Clinical Proteomics

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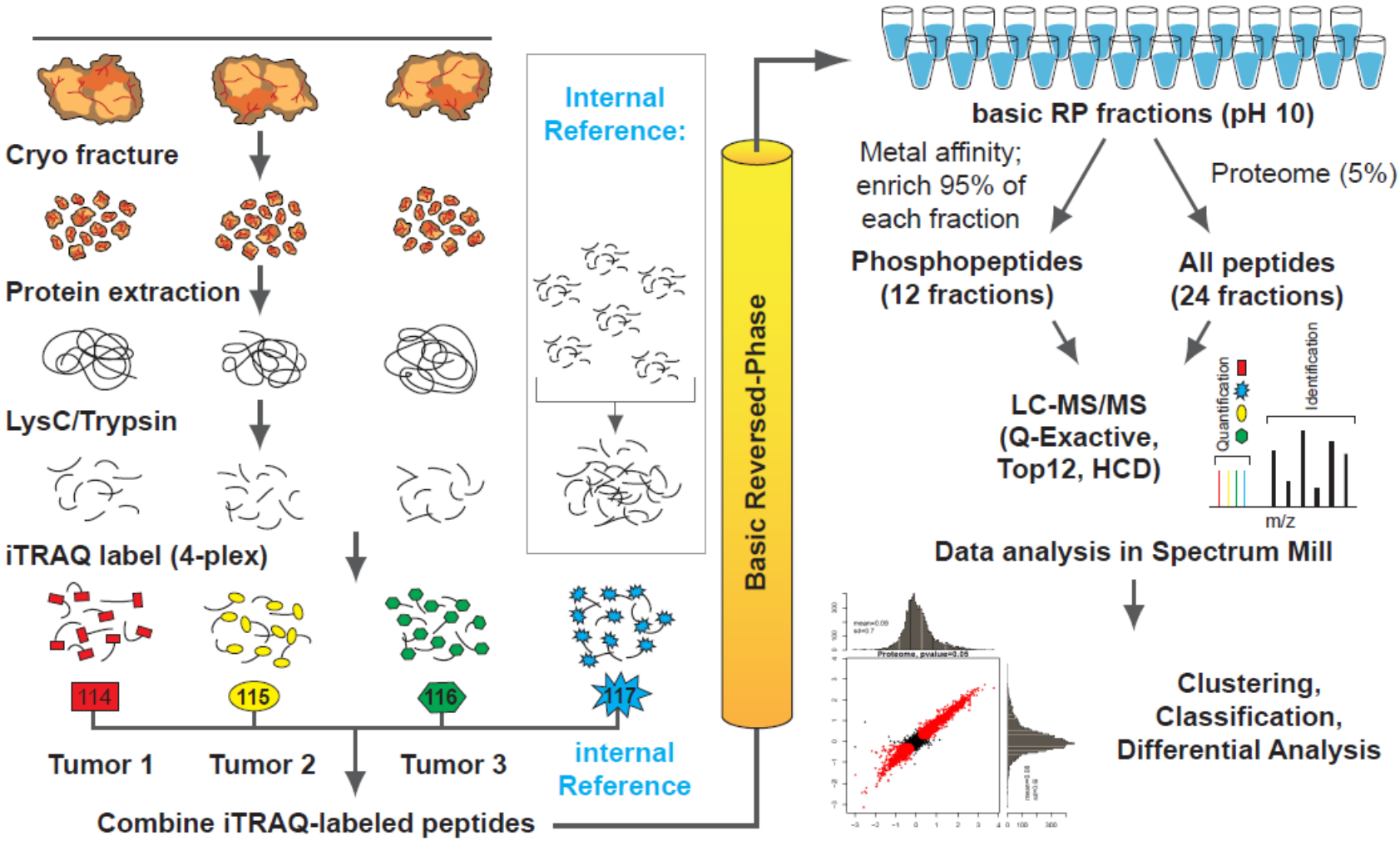
"Clinical proteomics" encompasses a spectrum of activity from pre-clinical discovery to applied diagnostics

- Proteomics applied to clinically relevant materials
  - "Quantitative and qualitative profiling of proteins and peptides that are present in clinical specimens like human tissues and body fluids"
- Proteomics addressing a clinical question or need
  - Discovery, analytical and preclinical validation of novel diagnostic or therapy related markers
- MS-based and/or proteomics-derived test in the clinical laboratory and informing clinical decision making
  - Clinical implementation of tests developed above
  - Emphasis on fluid proteomics
  - Includes the selection, validation and assessment of standard operating procedures (SOPs) in order that adequate and robust methods are integrated into the workflow of clinical laboratories
  - Dominated by the language of clinical chemists: Linearity, precision, bias, repeatability, reproducibility, stability, etc.

Ref: Apweiler et al. Clin Chem Lab Med 2009

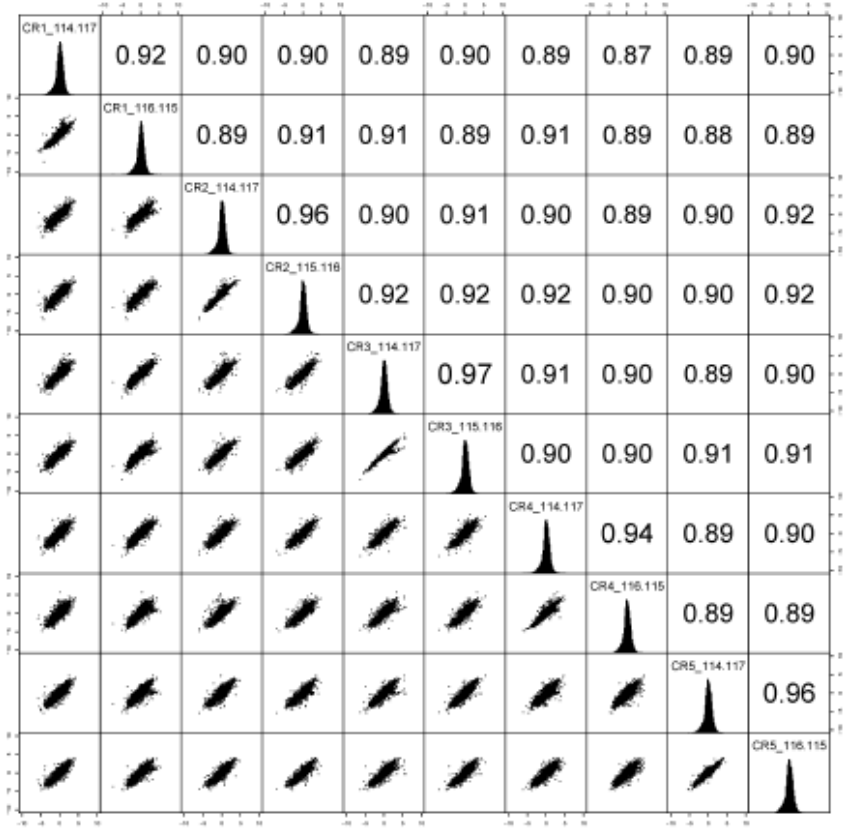
MS workflow allows precise relative quantification of global proteome and phosphoproteome across large numbers of samples



11,000 – 12,000 distinct proteins/sample
25,000 - 30,000 phosphosites/sample

Longitudinal QC analyses of PDX breast cancer sample demonstrate stability and reproducibility of complex analytic workflow

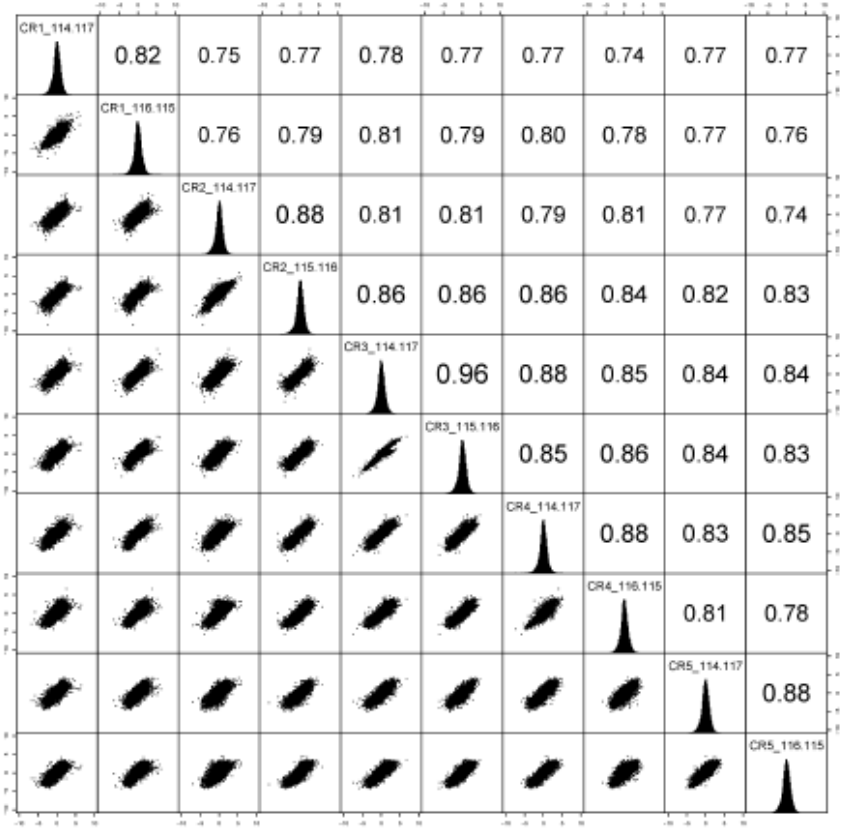
Proteome analysis of interstitial CompRef PDX samples



Log2 iTRAQ basal/luminal protein ratios

Average Pearson correlation
r = 0.91
12,687 proteins

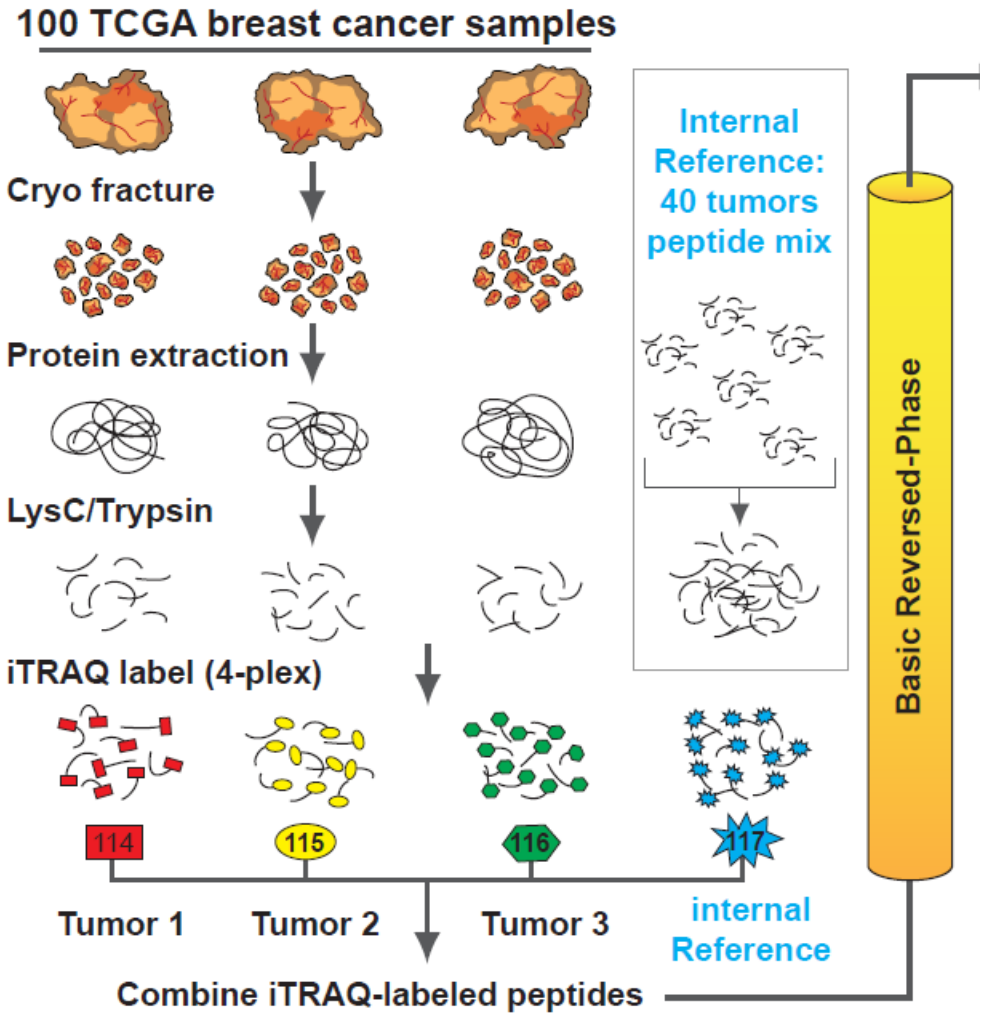
Phosphoproteome analysis of interstitial CompRef PDX samples



Log2 iTRAQ basal/luminal phosphosite ratios

Average Pearson correlation
r = 0.82
38,381 phosphorylation sites

Deep proteomic and phosphoproteomic annotation for 105 genomically characterized TCGA breast cancer samples

