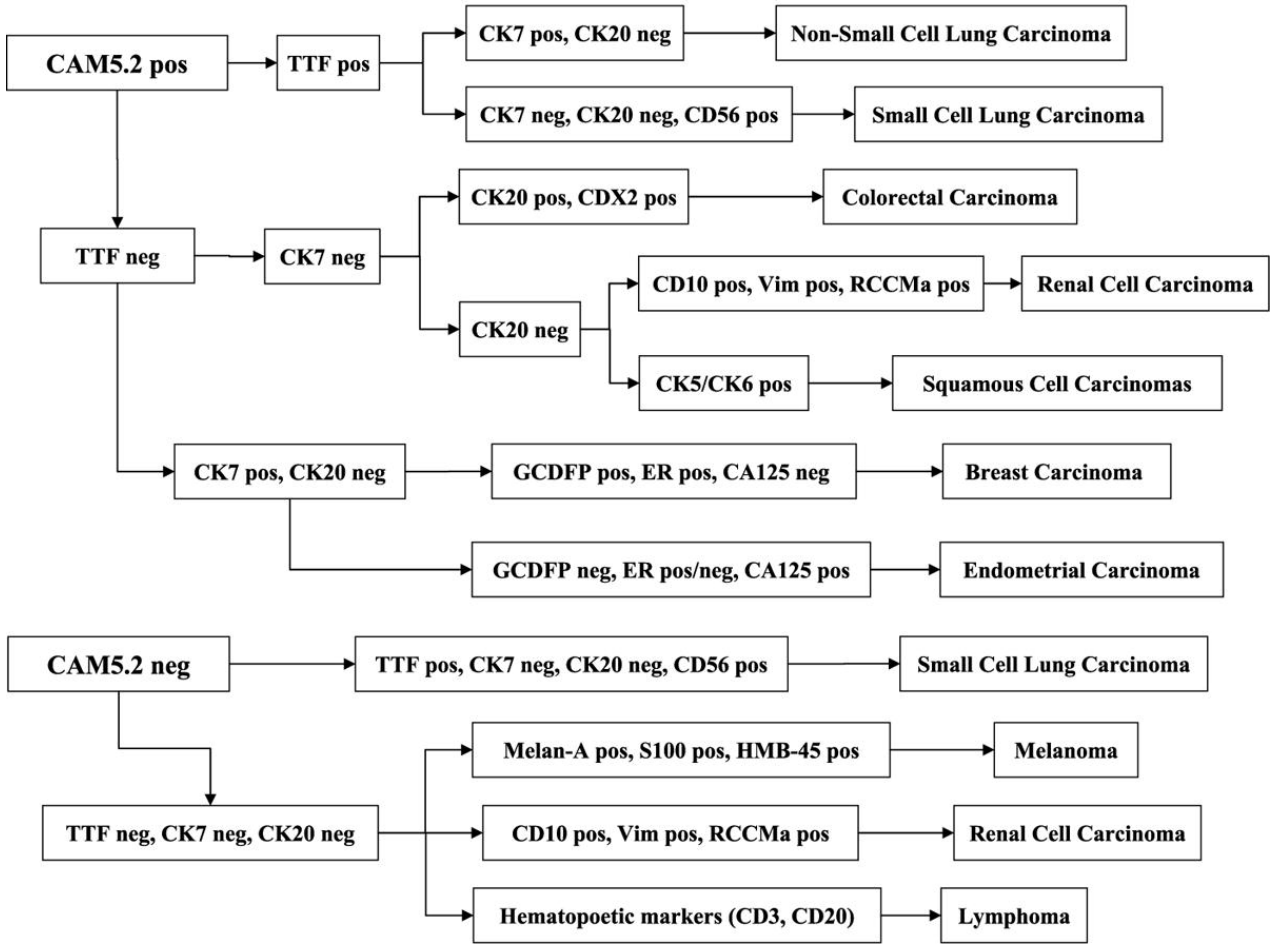
Wolf C Mueller, M.D.

Department of Neuropathology, Institute of Pathology,

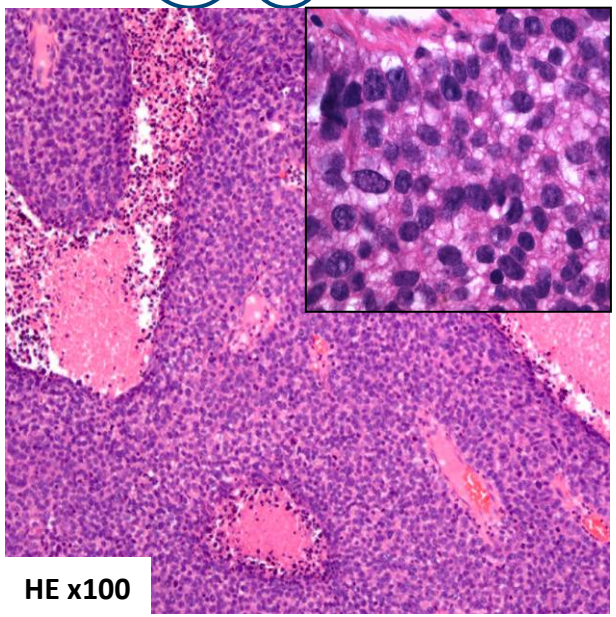
University Hospital Heidelberg, Germany

Primary Tumors	Morphology		Immunhistochemistry	
Lung (50%)	variable		TTF1 NAPSIN Surfactant	
Breast (15-20%)	Stroma		Mammaglobin ER PR HER2/neu	
Melanoma (5-10%)	Pigment Nucleoli		HMB45 S-100 Melan A	
Urogenital (Kidney) (5-10%)	„Plant cell- like“		CD10 PAX2 PAX8	
Gastrointestinal (5-10%)	Cytology		CDX2	
CUP (10%)	not indicative		not indicative	

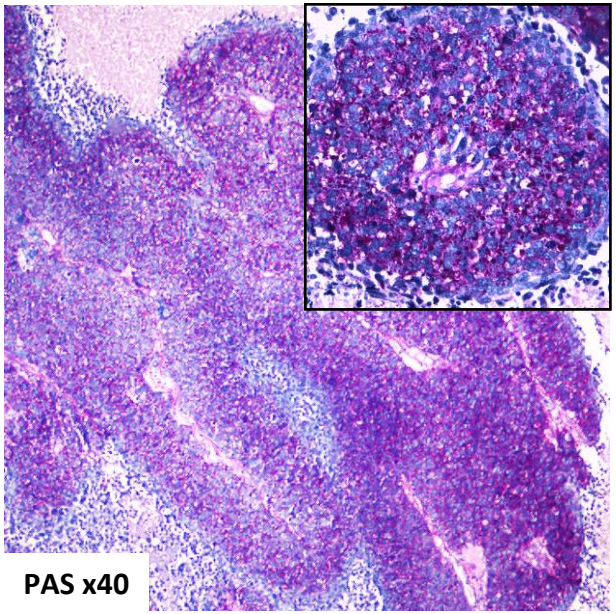
Identification of primary tumors based on IHC



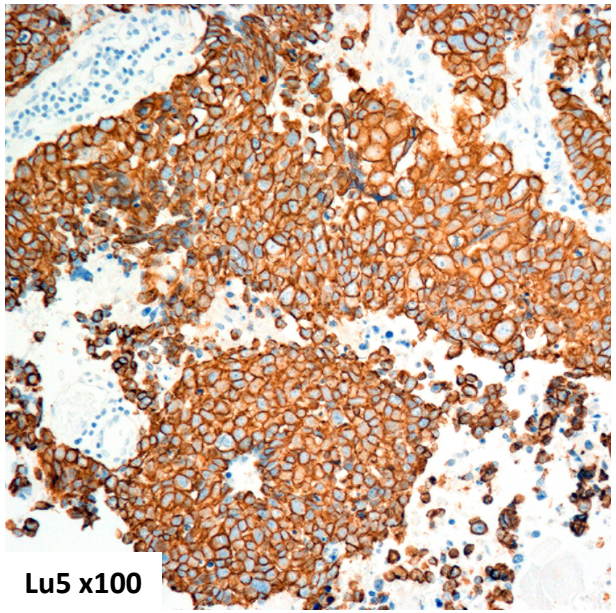
modified: Becher, M. et al. ***J Neuropath & Exp Neurol.*** 2006.



