

# Using Targeted Proteomic Assays in the Care of Patients

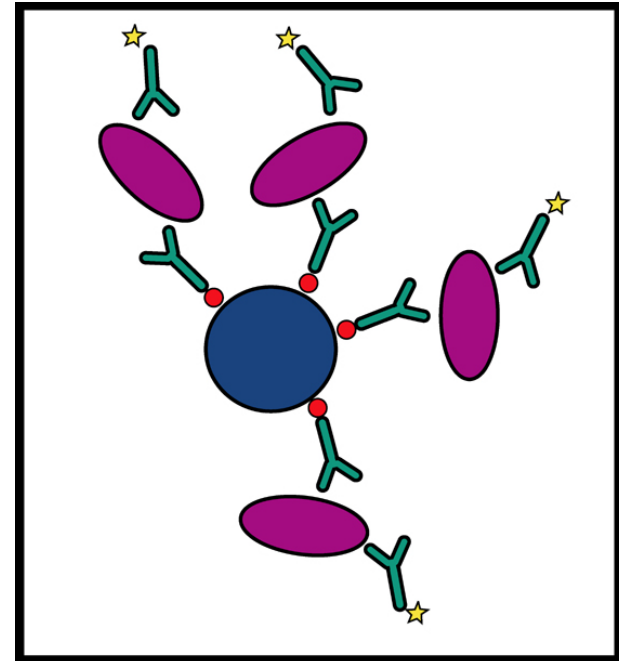


Andy Hoofnagle, MD PhD  
Department of Laboratory Medicine  
University of Washington

# Why Use Mass Spectrometry?

## Problems with clinical immunoassays

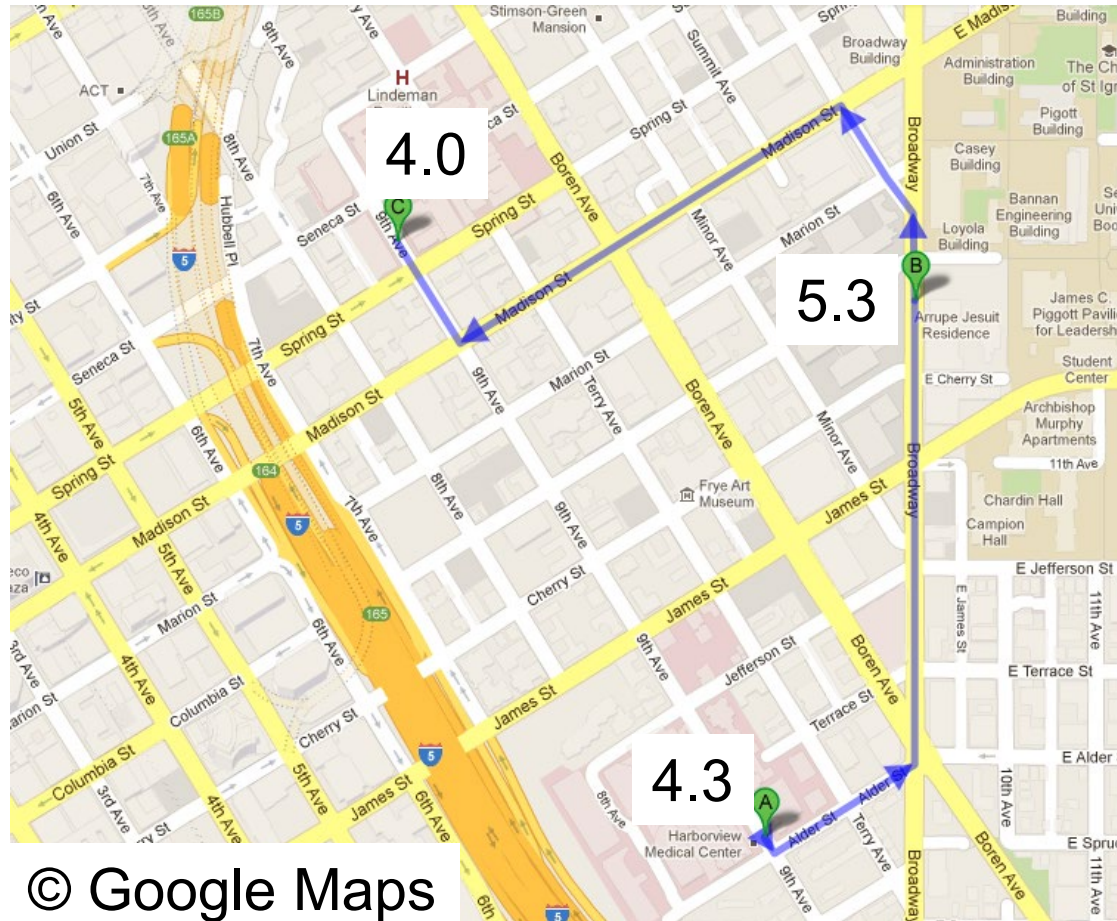
- Specificity
- Poor standardization
- Hook effect
- Anti-reagent antibodies
- Autoantibodies
- \* Microclots
- Single-plex