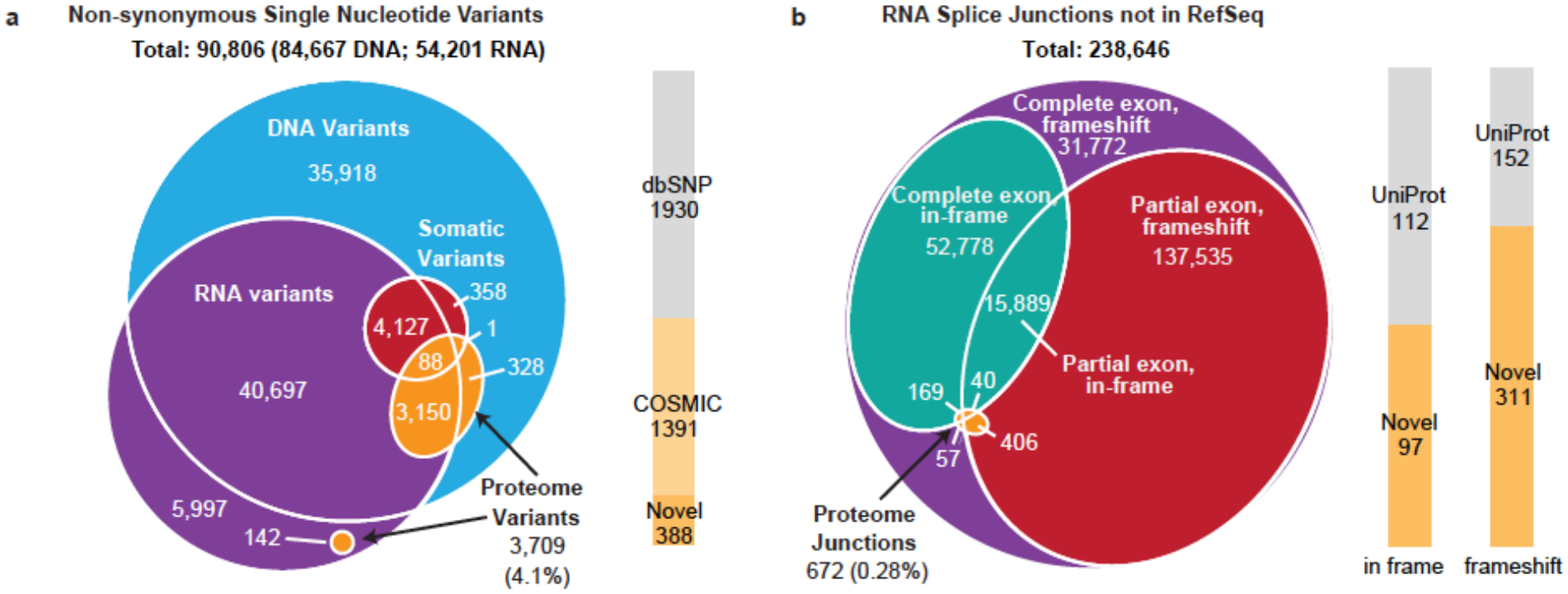


The Cancer Genome Atlas

- ~25 Cancer types
- 500 – 1000 tumors / cancer
- Comprehensive genomic characterization
  - WES or WGS
  - Array-based mRNA profiling or RNA Seq
  - CNV
  - DNA methylation
  - miRNA
- Protein characterization limited to RPPA

11,000 – 12,000 distinct proteins/tumor
25,000 - 30,000 phosphosites/tumor

Proteogenomic mapping using personalized databases facilitates functional annotation of genetic alterations in clinical samples

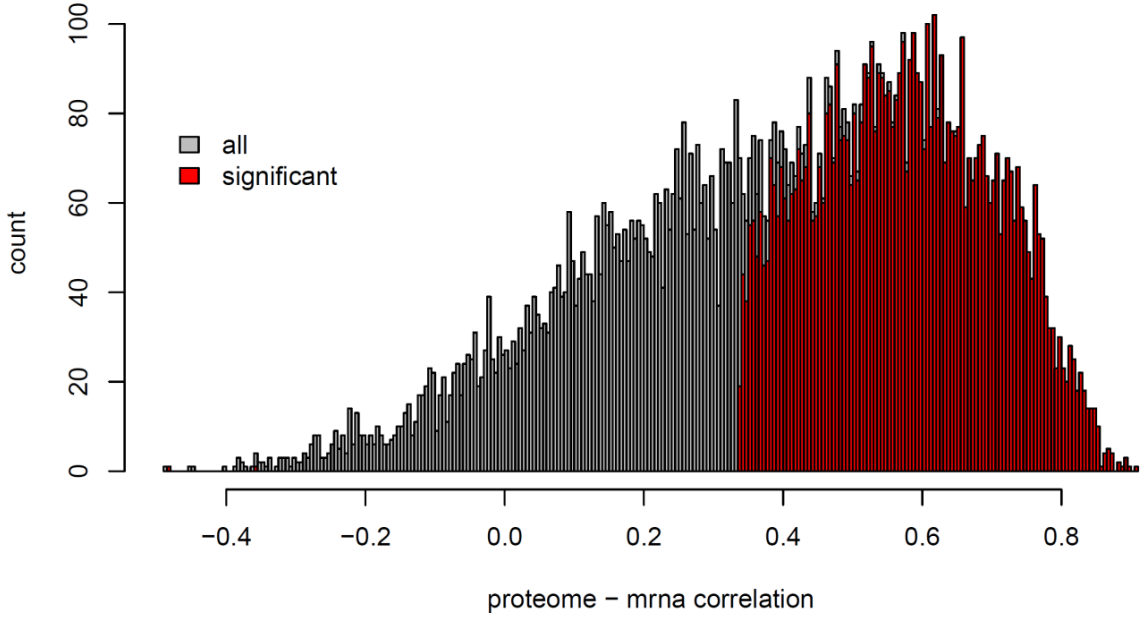


- **0.2-4.0% of frameshifts, alternative splices & single AA variants observable by proteomics**
  - For unobserved alterations, mRNA may be untranslated or translated at low abundance, or product may be unstable or targeted for degradation
  - Proteome coverage is deep but incomplete
  - ~30% of alterations would NOT be observable by proteomics (tryptic peptide length < 6 or >30)

(Phospho)proteomic data have comparable dimensionality to mRNA data

	total	average per tumor
Genes (mRNA)	17,814	17,811
Proteins quantified	12,529	11,307
Phosphosites quantified	79,767	27,779

RNA-Protein correlation is statistically significant and almost exclusively positive

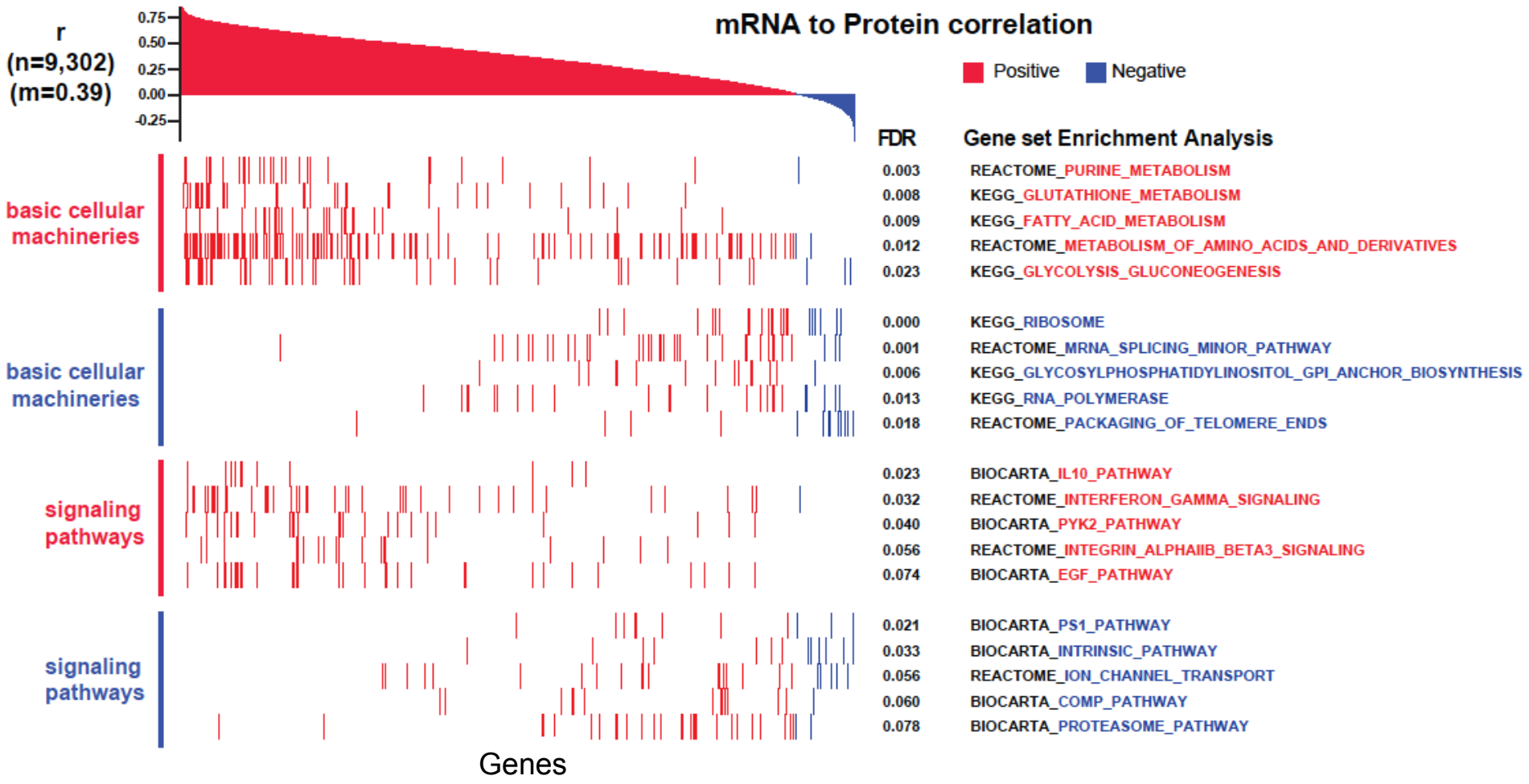


9,302 proteins/genes
Median correlation = 0.39
IQR=[0.21, 0.59]
Mean = 0.39

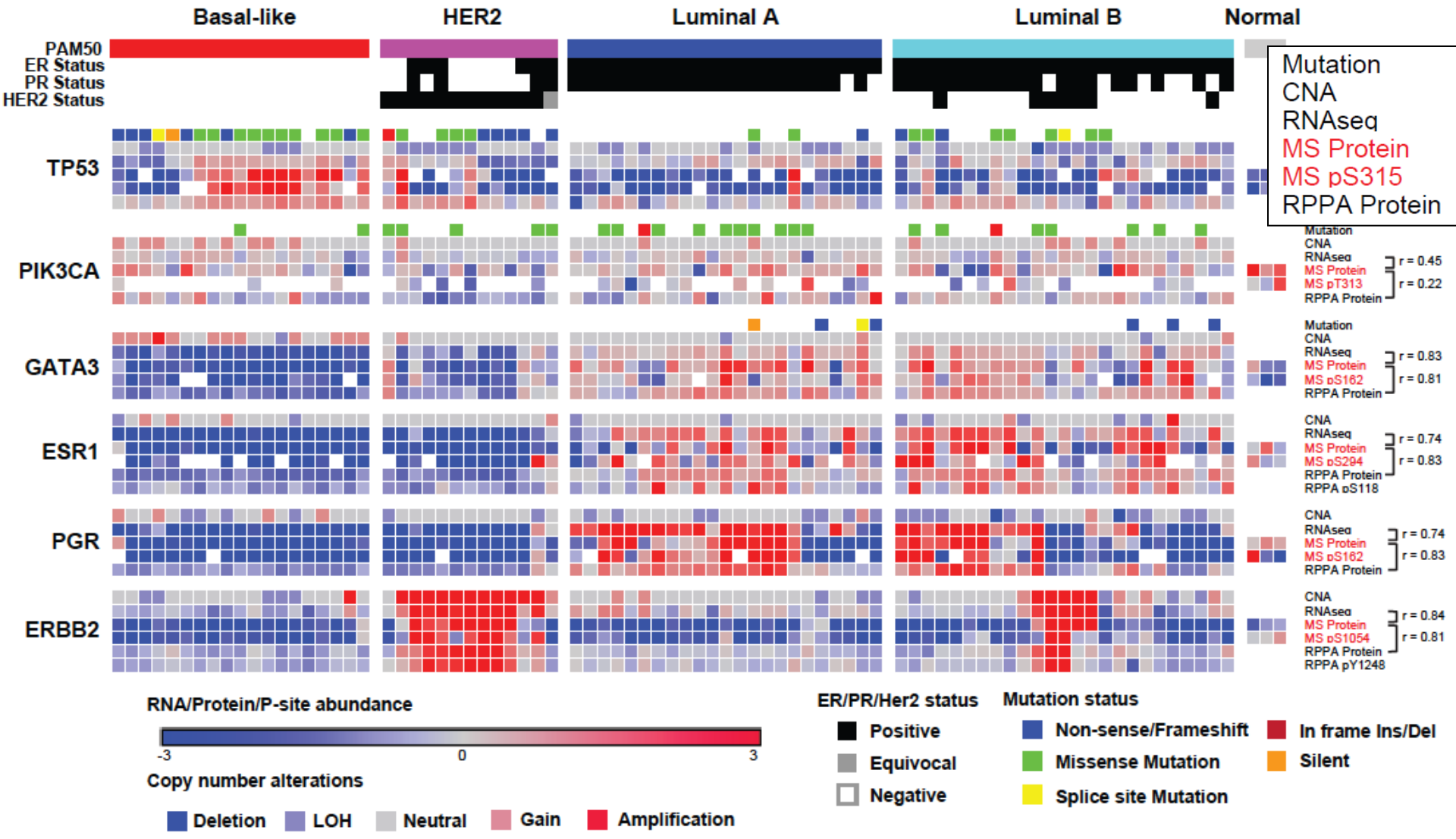
66.7% of all proteins/genes correlate*

*Jovanovic et al., Science 2015: “RNA levels explain 59-68% of protein abundance in baseline state” & “ribosomal proteins are regulated via post-transcriptional mechanisms”