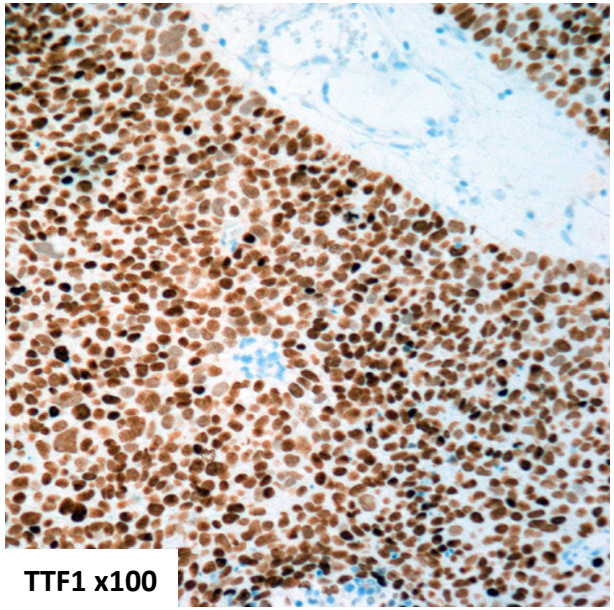
HE x100



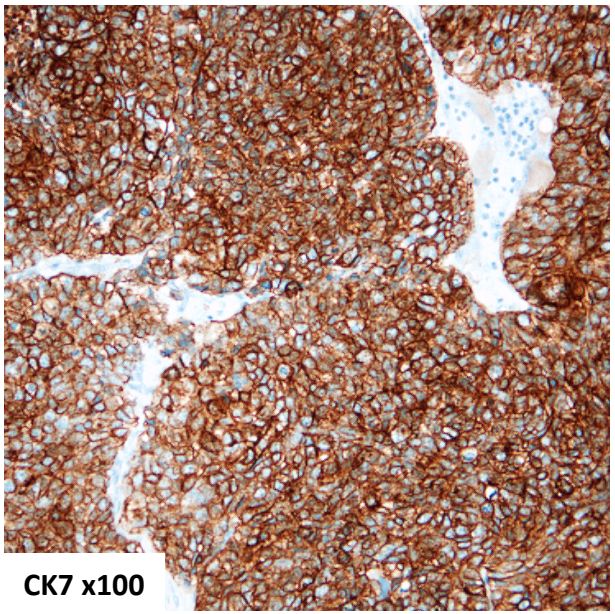
PAS x40



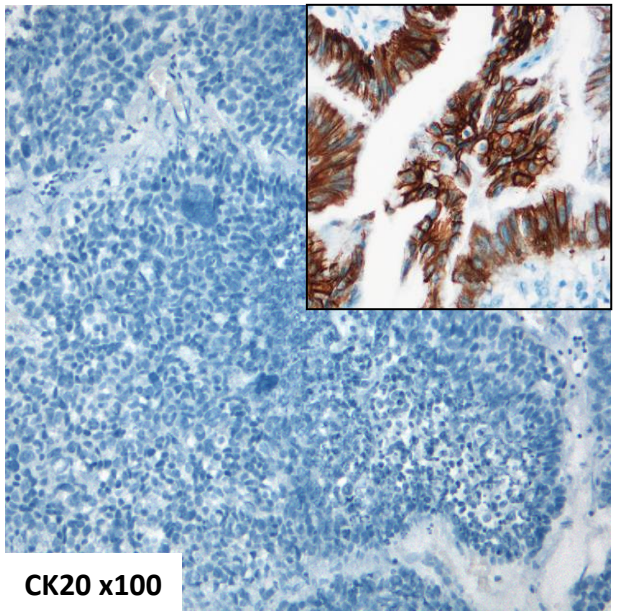
Lu5 x100



TTF1 x100

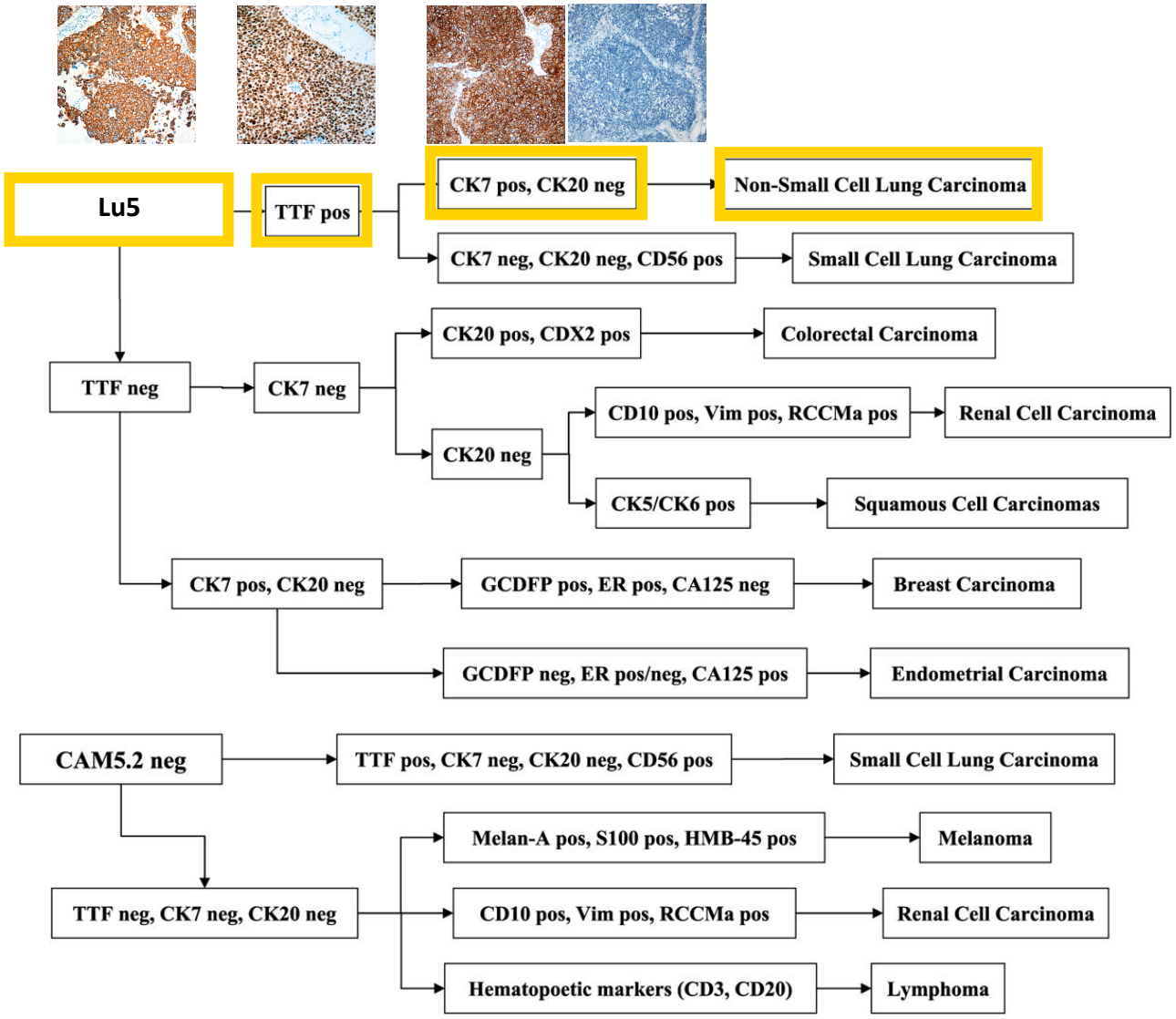


CK7 x100



CK20 x100

Identification of primary tumors based on IHC



Diagnosis

Histopathology/ IHC

Metastasis of an adenokarcinoma (PAS)