*Hoofnagle and Wener, J Immunol Methods (2009)*

*\* Strathmann, et al., AJCP (2011)*

# Hypothyroidism is Diagnosed with TSH

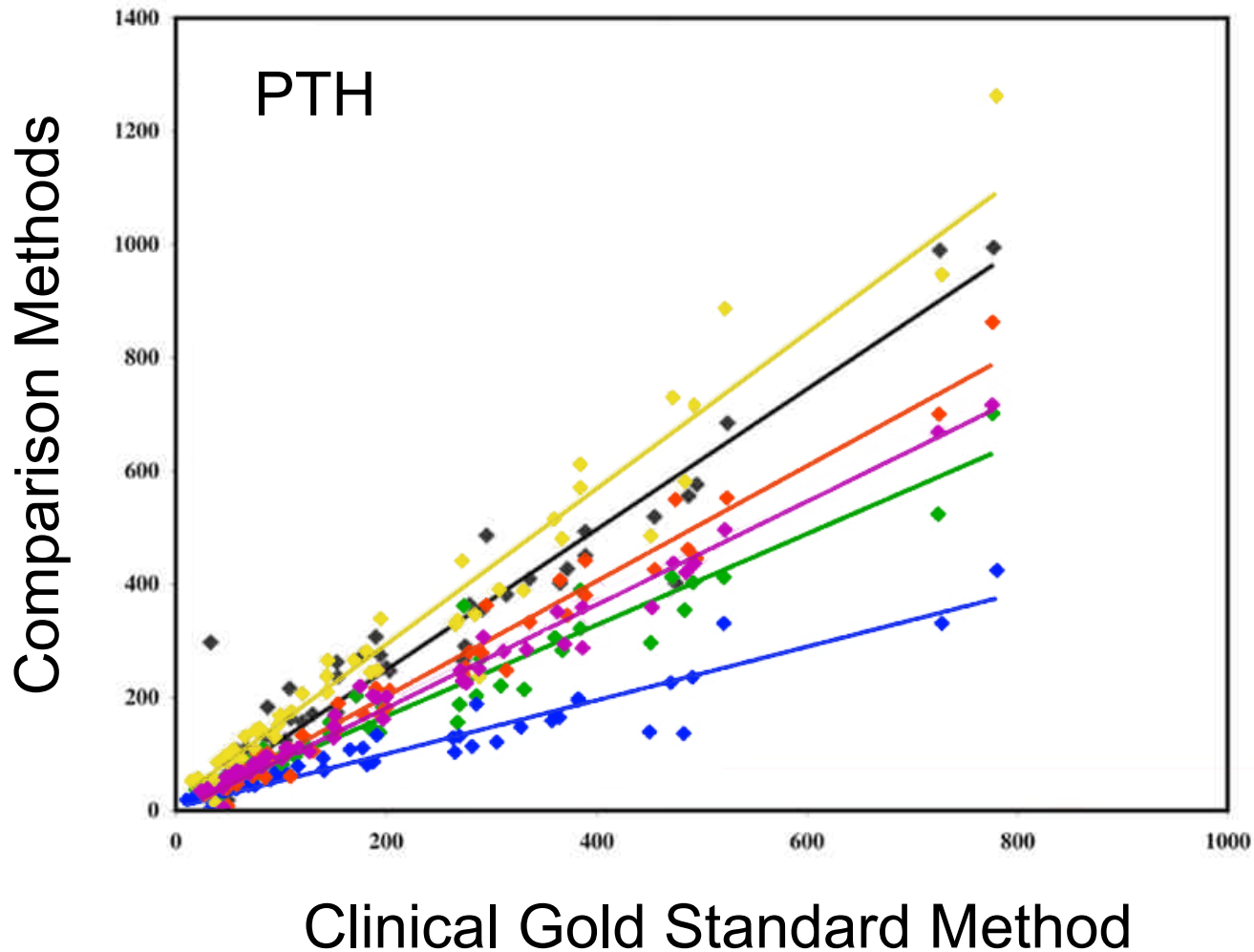