Certain areas of biology, such as signaling pathways containing E3 ligases and proteases, do not correlate on RNA and protein level



Major breast cancer driver genes can be accurately quantified on the protein and phosphorylation level



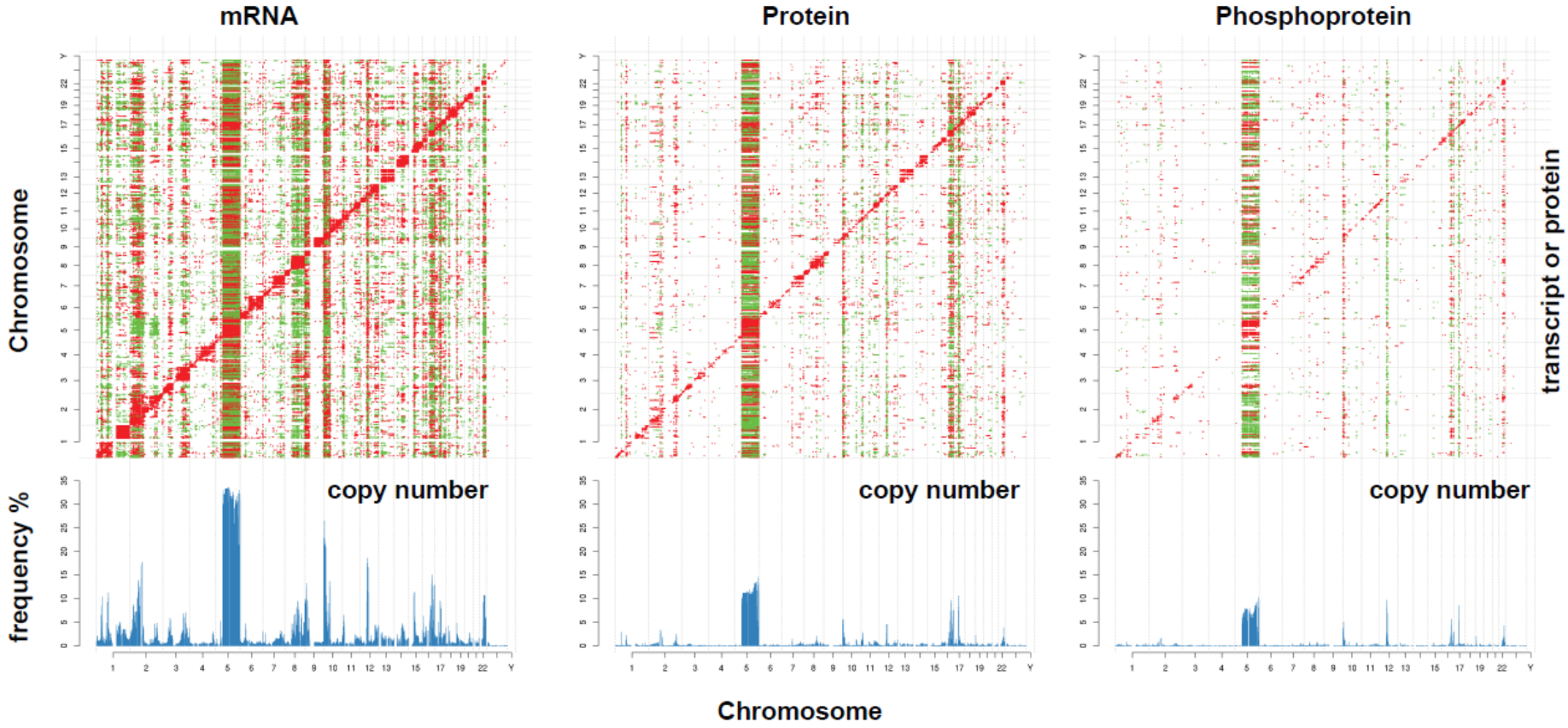
CNVs correlate positively with both mRNA and protein expression in CIS, and show many TRANS effects

Significant positive CIS effects

64% of CNA x RNA

31% of CNA x Protein

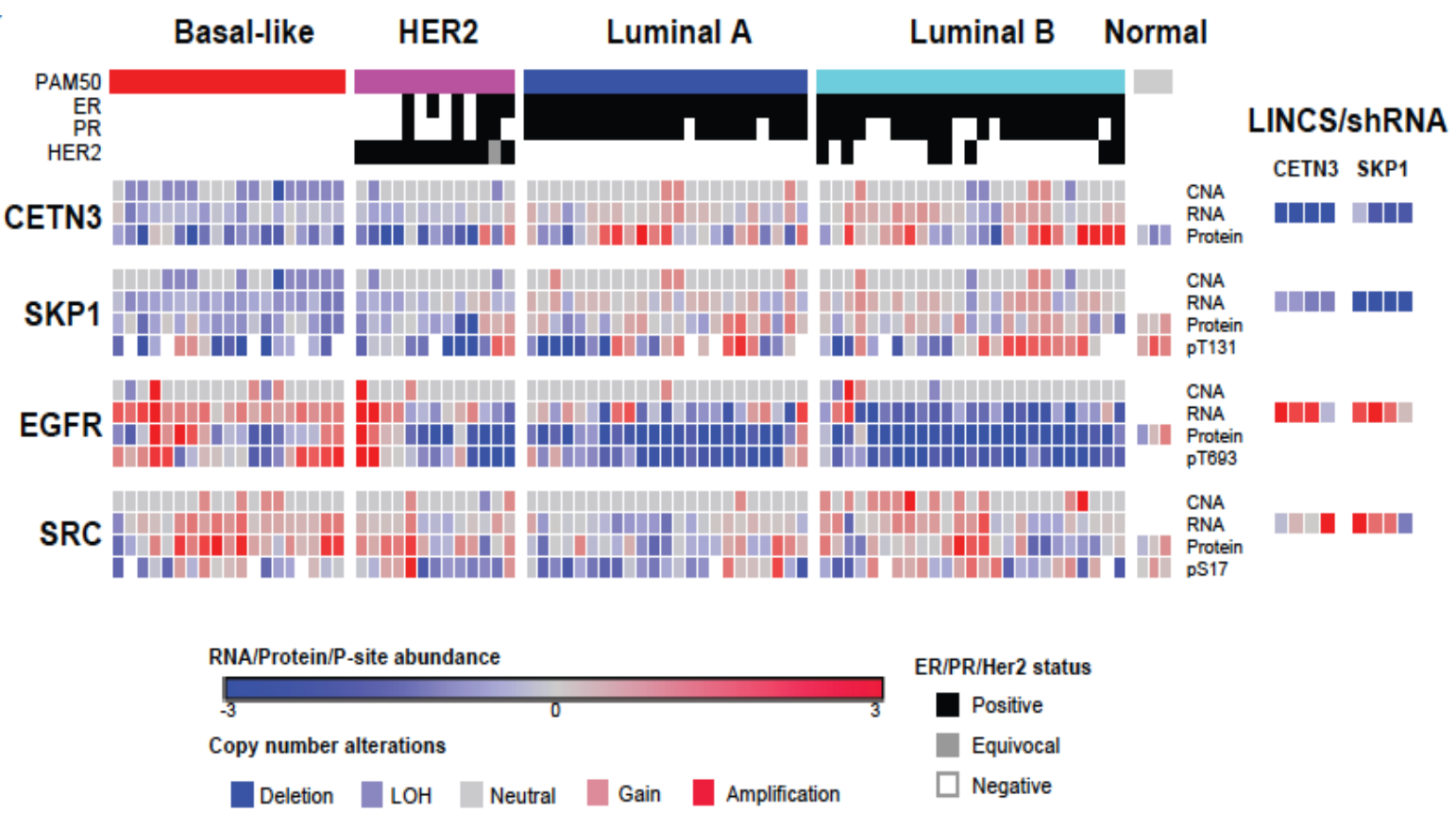
20% of CNA x Phosphoprotein

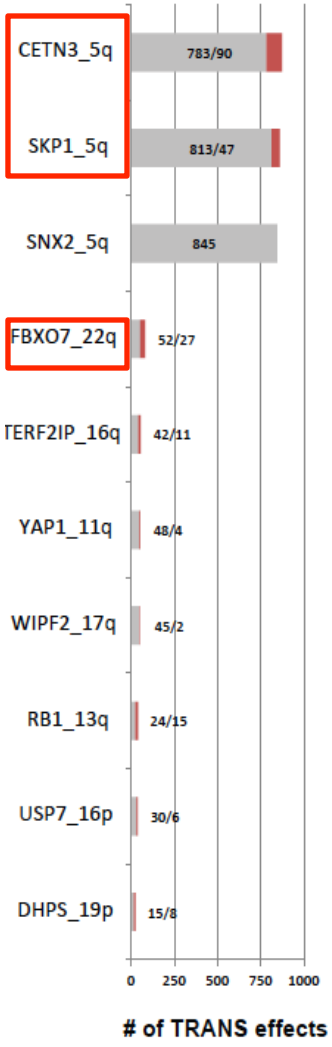


- CNA/protein correlations are a reduced representation of CNA/mRNA correlations in both CIS and TRANS
- Established oncogenes & tumor suppressors were significantly more likely to have both CNA/mRNA and CNA/protein correlation
- Correlations with CNA are more likely to be positive at the protein level

"Hot spots" of significant trans effects were found on chromosomes 5q, 10p, 12, 16q, 17q, and 22q

Comparison with LINCS knockdown data on ~3800 genes identifies SKP1 and CETN3 as causal candidates in 5Q deletion region Both negatively regulate EGFR expression

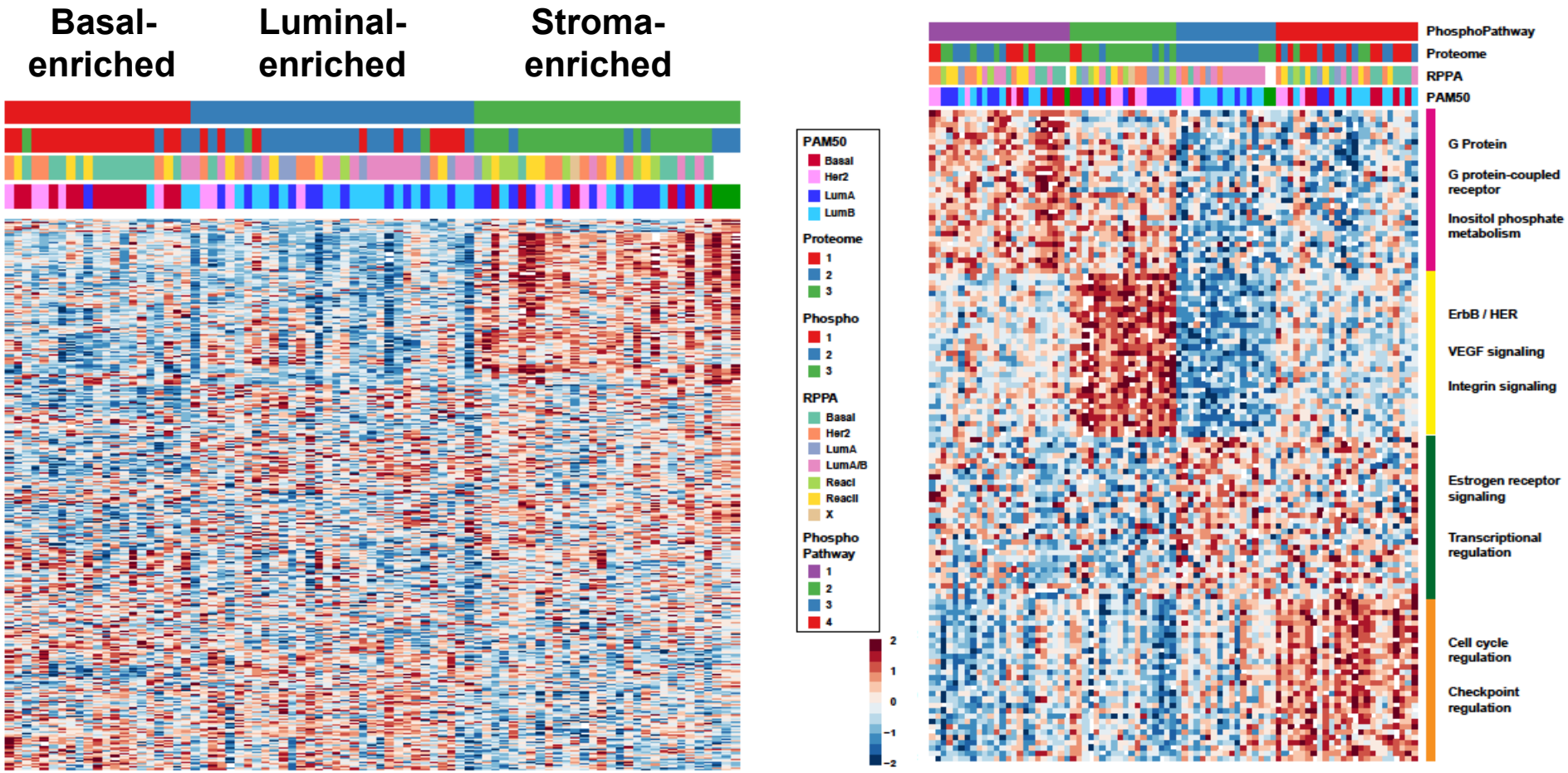




- Top 5q candidates were **CETN3** (part of TREX-2 complex; functions in mRNA export and chromatin positioning) and **SKP1** (part of a complex that mediates ubiquitination of proteins involved in cell cycle, signal transduction, and transcription)
- In a recent human interaction proteome study SKP1 and FBOX7 were interaction partners