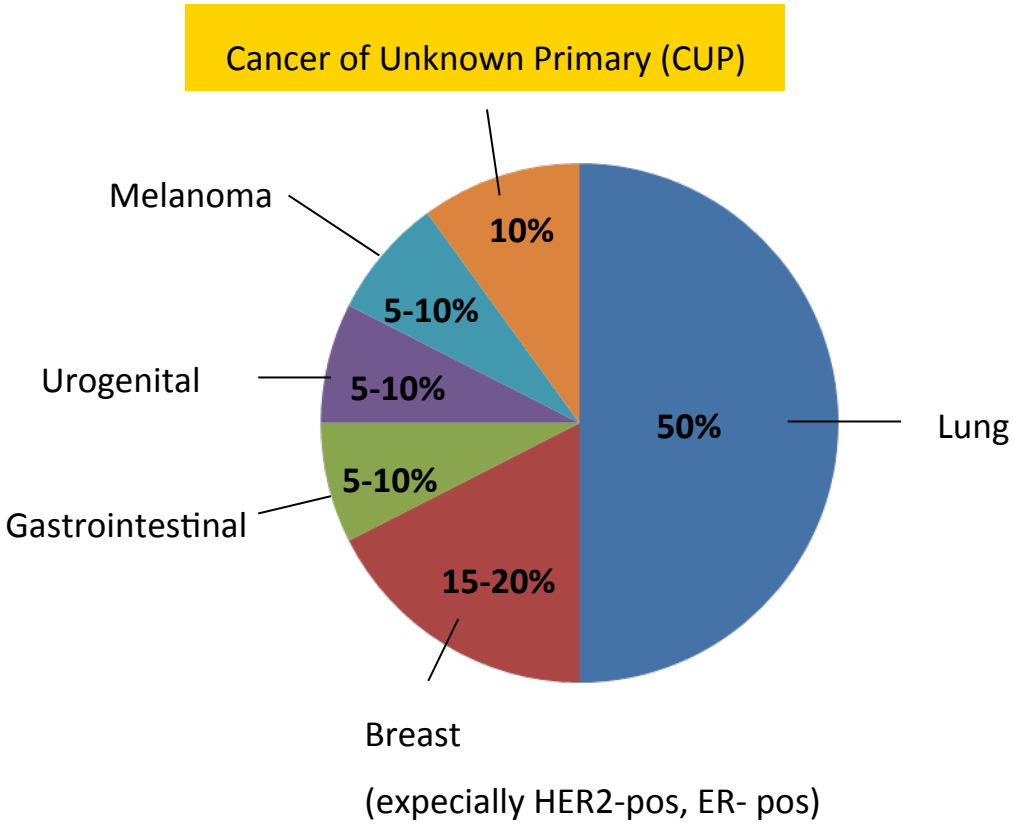
Primary tumor

Organ/Morphologie/IHC

Lung

modifiziert nach: Becher, M. et al. ***J Neuropath & Exp Neurol.*** 2006.

Distribution of primary tumors in patients with brain metastases



The Need

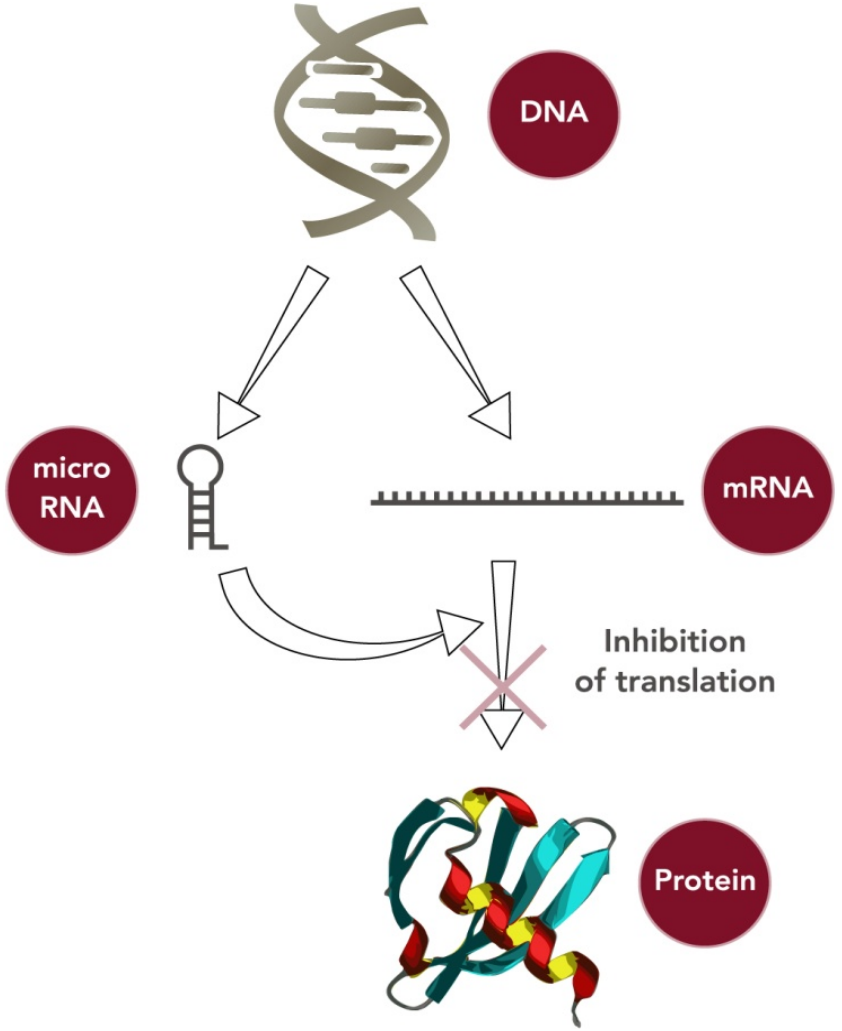
- Approx. 1/3 of all U.S. cancer patients (~400,000 patients) the tumor first identified is a metastasis
- approx. 5-10% cancer patients (~60,000, U.S.) the primary origin of the metastases is never identified, “Cancer of Unknown Primary” (CUP).
- Current primary tumor identification: costly, time consuming, and at times inefficient (physical examination of the patient, histopathology analysis of the biopsy, and imaging methods such as chest X-ray, CT and PET scans, endoscopy *etc*.)
- The average cost of full CUP diagnosis was estimated at $18,000*

Accurate identification of the primary tumor is critical for appropriate treatment administration and prognosis of patient.

*Schapira DV, Jarrett AR. Arch Intern Med. **1995**; 155:2050-2054.