## Three hospitals, 0.5 miles apart, three platforms, two different outcomes

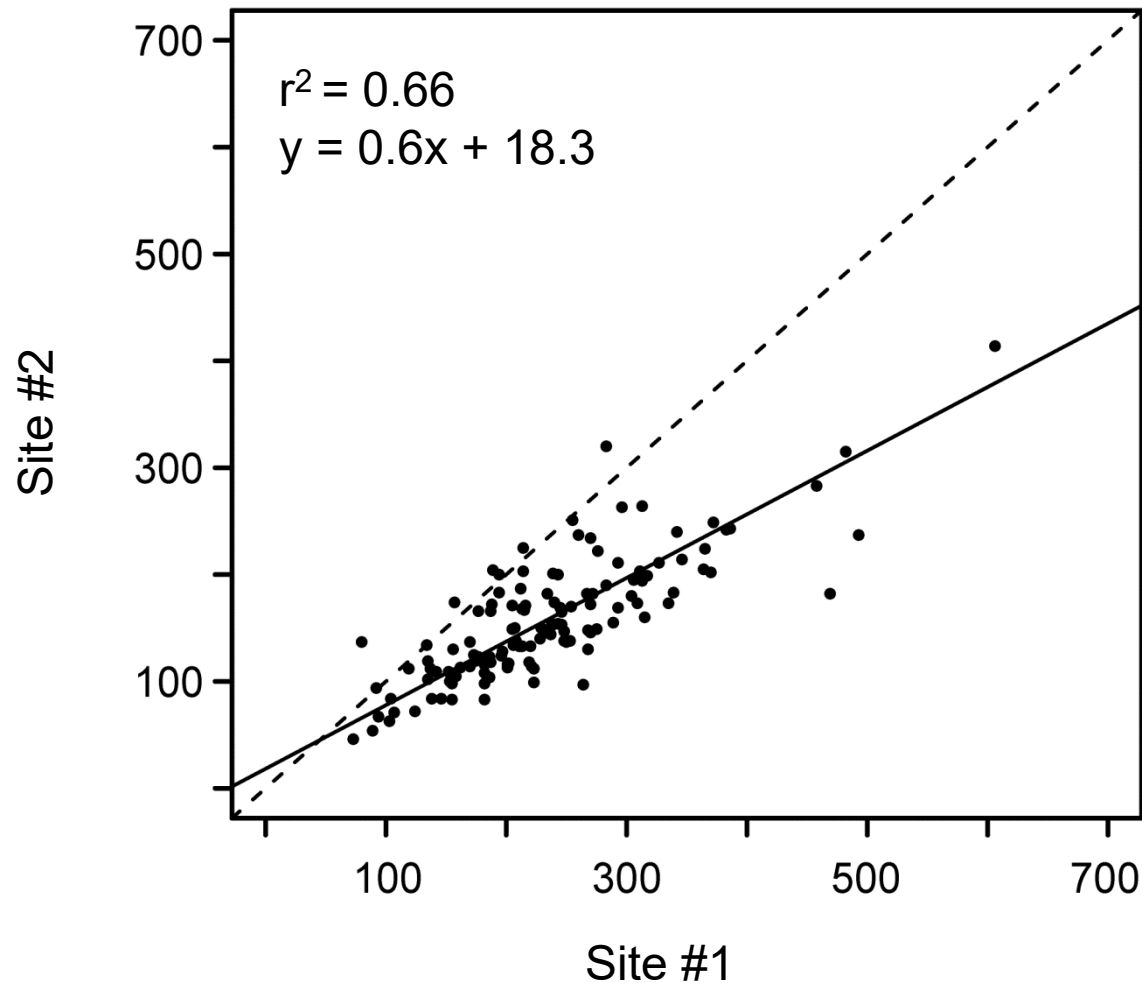
Roberts, Clin Chem, 2004

# This is Not Uncommon

*Seven