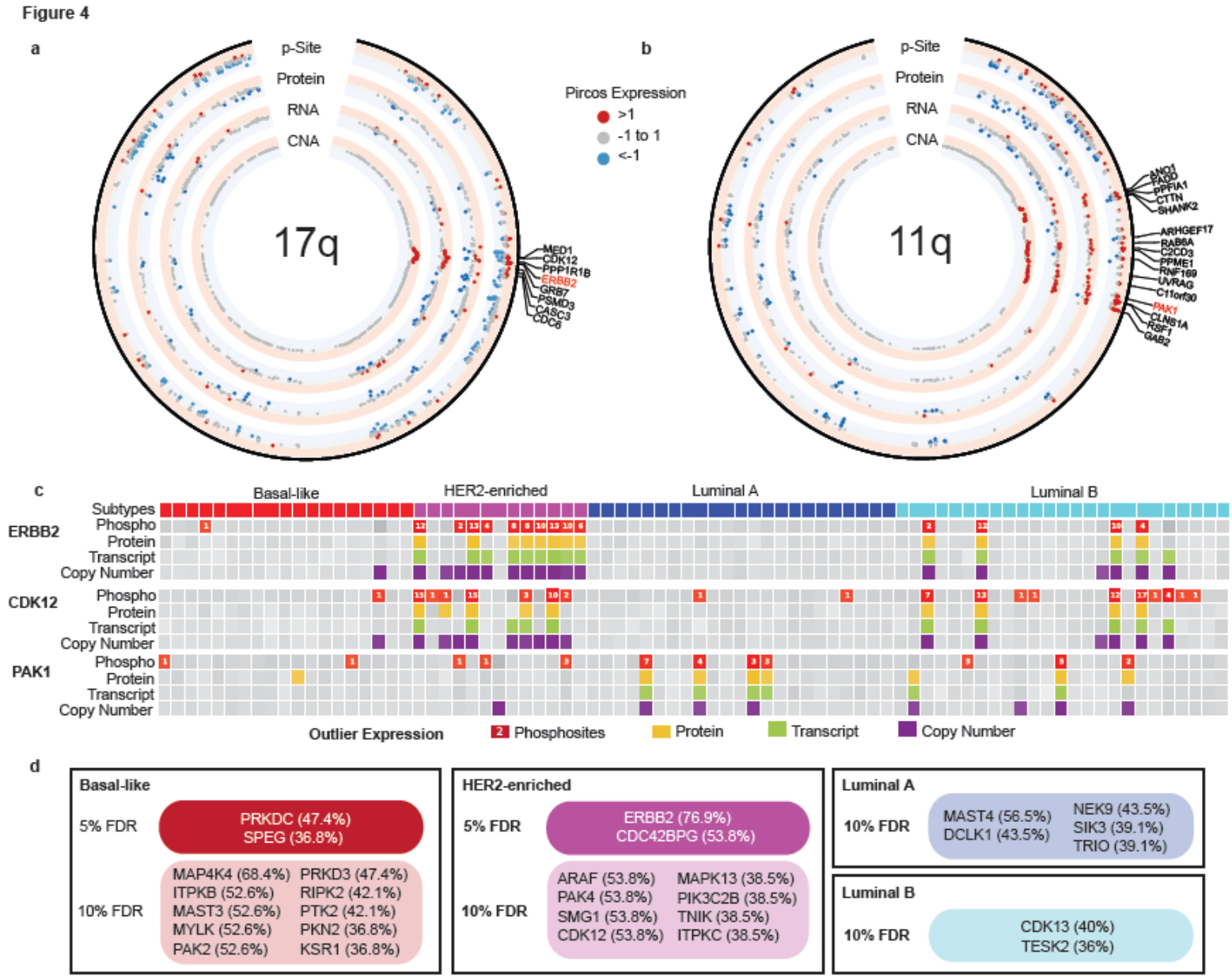
Regulated in CMap modT FDR<0.1

K-means clustering of proteome data yields three major groups Clustering on pathways from phosphopeptide-based ssGSEA yields a modified breast cancer taxonomy



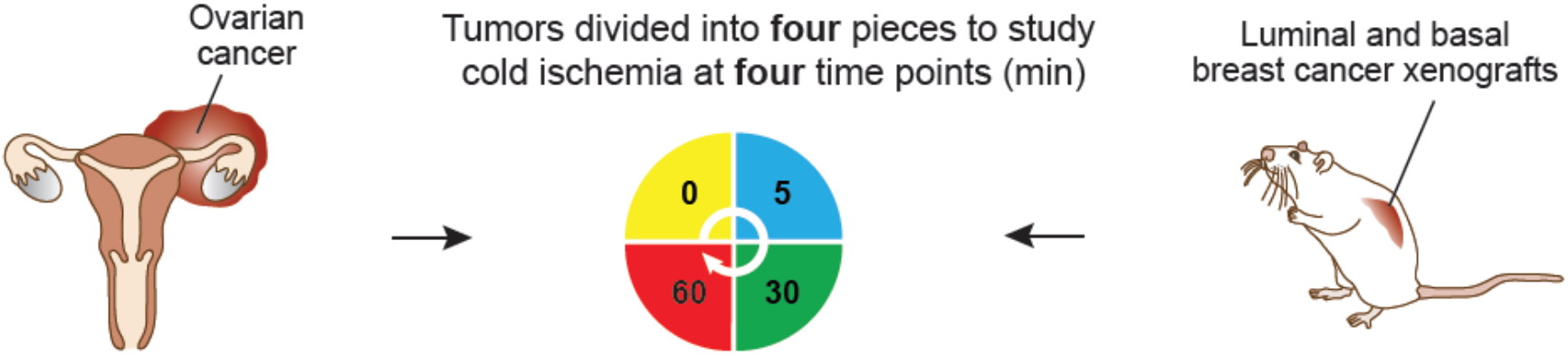
- Stroma-enriched proteomics subtype is highly enriched for Reactive I RPPA subtype
- Proteome clustering resembles PAM50 classification when instead of the most variable the most RNA/protein-correlated proteins are selected

“Pircos” plots map outlier kinase values onto genome, transcriptome and proteome and help nominate candidate drivers from CNA regions



Pre-analytical variability could have profound effects on posttranslational modifications

- Time between ligation, excision and freezing for the TCGA samples varied from minutes to ca. 1 hour
- Effects of ischemia on PTMs not well studied
- Activated kinases and phosphatases can act in seconds-minutes
  - Alterations in phosphosignaling in cancer well established



Samples: Four patient-derived ovarian cancer tumors and two xenografted human breast cancer tumors (basal-like; luminal-like; pools of 10 tumors)

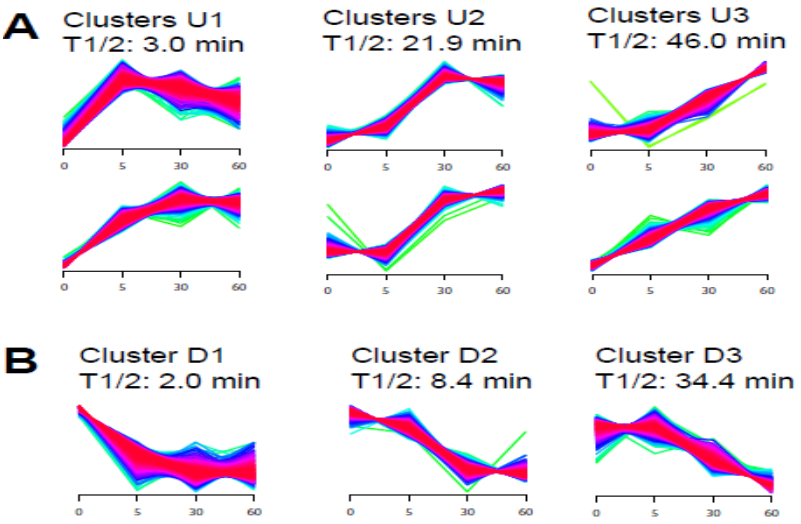
Collection: excision prior to ligation; immediate LN2

Analysis: 4-plex iTRAQ on high-performance MS instrumentation

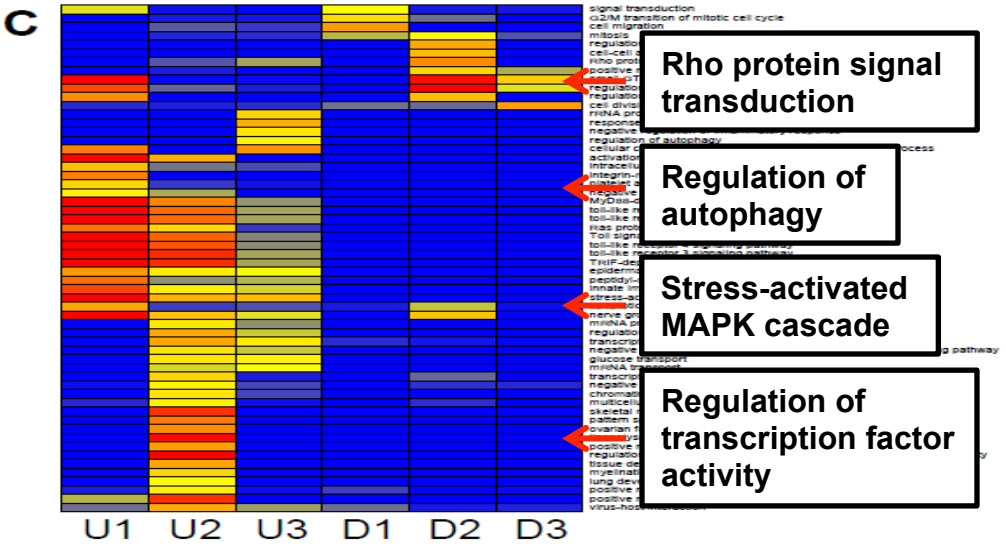
Cold ischemia times up to 1 hour cause no change in proteome but up to 24% change in phosphoproteome

	n tumor samples	Total	average per tumor	overlap in at least (n-1)	kinetics-based regression test*	moderated F-test*	union of both tests*	% regulated **
Phosphoproteome					#up/#down	#up/#down	#up/#down	#up/#down
Ovarian Cancer	4	23607	13156	9443	307/97	386/63	432/111	4.6/1.2
Basal Breast Cancer	3	38366	27668	26211	1252/948	1156/633	1493/1027	5.7/3.9
Luminal Brst Cancer	3	34327	25814	25102	4153/820	4220/962	4977/1139	19.8/4.5
Proteome								
Ovarian Cancer	4	9498	7550	6985	0/0	0/0	0/0	0/0
Basal Breast Cancer	3	17158	14989	14970	0/0	0/0	0/0	0/0
Luminal Brst Cancer	3	14224	12641	12679	0/0	0/0	0/0	0/0

Fuzzy c-means clusters of regulated phosphosites



Enriched GO Biological Process terms for temporal profiles



Mertins et al; Mol Cell Proteomics 2014

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- MS-based and/or proteomics-derived test in the clinical laboratory and informing clinical decision making
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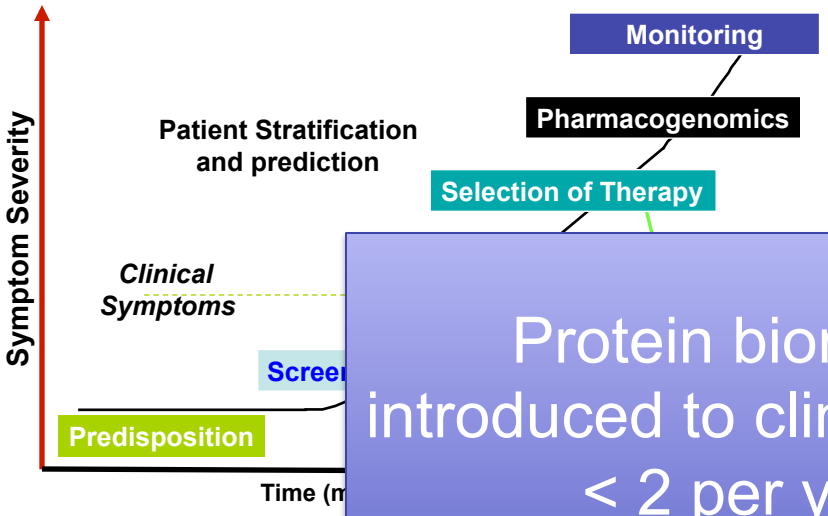
  - Includes the selection, validation and assessment of standard operating procedures (SOPs) in order that adequate and robust methods are integrated into the workflow of clinical laboratories

Fit-for-Purpose Guidelines have been established for MS-based assays

Tier and Areas of Application	Degree of Analytical Validation	Labeled Internal Standards	Reference Standards	Specificity	Precision	Quantitative Accuracy	Repeat-ability	Comments and Suggested References
Tier 1 **Clinical bioanalysis/ diagnostic laboratory test; single analyte or small numbers of analytes**	High, including batch-to-batch QC	Yes, for every analyte	Yes	High	High (typically <20-25% CV achieved)	Defining accuracy is a goal; true accuracy difficult to demonstrate.	High	Precise, quantitative assays; established, high performance; may need comply with FDA and CLIA guidance depending on use of assay Refs. 30, 41, 42, 53
Tier 2 **Research use assays for quantifying proteins, peptides, and post-translational modifications; 10's to 100's of analytes**	Moderate-to-high	Yes, for every analyte	Limited use	High	Moderate-to-high (typically <20-35% CV achieved)	Not applicable	High	Precise, relative quantitative assays; established performance; suitable for verification Refs. 30, 31, 36, 37, 40, 51, 70, 71
Tier 3 **Exploratory studies; 10's to 100's of analytes**	Low-to-moderate	None-to-limited	No	Moderate-to-high	Low-to-moderate: similar to label-free discovery	Not applicable	Moderate-to-high	Discovery in a targeted mode; performance not defined; results require further verification using quantitative techniques Refs. 36, 37, 86-89