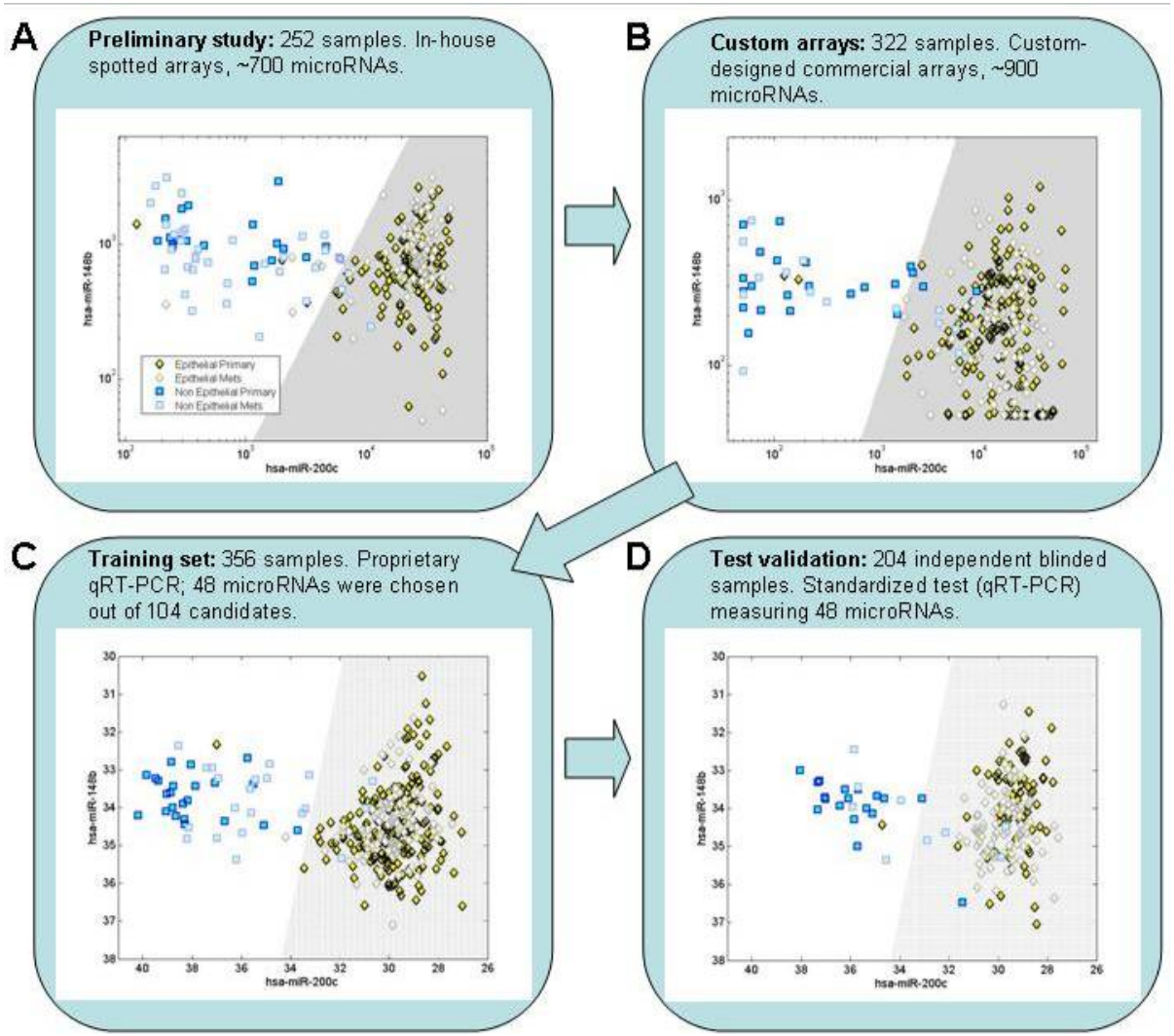
The molecule

- High tissue specificity
- Stable markers in body fluids and tissue samples
- Established role in cancer
- Can be profiled with high sensitivity and specificity



- microRNA is a ~22nt long single strand RNA
- Regulate the translation of thousands of protein coding genes
- There are ~1000 human microRNAs
- Central mechanism of post-transcriptional regulation
- Central to cell differentiation and development
- Associated with major diseases, including: cancer, obesity, diabetes

miRview mets™ Test: Development Stages

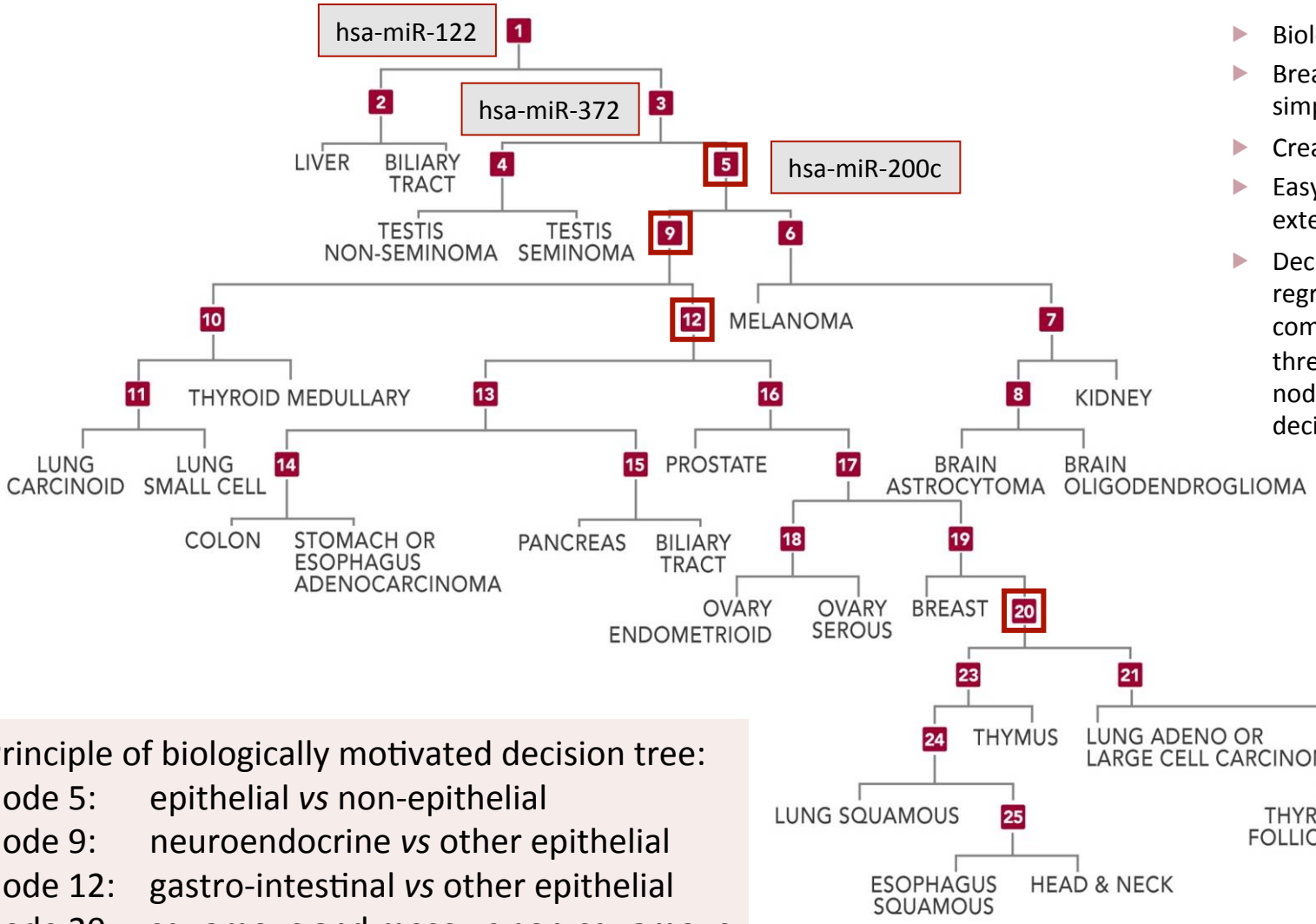


Rosenwald, Gilad et al. Modern Pathology 23: 814-823, 2010

The tool

- qRT-PCR test investigating the expression profile of 48 miRNAs in FFPE- tissue was trained to distinguish 17 tissue origins (organs) and 25 tumor classes (histological subtypes derived of the these 17 tissues) (Rosenwald et al., Modern Pathol. 2010).
- the 48 miRNAs were selected based on their biological relevance and specific expression profiles in preceding microarray and qRT- PCR- based validation studies (Rosenfeld et al., Nat Biotechnol. 2008; Rosenwald et al., Modern Pathol. 2010)
- classification combines a biologically motivated binary decision tree and a K- nearest neighbor algorithm (KNN) to improve robustness and accuracy (Rosenfeld et al., Nat Biotechnol. 2008; Rosenwald et al., Modern Pathol. 2010)

Classifier 1: Biologically motivated decision tree



- Biologically motivated
- Breaks up problem into simple decisions
- Creates context
- Easy to incorporate external data
- Decision tree uses logistic regression on combinations of one-to-three miRNAs in each node to make binary decisions.

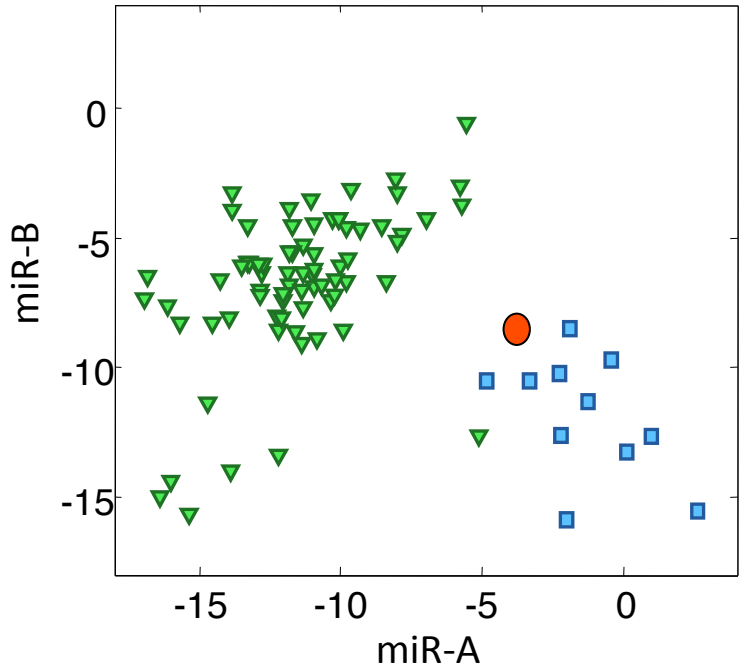
Principle of biologically motivated decision tree:

node 5: epithelial *vs* non-epithelial
node 9: neuroendocrine *vs* other epithelial
node 12: gastro-intestinal *vs* other epithelial
node 20: squamous and meso *vs* non-squamous