FDA-approved Immunoassays, One Laboratory*



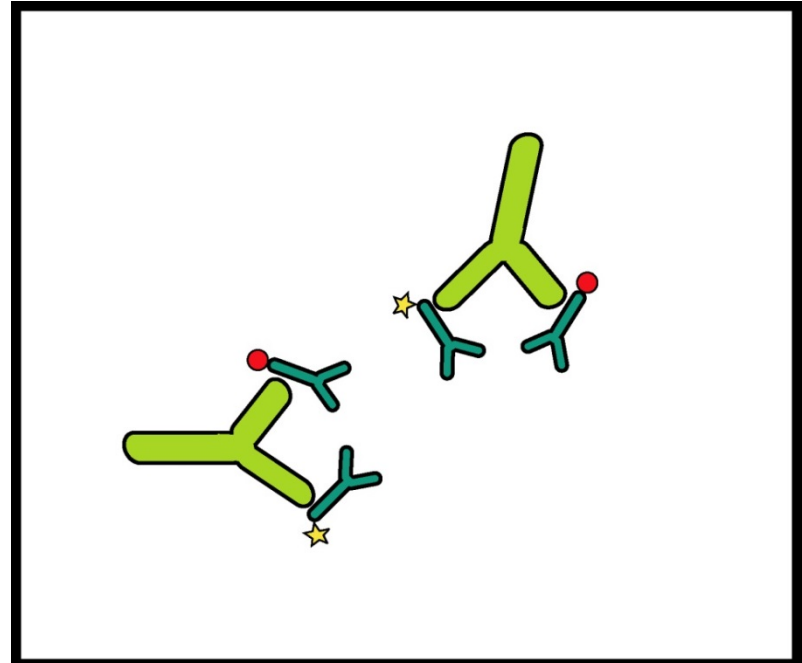
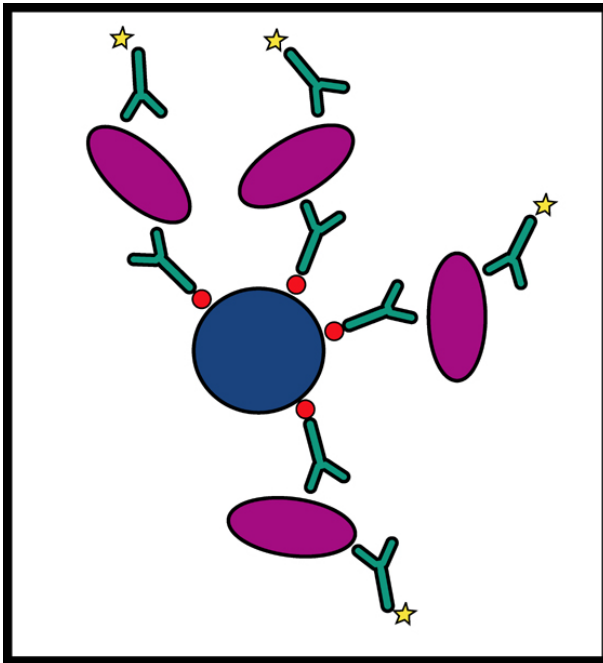
# Comparison of Immulite-1000 Assay at Two Sites