Targeted Peptide Measurements in Biology and Medicine: Best Practices for Mass Spectrometry-based Assay Development Using a Fit-for-Purpose Approach. Carr et al. MCP, 2014

Biomarkers have tremendous clinical utility
Investment in new candidates has been vast

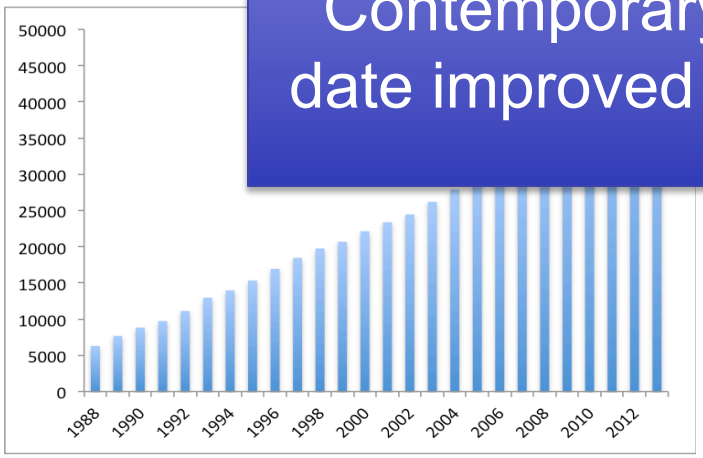


Period	**Decades**
Number of tests	**> 10 million/yr**
Instruments	**Ca. 100,000 machines in hospitals, labs**
Accuracy	**CV ~5-10% worldwide at ≥100pg/ml**

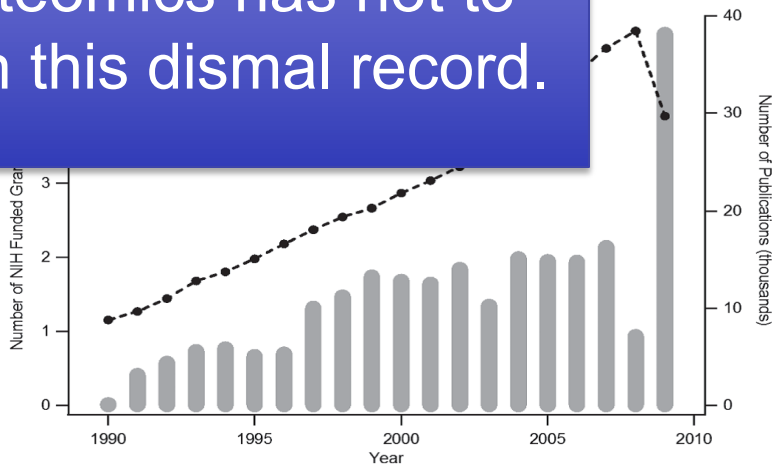
Cardiac damage	TnI, CK-MB, Mb, MPO, BNP
	he assays)
	gen, VWF
	s

Protein biomarkers have been introduced to clinical practice at a rate of < 2 per year for decades.

Contemporary proteomics has not to date improved upon this dismal record.



41,086 papers in 2013
> 370,000 in the past decade



Pharmaceutical Intelligence 2014

Factors leading to biomarker development failure

- Biology –
  - it is **hard** to find differences that are ***predictive***
  - it is **very hard** to find predictive markers in ***accessible fluids***
  - it is **ridiculously hard** to find accessible predictive markers that are ***not affected by related diseases*** -Josh LaBaer

Sample Selection ⇒ Sample Analysis ⇒ Observed Difference ⇒ Candidate Biomarker ⇒ Verified Biomarker ⇒ Validated Biomarker





Ref: Rifai N, Gillette MA, and Carr SA. *Nature Biotechnol.* (2006) 24: 971-983

Clinical proteomics demands a particular mindset

- If your clinical proteomics project focuses only on the proteomics, it will probably fail.
- You may get publications. You will not help patients.
- If you want to do clinical proteomics, THINK ABOUT THE CLINICAL BEFORE YOU THINK ABOUT THE PROTEOMICS

Start with a clinical question or need that is Important, Specific and Tractable

Important

- What do the end users (typically clinicians) need to know?
- What would be the expected clinical impact of knowing it?
  - Impact per patient
  - Total patients affected

Specific

- What sort of test is required?
  - Screening
  - Diagnostic
  - Prognostic
- What is the final diagnostic material?
  - Blood
  - Urine
  - Tissue
- What would happen based on a positive test?
  - Follow-on imaging
  - Invasive diagnostic procedure
  - Surgical intervention

Tractable

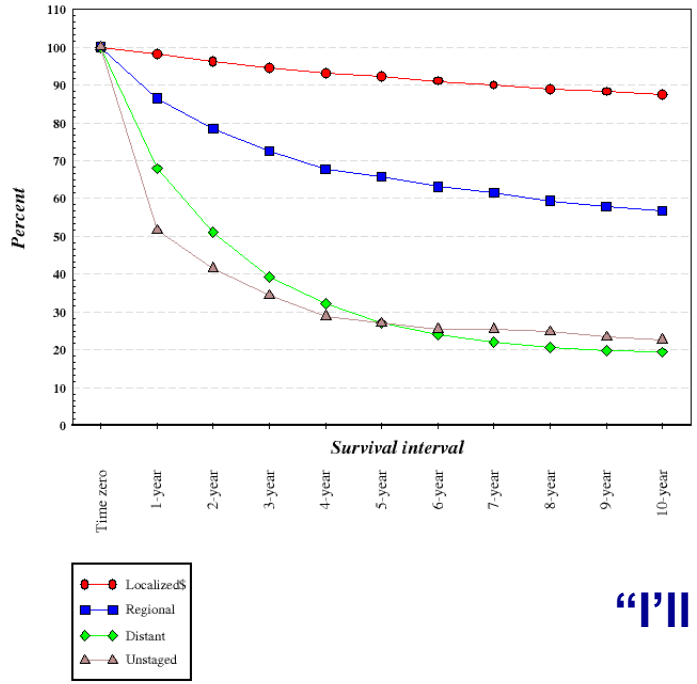
- Resources available for discovery and development
- Route to implementation

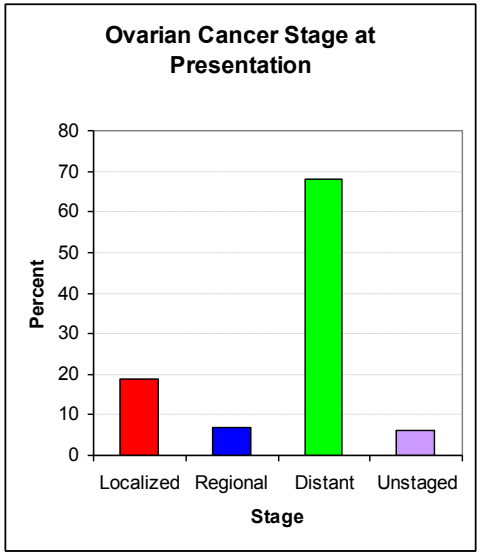
Start with a clinical question or need that is Important, Specific and Tractable

Ovarian Cancer

- 5th leading cause of cancer death among women
- 1.4% of women affected
- >14,000 US women will die of ovarian cancer in 2014
- No functional early detection method

Ovarian Cancer Survival By Stage (SEER)





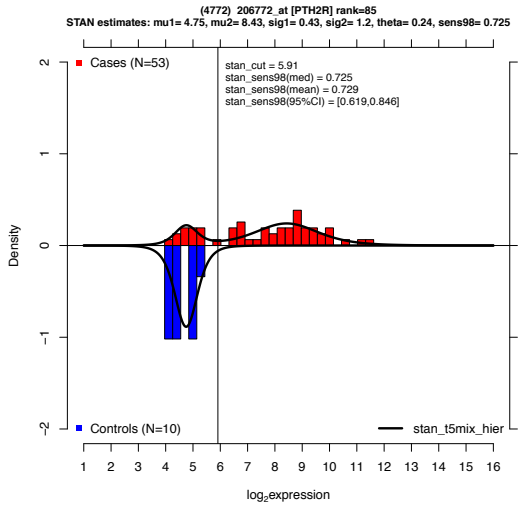
"I'll find biomarkers for ovarian cancer"

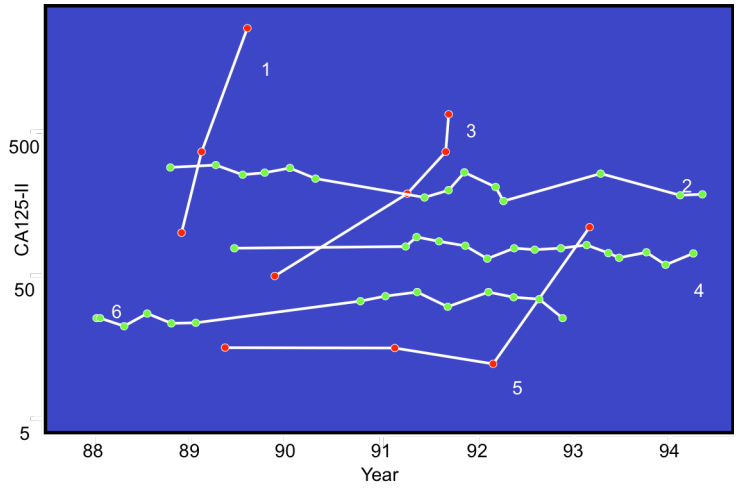
Start with a clinical question or need that is Important, Specific and Tractable. Involve all stakeholders.

Ovarian cancer marker panel with Sensitivity 100%, Specificity 90%
Annual incidence of ovarian cancer 1:2500 women
Positive Predictive Value = 0.4% => NOT CLINICALLY ACTIONABLE = USELESS!

Specific

- Type of test / material / initial follow-up: (Oncologist): Screening blood test to select patients for trans-vaginal ultrasound
- Consequence of positive test (Biomarker + ultrasound): Surgical biopsy
- Acceptable performance: (Oncological surgeon): 1 cancer / 5 biopsies
- Biomarker specifications: 98% specificity; maximize sensitivity





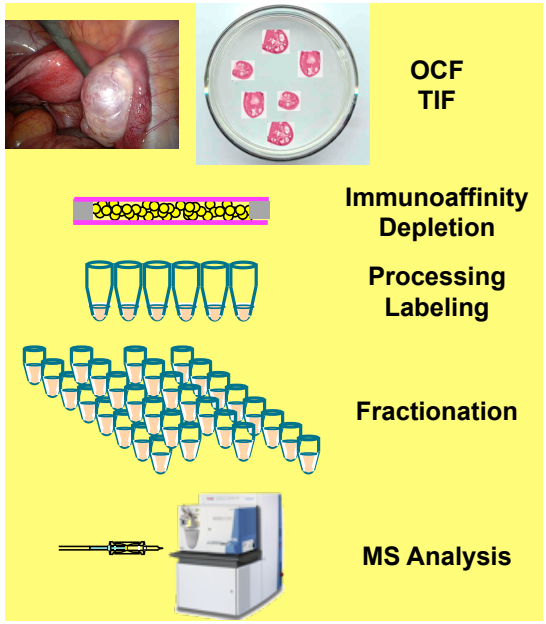
Tractable (Primary care physician; public health / policy expert; insurers)

- Annual / semiannual blood test on routine clinical visit

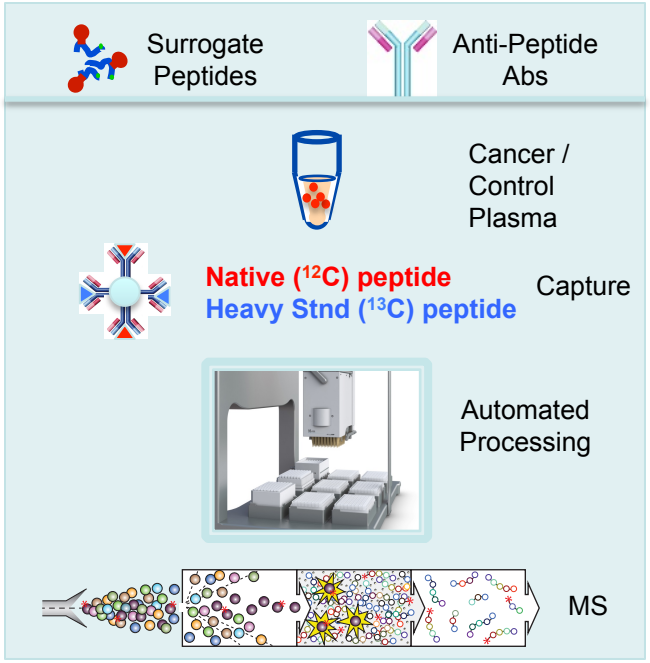
Clinical questions that are Important, Specific and Tractable drive meaningful biomarker candidate discovery

"Based on a hierarchical mixture model of marker distribution, identify candidate markers that maximize sensitivity at 98% specificity at least one year prior to clinical diagnosis of serous ovarian cancer in longitudinal plasma samples"