Classifier 2: K-Nearest Neighbors (KNN) Algorithm

KNN- algorithm:

- expression of all 48 miRNAs of the sample are compared to all samples in the training database (n=356)
- nearest seven samples from the training database are selected as compared to the sample to be classified
- majority vote of these seven samples measured by Pearson correlation finally classifies the unknown sample

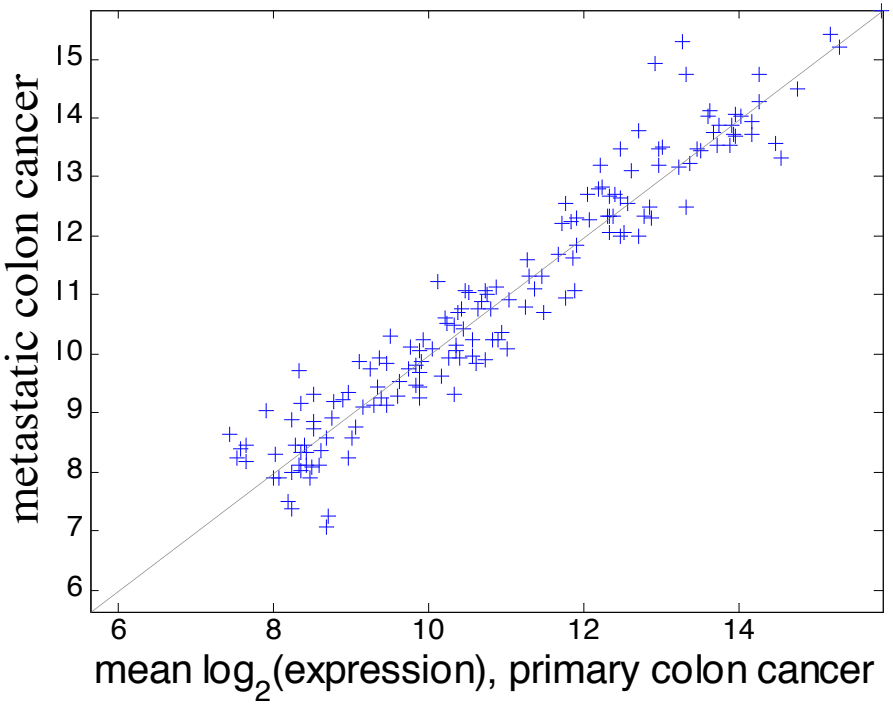


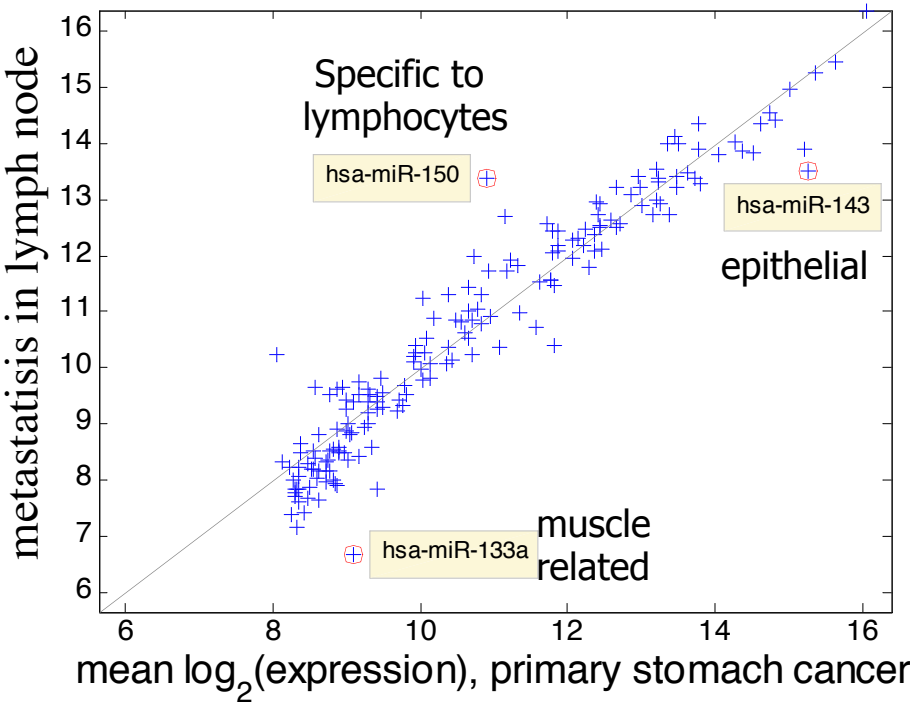
miRview™ mets – Accurate Molecular Classification

- Sensitivity: ~85% - >90%
- Specificity: 99%
- In 2/3 of cases the test reports a single high-confidence origin accurate in nearly 90% of cases
- In remaining cases two possible origins are reported
- Utilizes a relatively small number of molecular markers making the test more robust and transparent

Primary tumors *vs* their metastases

- Comparing primary to metastatic tumors:
  - Overall highly similar
  - Specific sites can have characteristic miRs: these have to be identified and excluded from future analyses.





Classification of brain metastases

First study of its kind to classify metastatic lesions confined to the brain implementing the miRview™ mets assay developed by Rosetta Genomics

Phase I

(Validation/ Feasibility study)

- Metastases to the brain
- primary tumors known to the pathologist
- investigating lab blinded to the primary tumor location and histology

Phase II

(true CUP- case analyses)

- Metastases to the brain
- primary tumor at time of diagnosis unknown
- ambiguous IHC in pathology
- full clinical work-up or tumor progression later identified primary tumor location

Samples in the assay – Phase 1

- 101 samples were tested
- 93 of these were metastases to the brain.
- 8 of these were metastases to the spinal bone or spinal cord

- 12 of the 101 samples were metastatic lesions of prostate cancer (later to be excluded from the analysis)
- **89** samples derived from **11** primary tumor sites remained for a full analysis

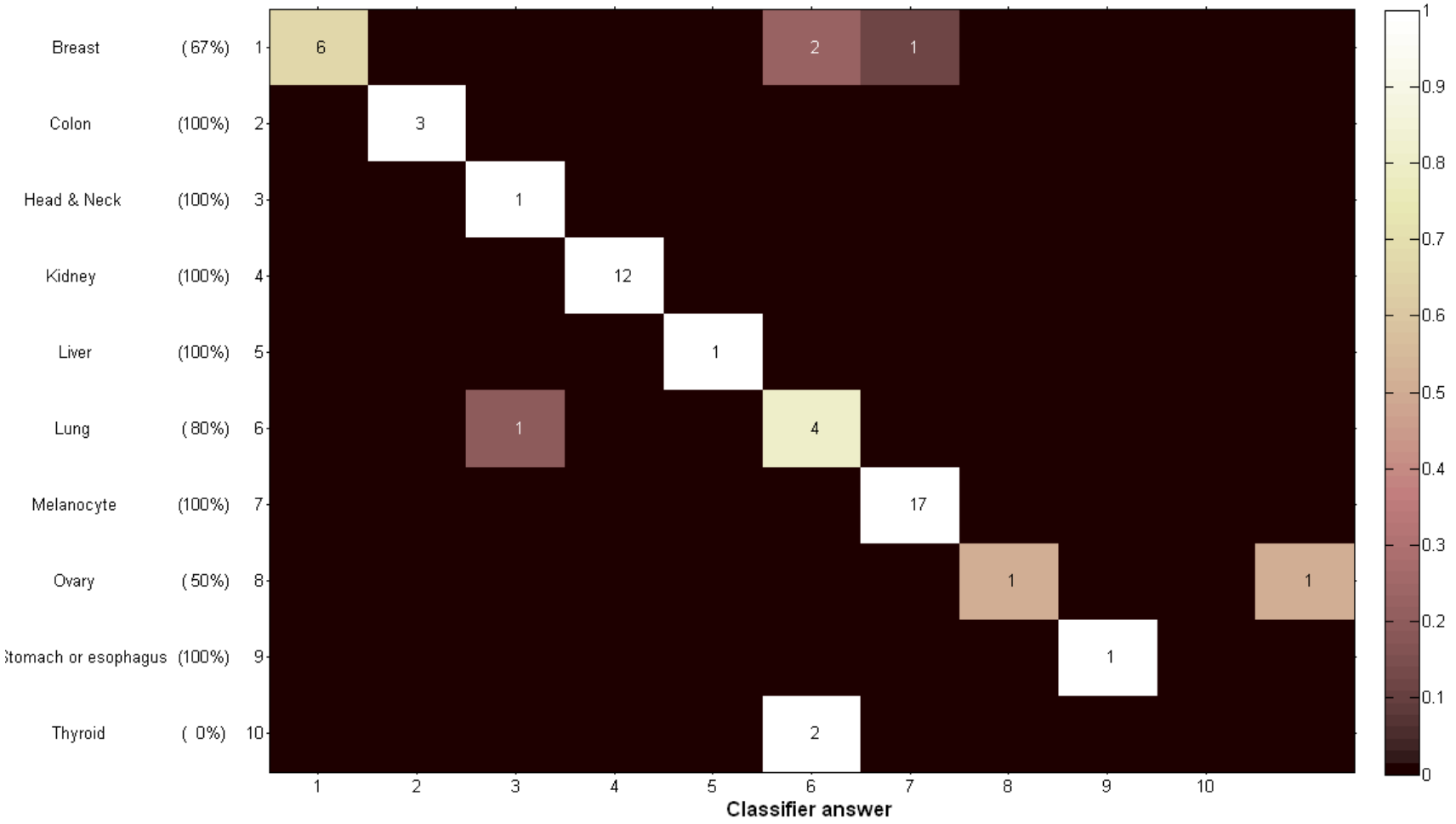
Performance (all samples without 12 prostate cancer samples)