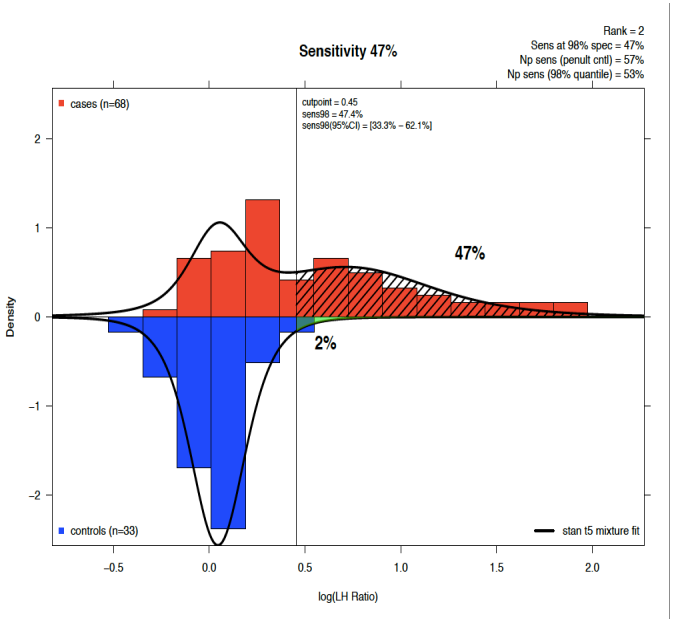
Discovery (Proximal Fluids)



Verification (Plasma)



Biomarker Candidate for Validation

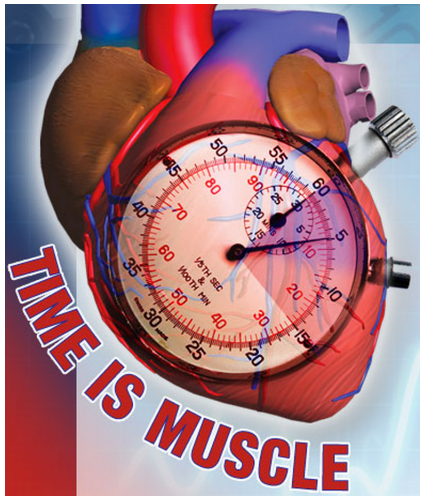


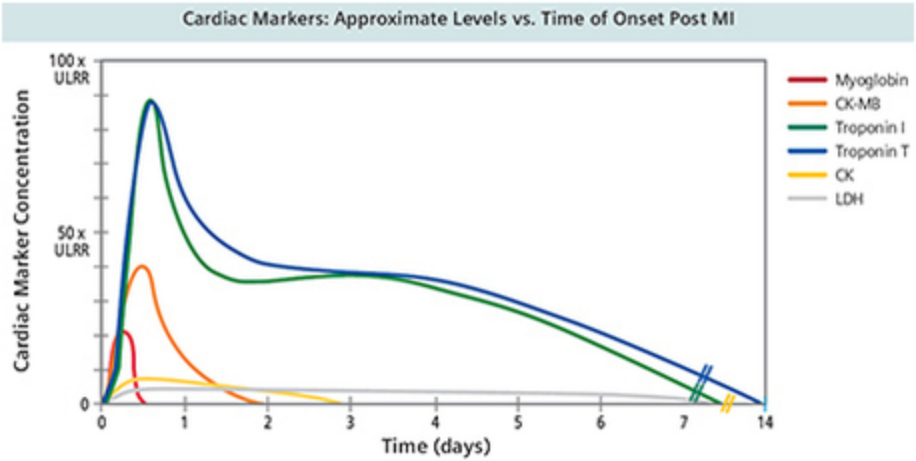
Sample Type, Quality and Suitability are of preeminent importance

- **Type:** Robust difference signal detectable with unbiased approach
  - Often not the same material as that for the final clinical test
- **Quality** refers to minimization of pre-analytical variability and maximization of the degree to which the sample represents native biology
  - Time of day
  - Position of patient
  - Technique of acquisition
  - Suitability and standardization of processing
  - Timing and technique of storage
- **Suitability** refers to the degree to which the samples reflect the population to which the clinical test would be applied
  - Suitability applies to cases **and** controls and emphasizes avoidance of systematic bias
  - Suitability includes sufficiency of sample annotation

"Samples of convenience" are rarely ideal and often inadequate. They *may* be tolerable for discovery but should generally be avoided for verification.

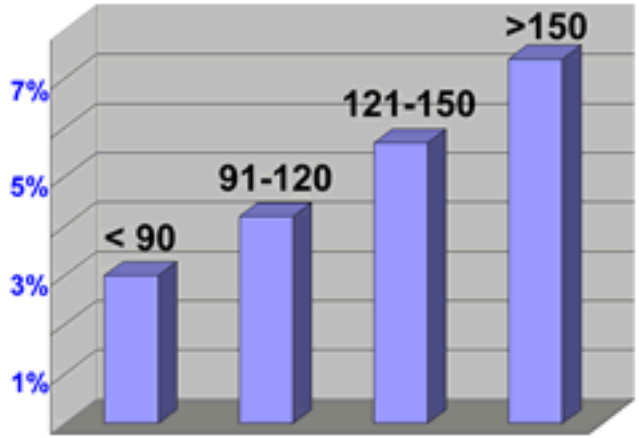
Improved markers of early myocardial injury are needed



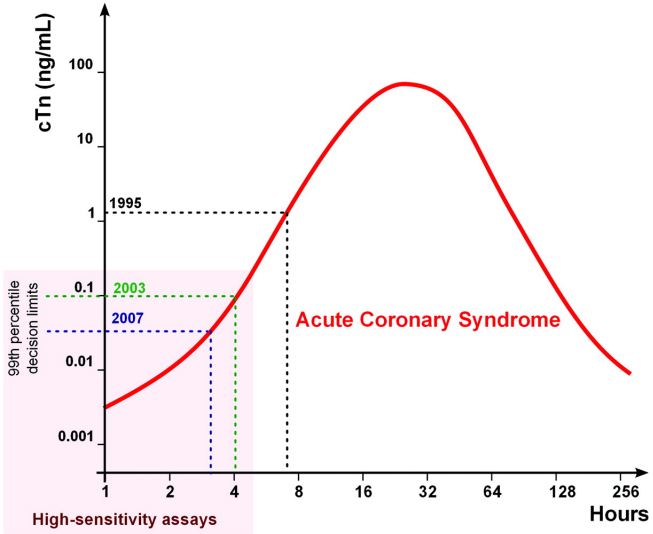


Siemens

In-hospital mortality by time from symptom onset to PTCA



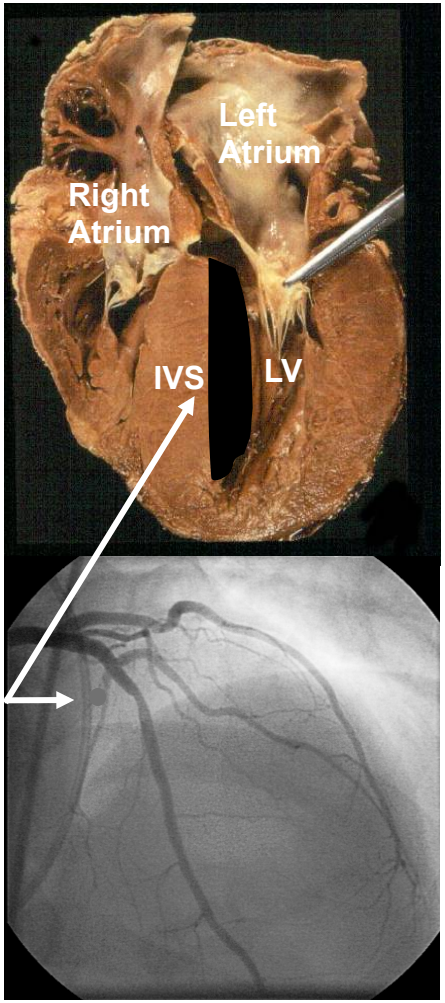
PTCA.org



cTn Assay	Diagnostic cutoff	Implementation
TnI	≥ 1.5 ng/mL	1995
cTnI	> 0.10 ng/mL	2003
TnI-Ultra	> 0.04 ng/mL	2007

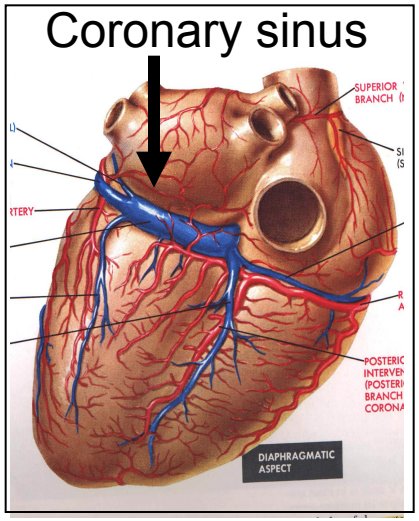
Plasma-based Discovery Using a Human Model of Myocardial Injury

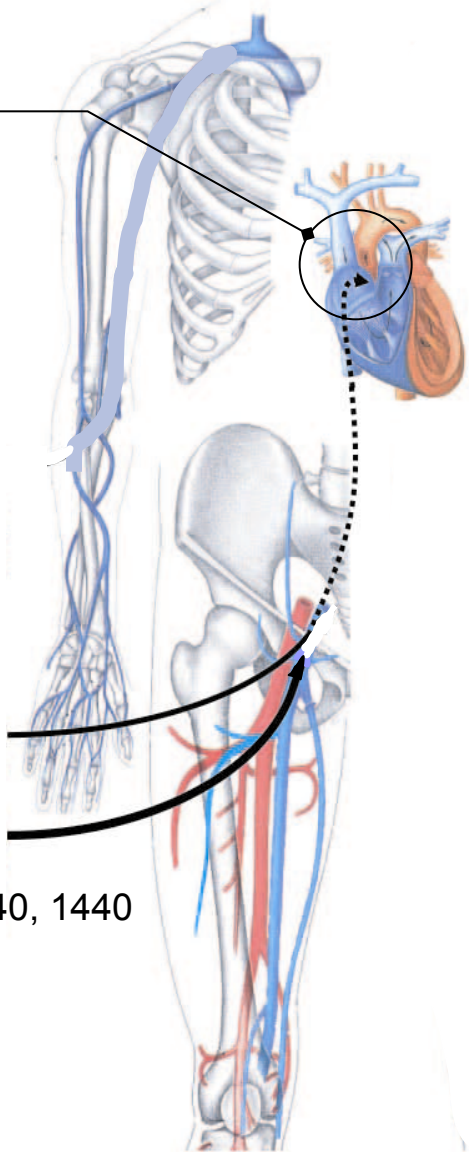
Hypertrophic Obstructive Cardiomyopathy (HOCM)



Planned therapeutic MI by alcohol ablation

PLASMA as a proximal fluid



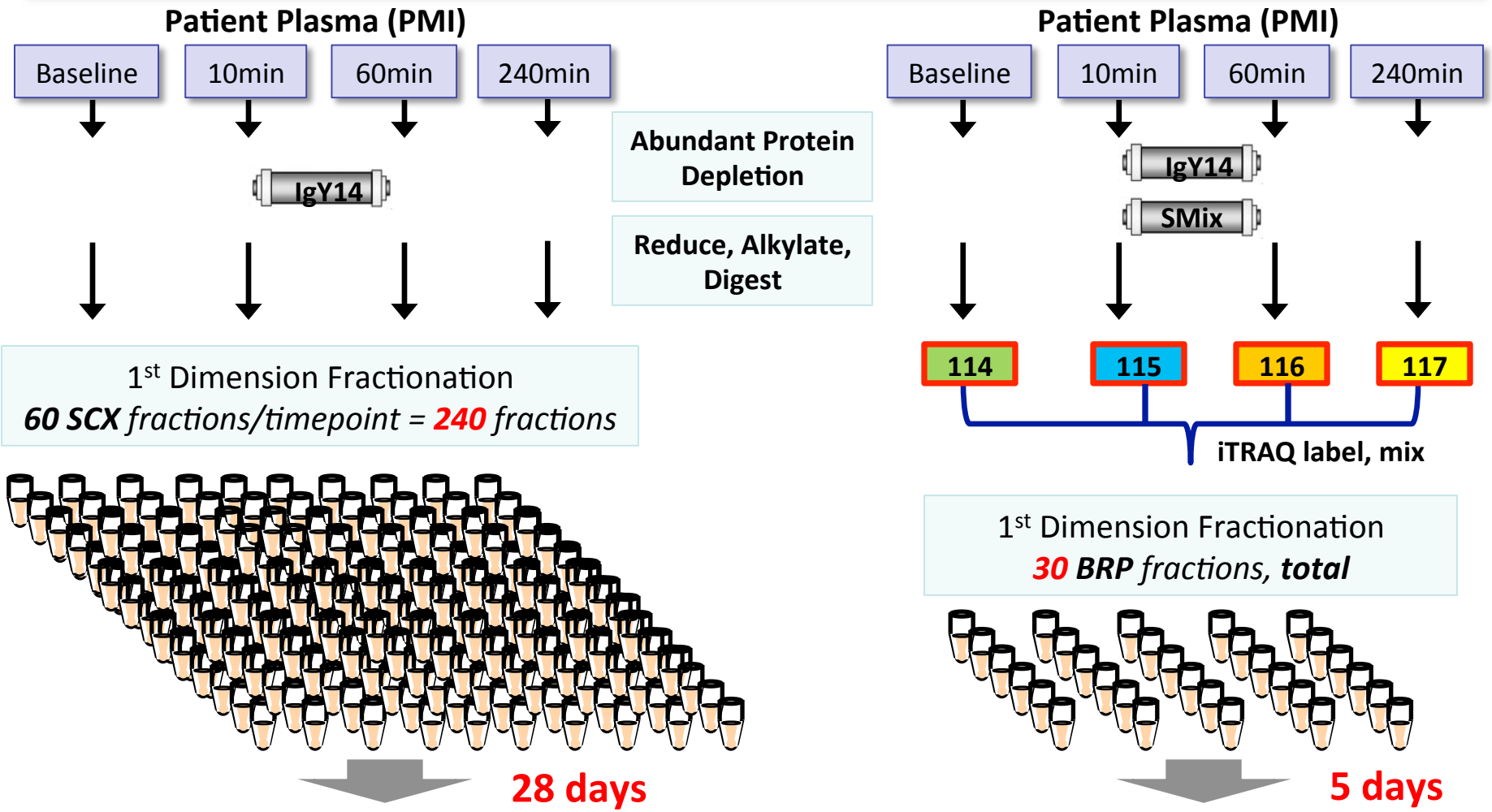


Coronary Sinus Samples
Time (min): Baseline, 10, 60

Femoral Vein Samples
Time (min): Baseline, 10, 60, 120, 240, 1440

Collaboration with Robert Gerszten, MGH and Marc Sabatine, BWH

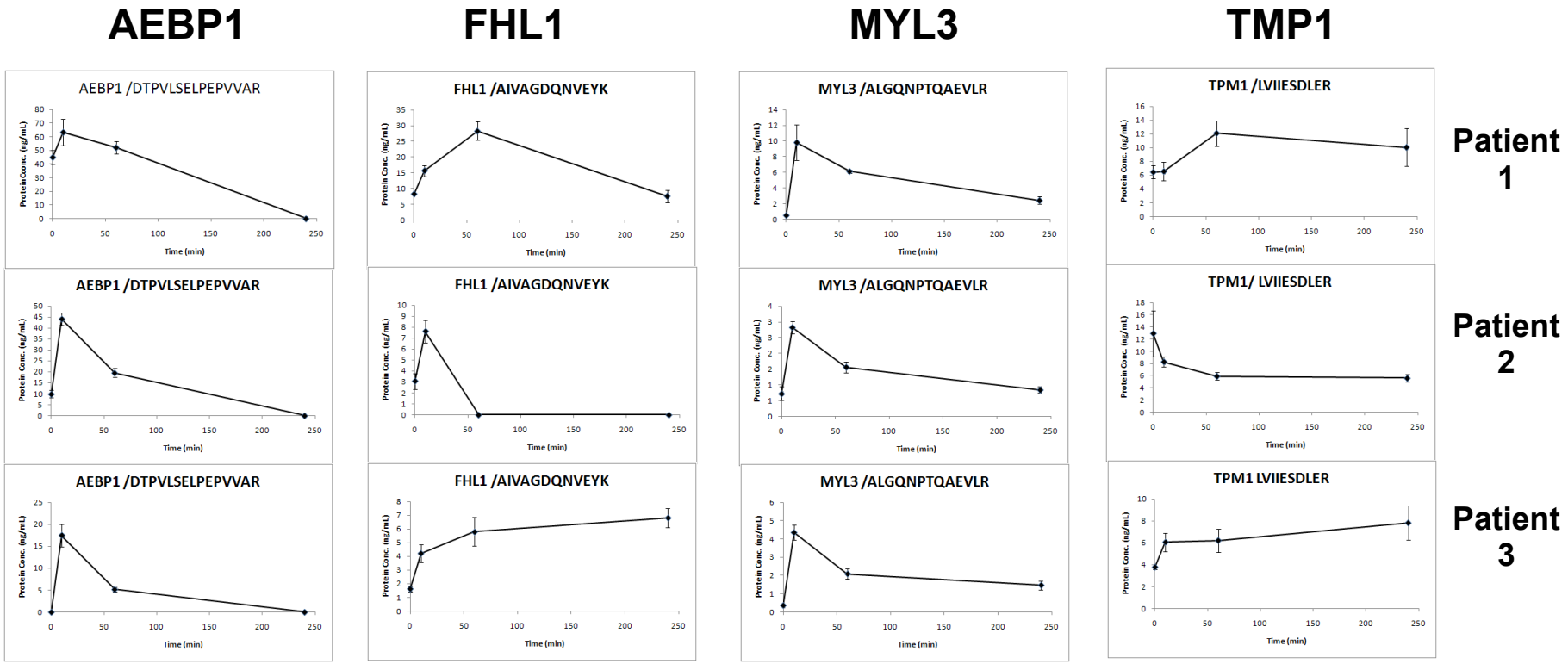
Optimized plasma processing has become at least 6X faster and 4X less expensive … and performs better



< 1000 proteins / sample
< 700 proteins measured in all samples
Troponins not quantified

~ 5000 proteins / sample
3800 proteins measured in all samples
Troponins robustly quantified

MRM-MS assays ("Tier 2") for four novel candidate biomarkers of MI in peripheral plasma of PMI patients showed promising temporal profiles



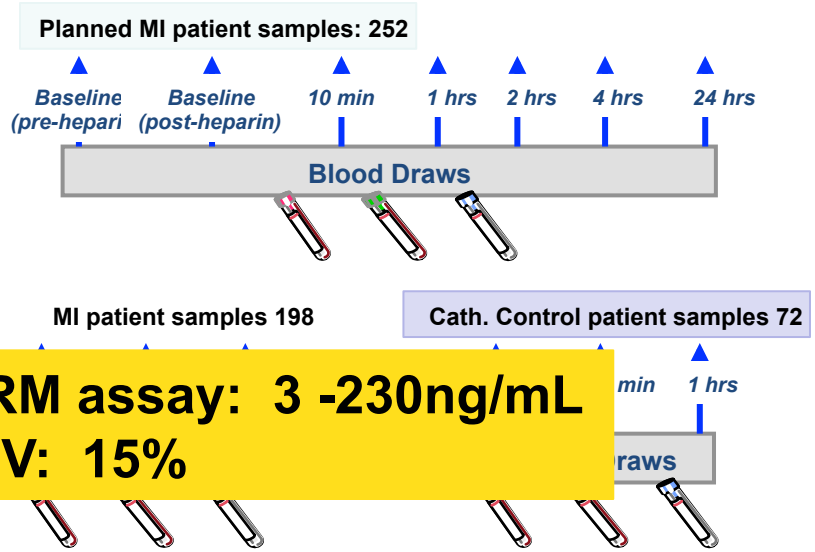
- All at low ng/mL range
- 4 time points/patient
- CVs for biological replicates under 20%

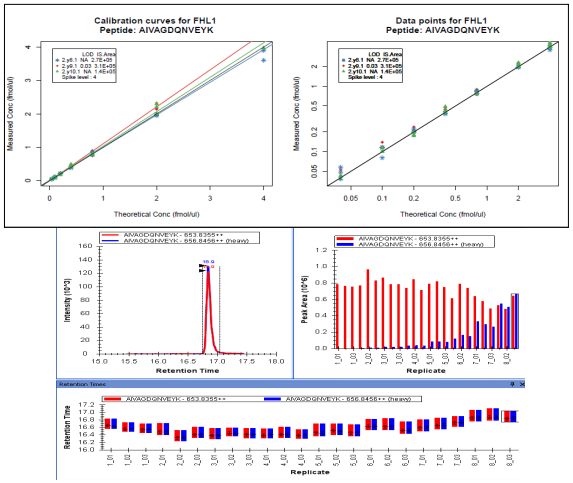
Addona et al. *Nature Biotechnology* **(2011)** 29: 635

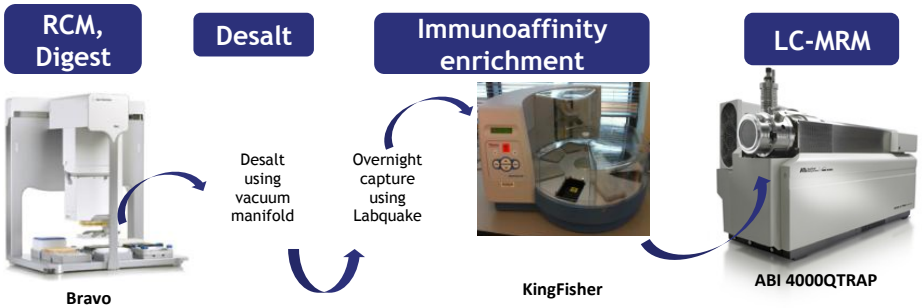
23-plex immunoMRM assay for CV disease biomarker candidates used to assay 522 patient samples in 2 months

Protein	Peptide	LOD (fm/ul)	LOQ (fm/ ul)	LOQ (ng/ mL)
Troponin I	NITEIADLTQK	0.16	0.48	11.60
IL 33	TDPGVFIGVK	0.07	0.21	6.56
	VLLSYYESQHPSNESGDGVDGK	0.07	0.22	6.62
ACLP Aortic carboxypeptidase-like protein 1	ILNPGEYR	0.04	0.11	14.33
	DTPVLSELPEPVVAR	0.60	1.81	237.21
FHL1 four and a half LIM domains 1 isoform 5	AIVAGDQNVEYK	0.03	0.10	3.26
	NPITGFGK	0.04	0.13	4.39
MYL3 Myosin light chain 3	AAPAPAPPPEPERPK	1.79	5.38	118.02
	ALGQNPTQAEVLR	0.06	0.19	4.16
	HVLATLGER	0.23	0.70	15.43
TPM1 Isoform 4 of Tropomyosin alpha-1 chain	LVIIESDLER	0.08	0.25	8.23
	SIDDLEDELYAQK	na	na	na
	HIAEDA[illegible]			
ITGB1 Isoform Beta-1C of Integrin beta-1	GEVFN[illegible]			
TAGLN2 Transgelin-2	ENFQN[illegible]			
TAGLN1 Transgelin-1	AAEDYG[illegible]			
FGL2 Fibroleukin	ELESE[illegible]			
	EEINVL[illegible]			
SCUBE2 Signal peptide	GSVACECRPGFELAK	0.05	0.15	16.41
FSTL1 Follistatin-related protein 1	IQVDYDGHCK	0.59	1.77	61.76
	LDSSEFLK	0.62	1.85	64.66
SPON1 Spondin-1	VEGDPDFYKPGTSYR	0.04	0.11	9.91
	AQWPAWQPLNVR	0.13	0.39	35.30

LOQ range in 23-plex iMRM assay: 3 -230ng/mL
Median CV: 15%







"Clinical proteomics" encompasses a spectrum of activity from pre-clinical discovery to applied diagnostics

- Proteomics applied to clinically relevant materials
  - "Quantitative and qualitative profiling of proteins and peptides that are present in clinical specimens like tissues and body fluids"
- Proteomics addressing a clinical question or need
  - Analytical and clinical validation and implementation of novel diagnostic or therapy related markers identified in preclinical studies
- MS-based and/or proteomics-derived test in the clinical laboratory and informing clinical decision making
  - Emphasis on fluid proteomics
  - Includes the selection, validation and assessment of standard operating procedures (SOPs) in order that adequate and robust methods are integrated into the workflow of clinical laboratories

Validation requirements for a "Level 1 Clinical Assay" set a very high bar

***Simplified* example of what might be acceptable to FDA:**

Precision: <8% within-day variability, <12% between-day variability

Bias: <5% on each of five days

Calibration curve slope: <5% difference over five days

Interference and Matrix effects: Blank samples (with no spiked internal standard peptide) and double blanks (with no spiked peptide or spiked internal standard peptide) contribute less than 5% of LLOQ signal, recovery of analyte spiked into 60 samples is 85-115% for all samples, three transitions monitored and the two transition ratios are within 25% of mean for all 60 samples and are monitored for all samples in production as QA

Simplified example of what might be acceptable to FDA (cont'd):

LLOQ validation: A sample run consecutively for 25 days at a level 50% above the LLOQ has a precision <15%

Carryover: Blank samples run after a matrix-matched highest calibrator have less than 5% of the signal at the LLOQ for the endogenous peptide and internal standard channels

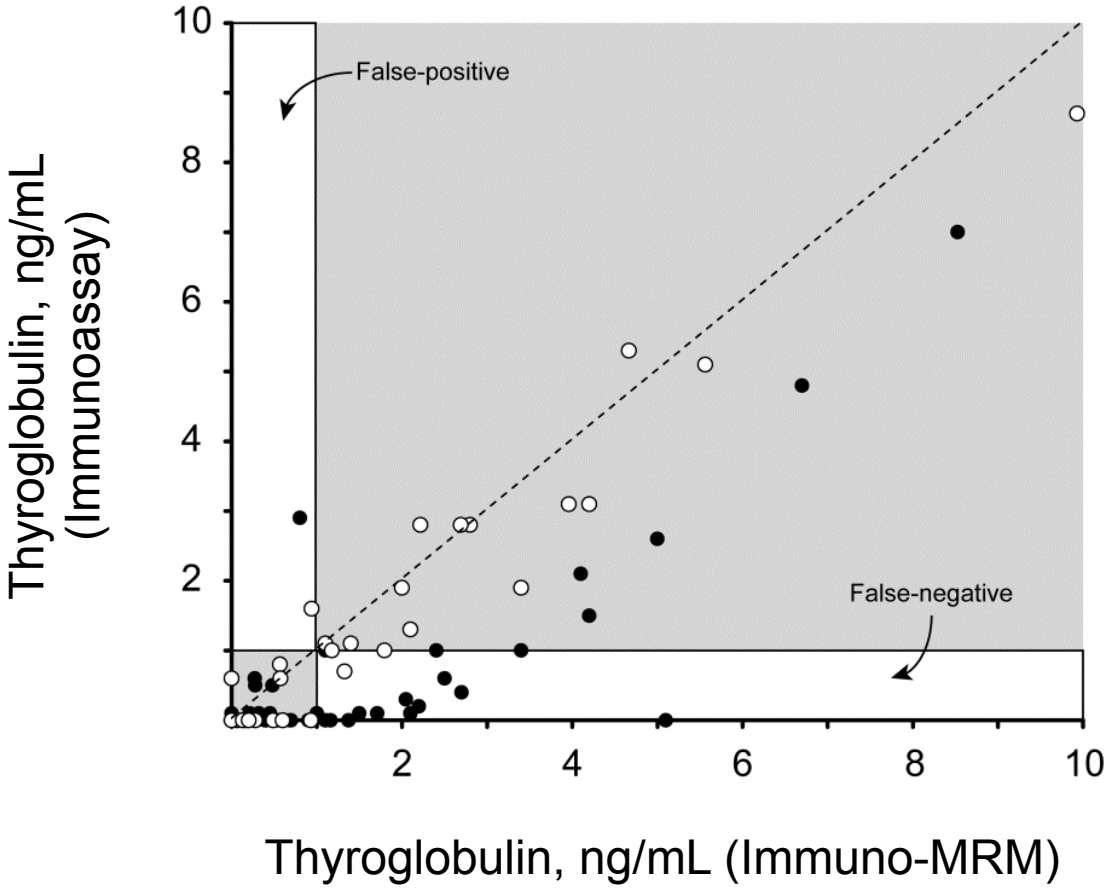
Stability and sample type: different collection and storage conditions are evaluated for the effect on the measurement of the endogenous anlayte concentration, no effect is >15%