Results of assay on 89 samples

Tissue	N (successful samples in test set)	Sensitivity (%)	Specificity (%)	N in single answer	Sensitivity of single answer	Specificity of single answer
Biliary tract	1	**0**	100	0	**-------**	100
Breast	18	**72.22**	95.8	9	**66.67**	100
Colon	4	**75**	95.3	3	**100**	100
Head & Neck	4	**100**	89.4	1	**100**	98.1
Kidney	17	**94.12**	98.6	12	**100**	100
Liver	1	**100**	100	1	**100**	100
Lung	16	**87.5**	78.1	5	**80**	91.7
Melanoma	17	**100**	90.3	17	**100**	97.2
Ovary	5	**60**	97.6	2	**50**	100
Stomach or esophagus	4	**100**	98.8	1	**100**	100
Thyroid	2	**0**	97.7	2	**0**	100

Performance (single answer without 12 prostate cancer samples)

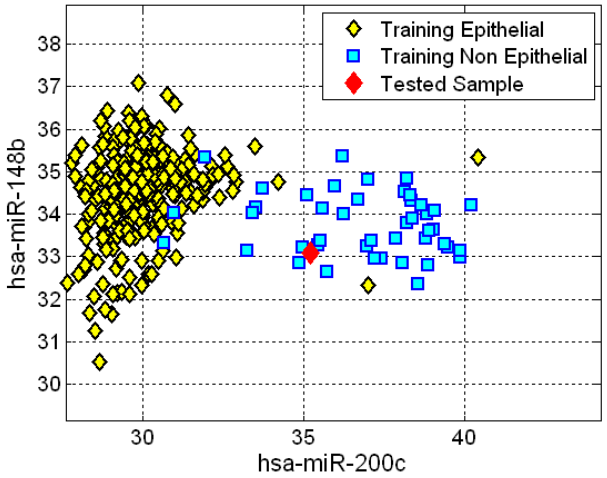
Classifications of 53 single answer samples



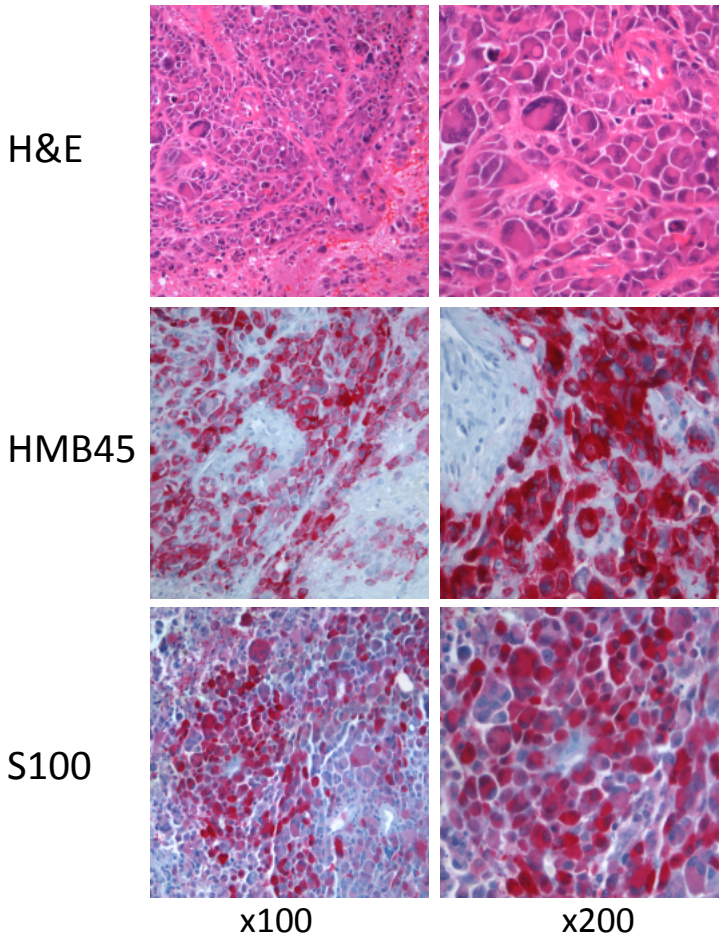
Special Case

- Suspected breast cancer metastasis that turn out melanoma in the epithelial node (Decision tree analysis, node 5)

A



B



Wolf C. Mueller, Heidelberg

Conclusions: Phase I

- miRview™ mets assay reaches high sensitivity and specificity for the majority of tested primary tumors sites
- Sensitivity is further increased in cases with single answers
- As an unbiased test, miRview™ mets assay can indicate clinically unsuspected or “neglected” primary tumor sites (see malignant melanoma *vs* breast cancer)
- **BUT** – for further refinement and extension of primary tumor sites miRview™ mets assay would profit from further training on metastases derived from less frequently observed primary tumor sites in the CNS (*i.e.* ovary, thyroid)