Clinical validation: safe and effective (PMA, 100s-1000s of samples), equivalence (510k, 100s of samples)

Somewhat lower levels of analytical validation could be clinically implemented under CLIA (Clinical Laboratory Improvement Amendments)

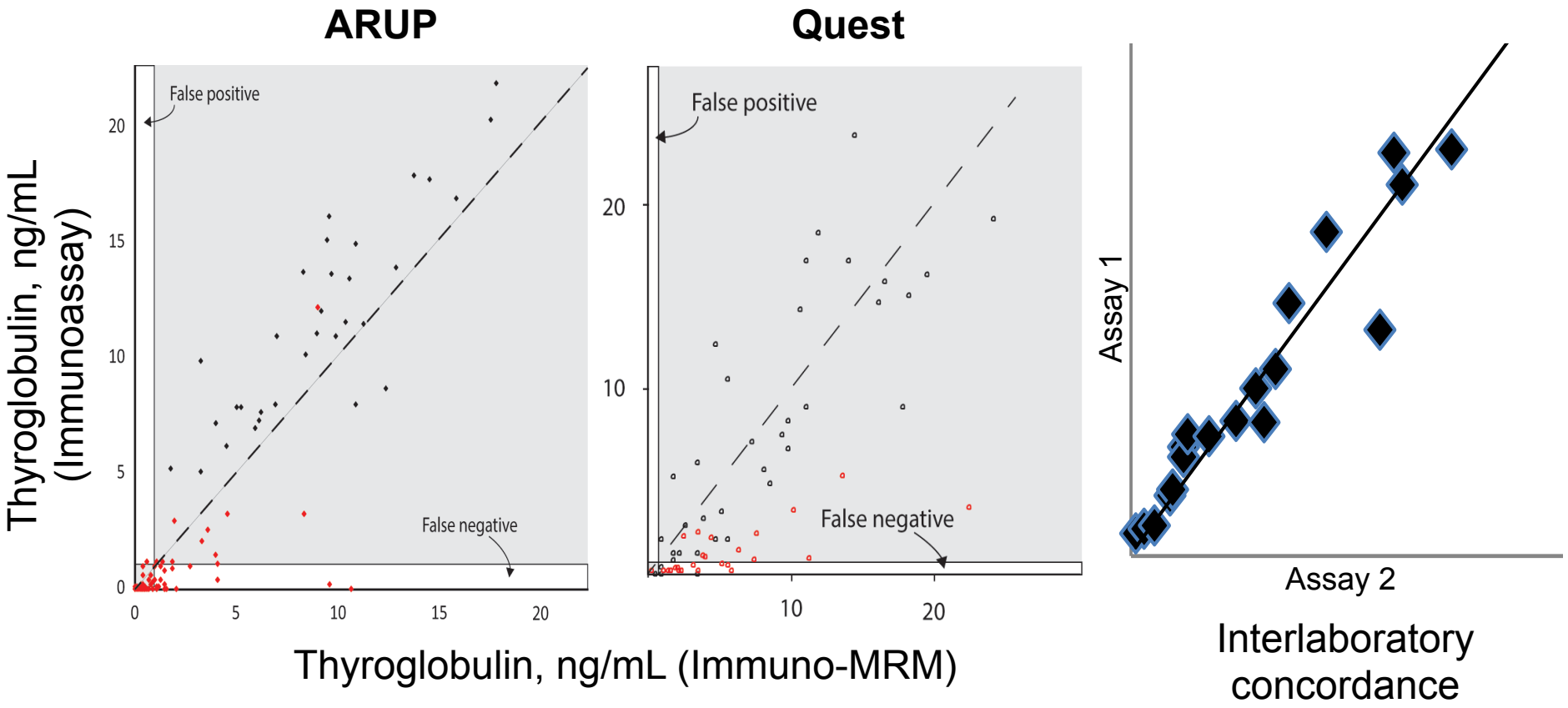
A. Hoofnagle

Clinical iMRM assay for thyroid cancer marker to address interference from autoantibodies



Hoofnagle, JCEM, 2013

Assay was replicated at National Reference Labs with high interlaboratory concordance

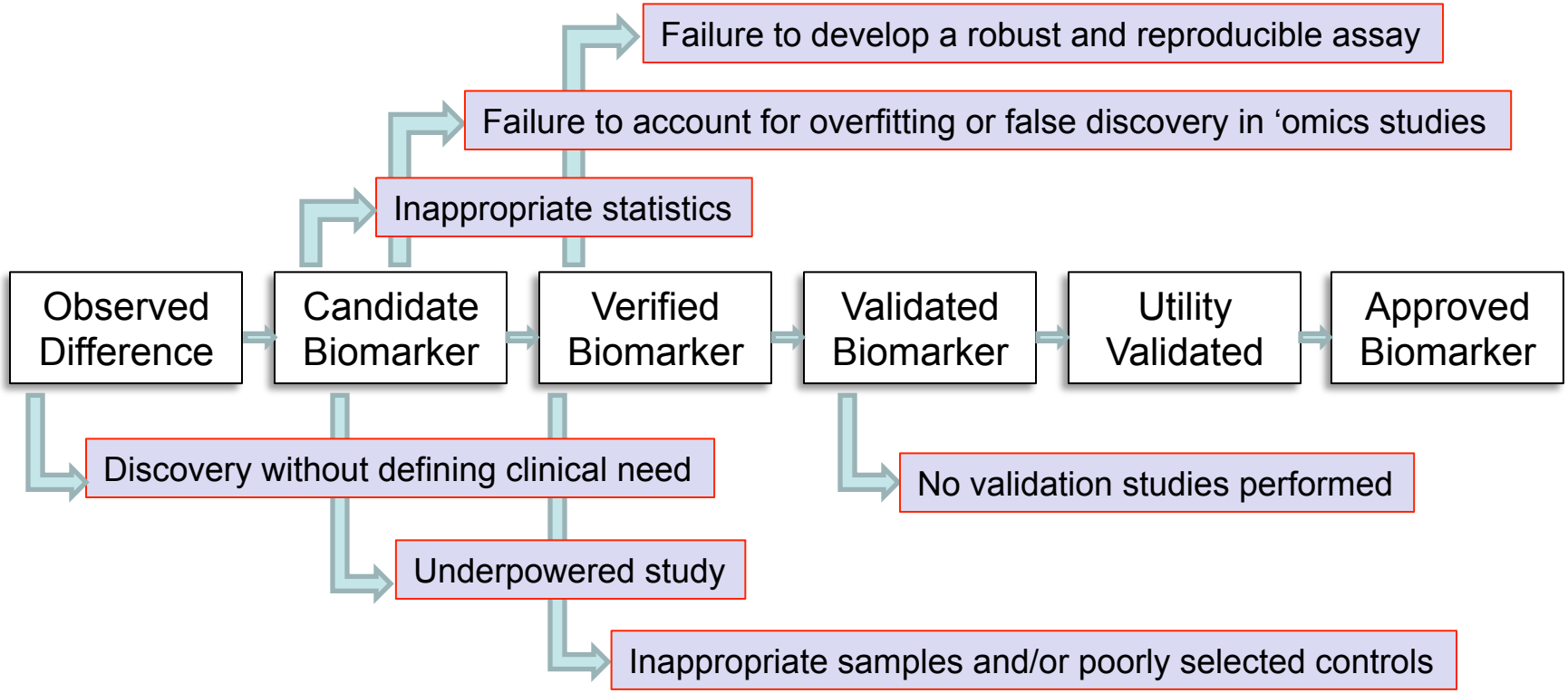


Kushnir, Clin Chem, 2013

Clarke, J Inv Med, 2012

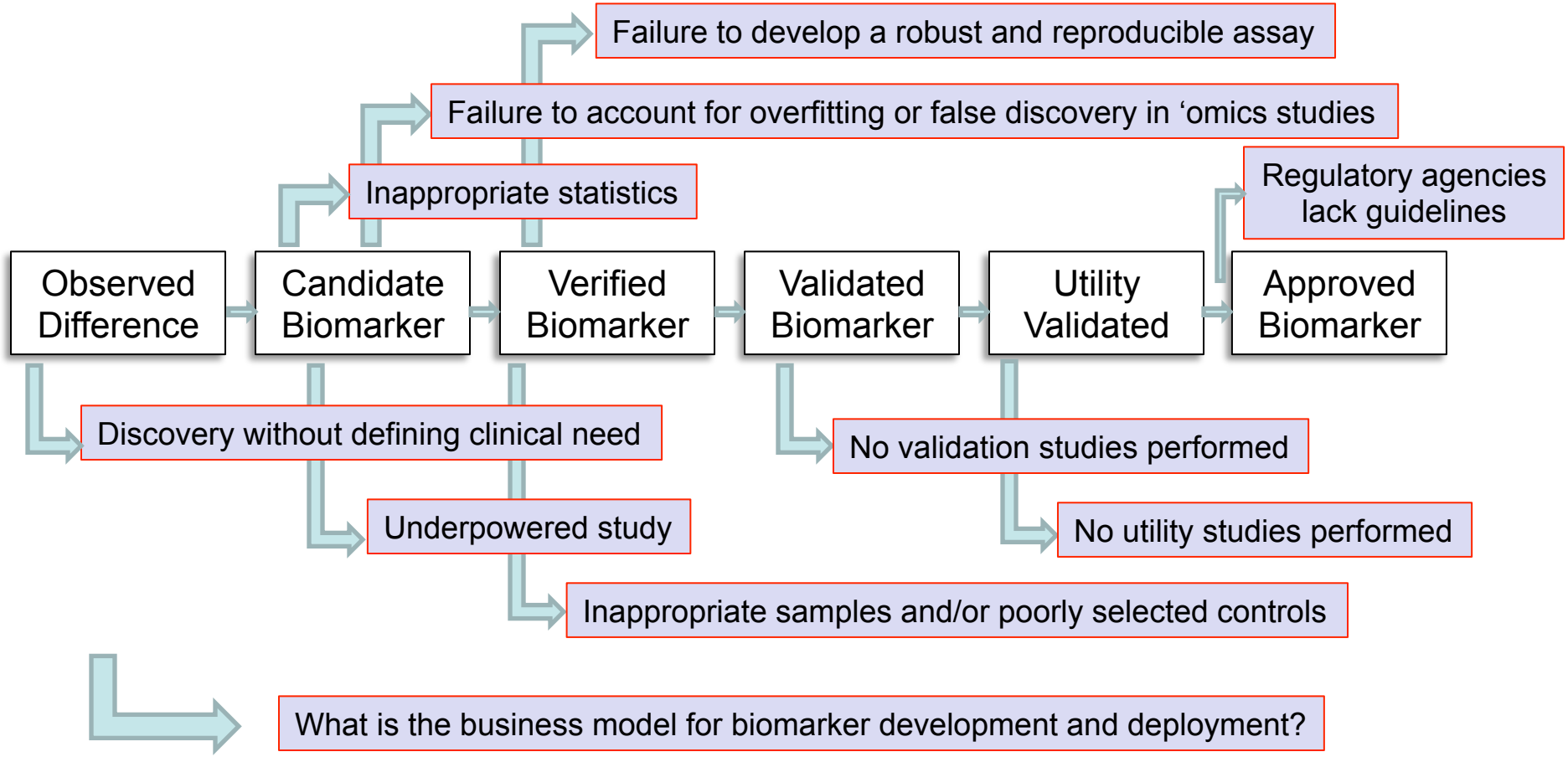
Hoofnagle, personal communication

Biomarker development is fraught with common pitfalls



Modified from Josh LaBaer

Biomarker development is fraught with common pitfalls



Modified from Josh LaBaer

Clinical proteomics is hard, but it's not as hard as this.
We'll succeed if we systematically identify and address the challenges.

Conclusions

- Clinical proteomics begins with “Clinical” – invest in defining the question or need and finding the right samples
- Modern proteomic approaches and technologies when coherently integrated can yield new biological insights and novel, sufficiently credentialed biomarker candidates that merit real clinical evaluation
- New, targeted MS-based methods enable highly specific and sensitive quantitative measurement of proteins and their modifications in high multiplex
  - MRM-MS is becoming the new workhorse technology
  - Broad availability of this resource will change paradigms for how experiments are planned and executed
  - With technological evolution, convergence of discovery and verification is likely

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