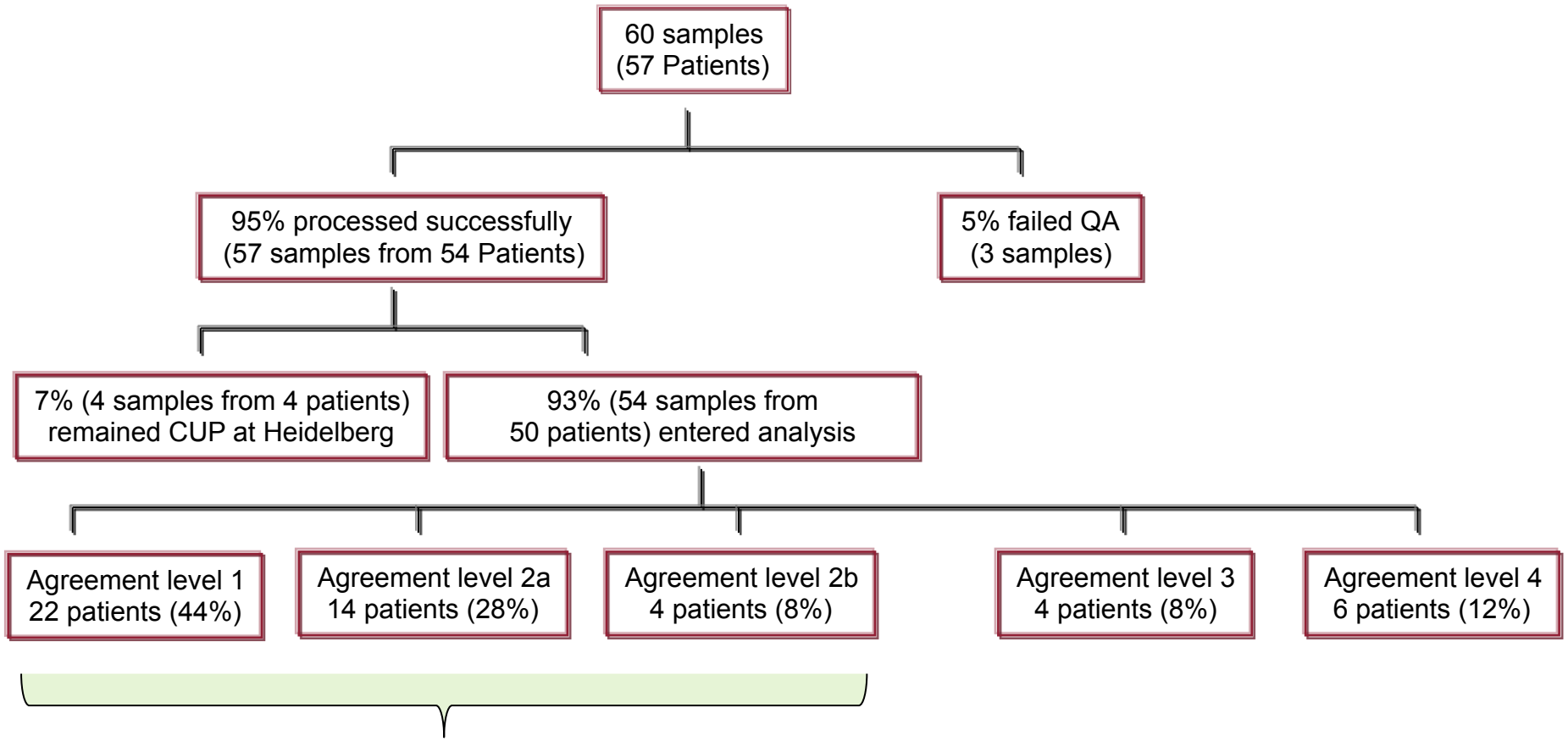
Phase II - Study description

- 60 samples representing 57 patients with CNS metastasis from unknown origin were sent to Rosetta Genomics
- 47: metastases to the brain.
- 10: metastases to spinal bone or spinal cord
- Processed (blinded) at Rosetta's CLIA laboratory (Philadelphia, USA)
- Results were crossed with IHC results and with patient's clinical and follow up data

Phase II - Results

- prediction of miRview™ mets was compared with results of a full workup:
  - Pathologic workup
  - Imaging
  - Clinical examination
- Each prediction received a score:
  - Agreement **level-1 – Clinical-Match**, assay result confirmed by both clinic and pathology
  - Agreement **level-2 – Pathology-Match** (no clinically verified primary tumor to date)
    - Level-2a: pathology findings were “consistent-with” the test results
    - Level-2b: pathology findings could not ”rule-out” the test results
  - Agreement **level-3– Pathology-Mismatch** (no clinically verified primary tumor to date), pathology work-up was not typical for the test diagnosis
  - Agreement **level-4 – Clinical-Mismatch**, clinical diagnosis was discordant with the test result

Phase II Results



80%
Concordance with clinical presentation and pathology

Conclusions: Phase II

- miRview™ mets assay reaches agreement with clinico-pathology data of true CUP patients in the majority of analyzed cases (80%)
- true performance of miRview™ mets assay in cases with “pathology-mismatch” remains unresolved due to still unrecognized primary tumor sites in these cases
- Performance of miRview™ mets assay in cases with “clinical-mismatch” necessitates further refinement of the assay with additional samples from these sites

miRview™ mets assay will not replace histology/IHC work-up of metastatic lesions to the brain – but is a powerful tool to guide clinical work-up in cases with ambiguous clinico-pathological findings. (Mueller et al. **Oncologist**. 2011;16(2):165-74)

miRview® mets^2 overview

Wolf C. Mueller, Heidelberg

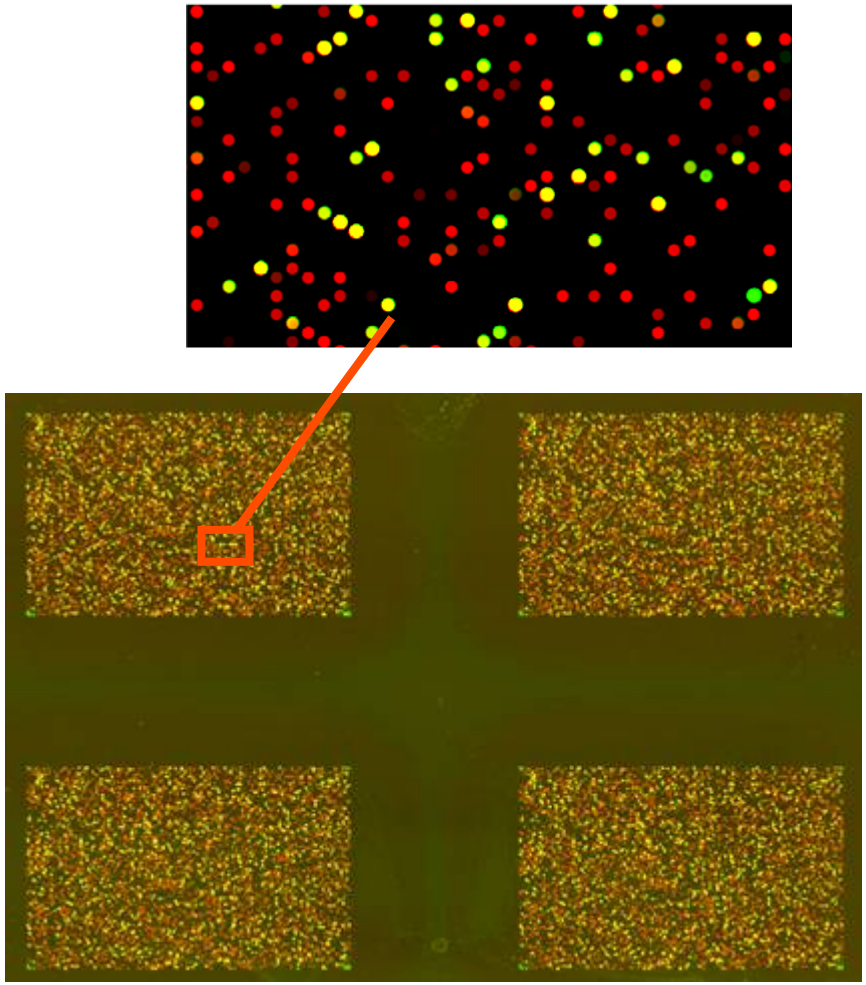
miRview® mets^2 overview

- Identifies the tissue-of-origin for 42 different types of tumors[1] which represent over 92% of all cancer prevalence
- Leverages proven proprietary microRNA technology to measure the expression level of 64 microRNA biomarkers
- ~1300 samples used for developing the assay
- The test returns either a single tissue of origin or two such origins
- Performance characteristics based on blinded validation set of 509 samples:
  - Overall sensitivity of 85%
  - Overall specificity of 99.3%
  - Vast majority of reported outcomes is a single tissue of origin
  - Sensitivity for single answer is 90%

miRview® mets^2 overview

Microarray platform

- Contains > 900 human microRNAs
- Probes are printed in triplicate
- Negative controls
- Positive controls:
  - Synthetic small RNA oligonucleotides
  - Small RNA – 6 probes
- Very reproducible results
- High sensitivity
- Dynamic range of more than 3 orders of magnitude



miRview® mets^2 overview

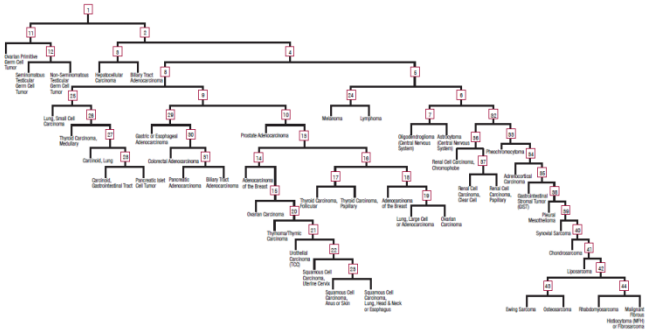
Complementary classifiers

Array results

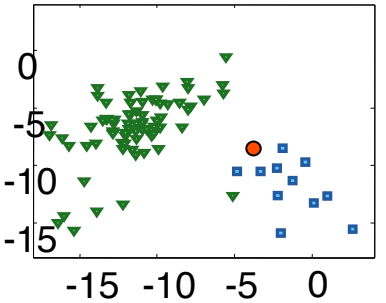
Signal Processing

Two classifiers are employed to interpret the expression of 64 microRNAs as a combined classifier

Decision Tree



KNN



Decision Maker

miRview® mets^2 overview

Assay Methodology

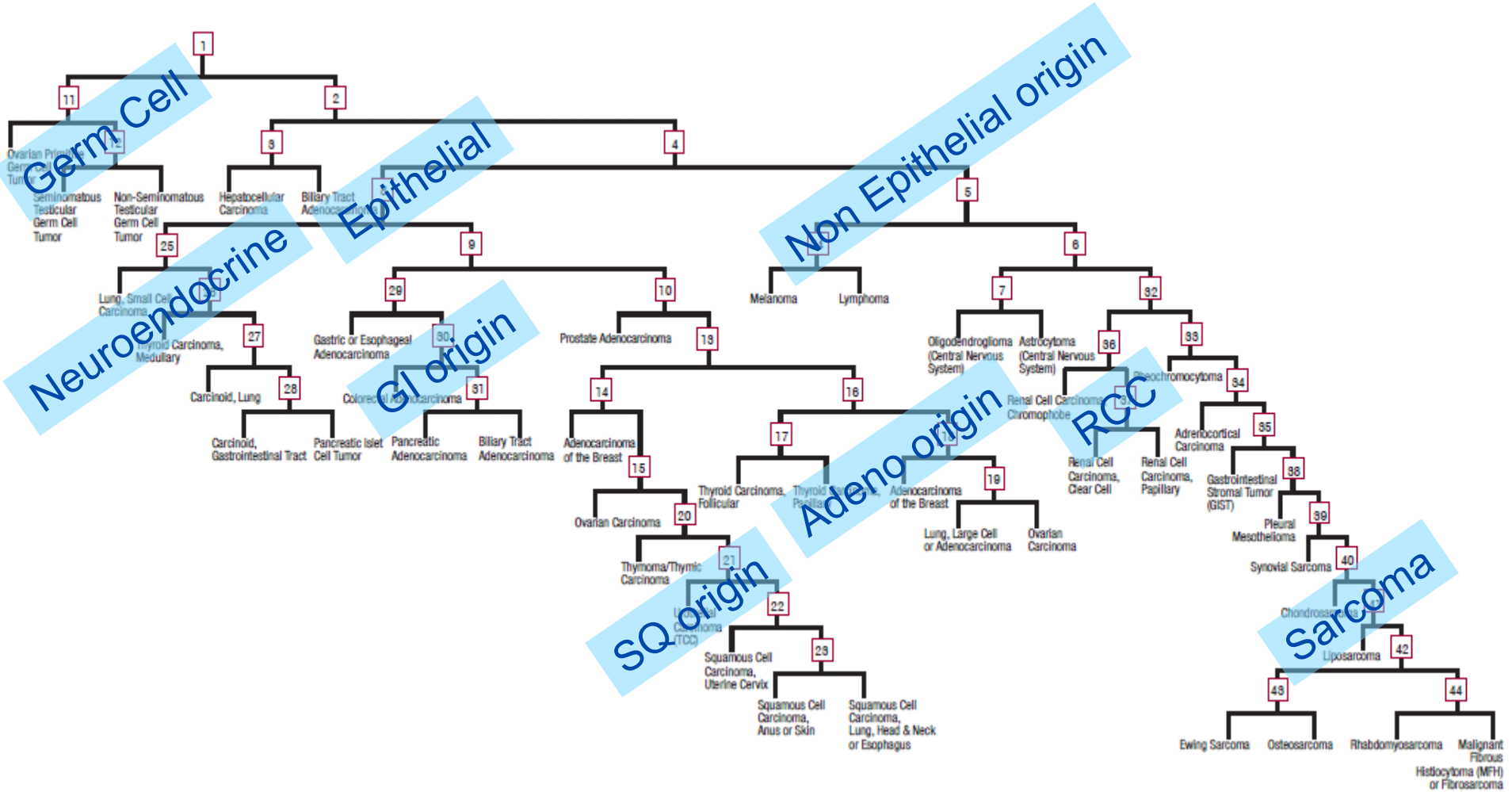
- microRNA expression levels in the sample are compared to the assay's training samples using the K-nearest-neighbor (KNN) classification algorithm[1]
  - KNN predicts tissue origin based on the most similar training samples

- microRNA expression levels are also quantified and applied to a binary tree classification system[1]
  - The tree breaks up the complex multi-tissue classification problem into a set of simple binary decisions, each involving 1-3 microRNA

[1] Rosenfeld, N., R. Aharonov, et al. (2008). "MicroRNAs accurately identify cancer tissue origin." *Nat Biotechnol* 26(4): 462-9.

miRview® mets^2 overview

Decision tree structure



miRview® mets^2 overview

- Each classifier can return any answer out of 49 possible answers (the 42 classes +7 additional answers)
  - The additional 7 possible answers are unifications of certain tumor categories
- Both classifiers answer or just one of them will be reported depending on the confidence of the result reported by each classifier
- Possible result - Completed Analysis, But No Result Generated
  - The microRNA expression pattern of this sample does not match any of the expression patterns in our panel closely enough

Comparing the two miRview® mets assays

Overview

- Our first generation assay identifies the tumor-of-origin based on 48 microRNAs measured on a qRT-PCR platform differentiating 25 tumor types
- We have developed a 2nd generation assay that identifies 42 tumor types using a custom microarray, based on 64 microRNAs
- The 2nd gen assay development heavily relied on the knowledge, cohorts, results and experience gained from the 1st gen, and is overall very similar
  - 30 of the 48 microRNAs of 1st gen are used in 2nd gen
  - Both assays work in a similar fashion: microRNA expression is measured and "fed" into two algorithms: A binary decision tree and a KNN. The two answers are then compared and combined and a test result is generated
  - The algorithms are essentially the same, with some improvements and the incorporation of more tumor types

Mets^2 Improvements

- Identifies 42 different tumor types, including carcinomas, sarcomas, lymphomas, and germ-cell tumors, as opposed to 25 in first generation
- 2nd gen assay gives a single result in 82% of cases as opposed to 67% in first generation
- 2nd gen assay can generate a “no result” in cases where the patient sample is not similar enough to any of the 42 tumor types (in 1st gen, the assay always selected from the 25 tumor types)
- In 2nd gen, if two results are reported, they are ordered based on PPV (likelihood of being correct), whereas in first gen a fixed order (KNN first)
- 2nd gen assay is not limited to metastases (can send also if not sure if the tumor is a primary or a metastasis)

Assays Comparison

	Platform	# of microRNA biomarkers	Tumor Panel Size	Accuracy (single answer accuracy)	% Single call	Sample Type	RNA amount
mets	PCR	48	25	85% (90%)	67%	Biopsies and resections preserved as FFPE	1 µg
mets[2]	Custom designed arrays	64	42	85% (90%)	82%	Bx, resections, BM bx, FNAs, bronchial washings and brushings and decalcified specimens preserved as FFPE	For biopsies and resections: 0.25 – 1.0 µg (decalcified samples 1-3 µg) For FNA samples: no limit on RNA amount but tumor cell % >50%.

Test availability & costs

Link to order: (all European countries except for Greece and Turkey)

http://www.mirviewdx.com/promets_HowToOrder.html

Price: $3,960
(similar to other commercially available molecular CUP- assays on the US market)

TAT: 1 week

Contact:
Steven P. Miller
Director of Marketing and Reimbursement Rosetta Genomics, Inc.
Email: steve_mi@rosettagenomics.com

Acknowledgements

Rosetta's CLIA laboratory
3711 Market St., Suite 740
Philadelphia, PA, 19104

Tina Bocker Edmonston
Brianna St.Cyr

Corporate Headquarters and R&D
10 Plaut st.
Rehovot
Israel, 76706

Yael Spector
Ranit Aharonov
Shai Rosenwald
Ayelet Chajut

Department of Neuropathology – University Heidelberg
Diana Jäger
Margot Werner
Katharina Pilot
Claudia Decker