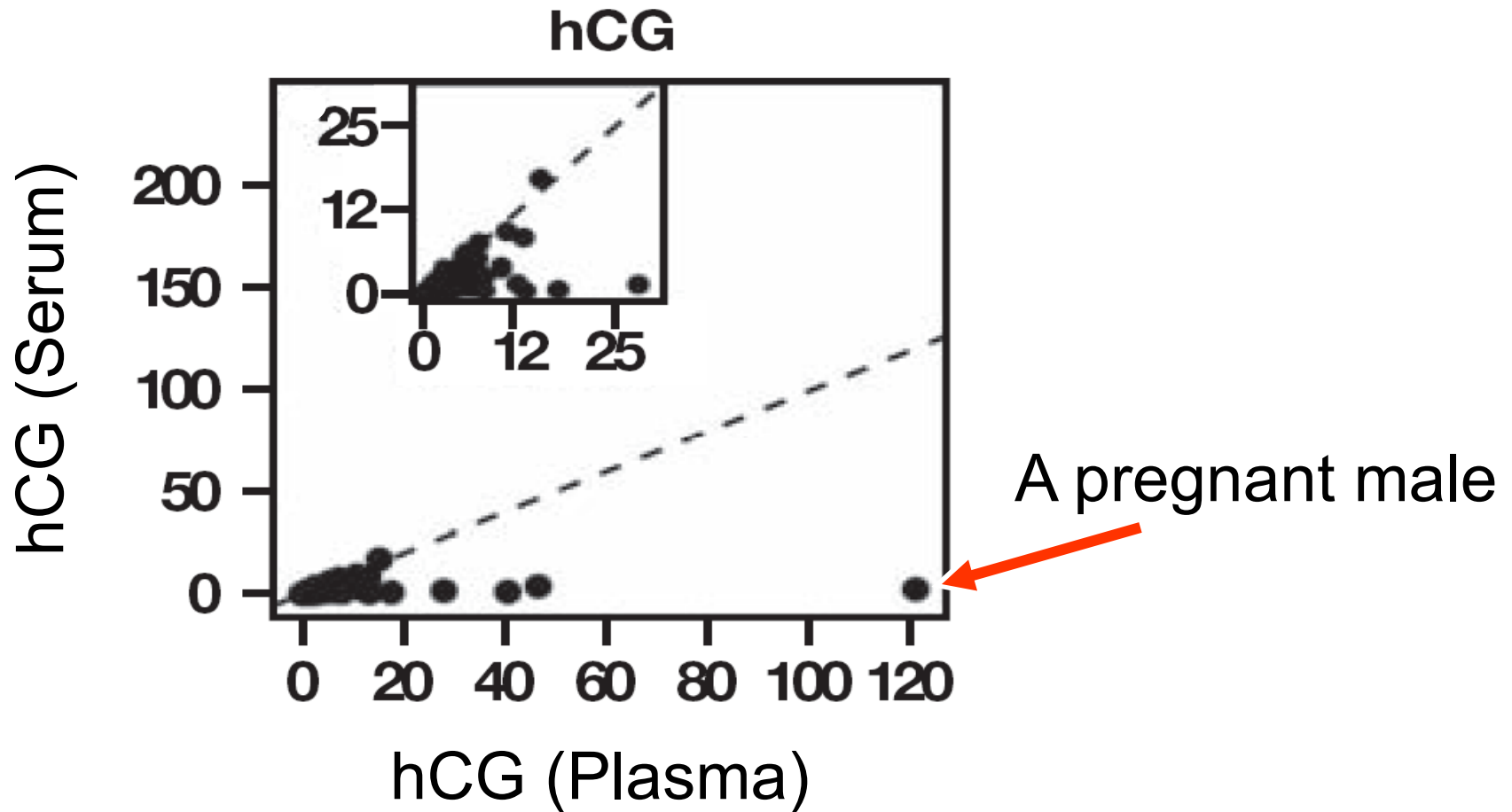
# Anti-reagent Interferences

## *Bridging the Gap*

Non-specific anti-reagent antibodies  
can cause false positive results

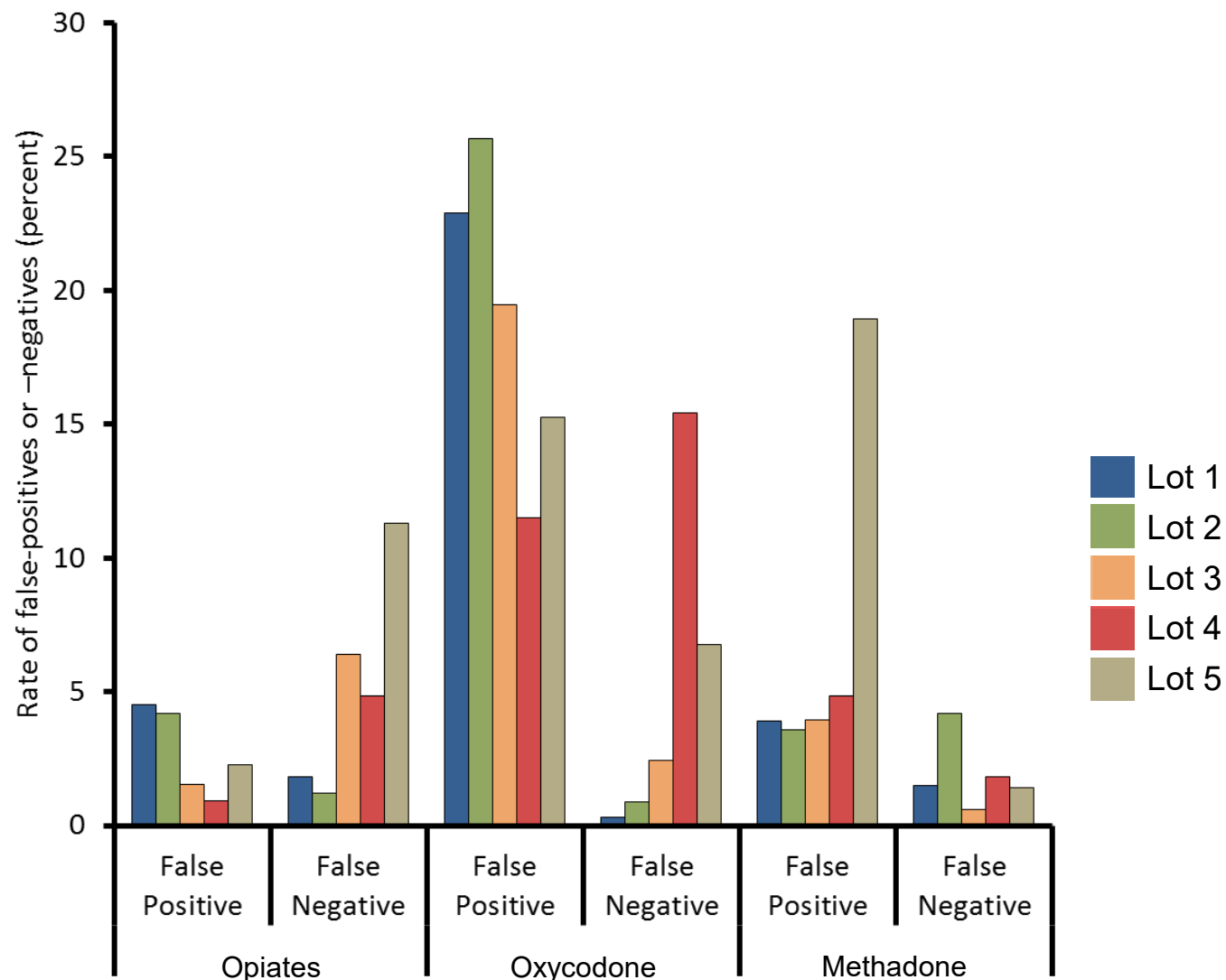


# Microclots



Something in plasma leads to false positives (not in serum)

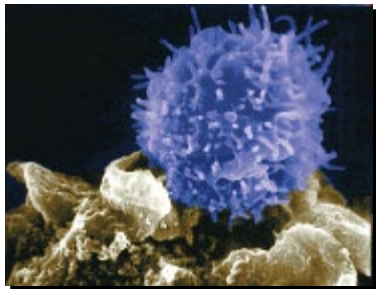
# Rates of False Negative and False Positive Results Change with Immunoassay Lot Number



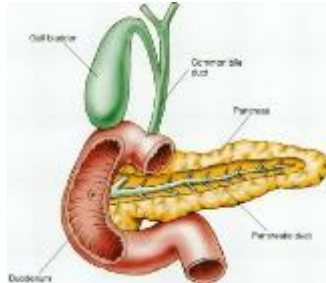
# Hypothesis-driven Method Development

*Vitamin D Biology*

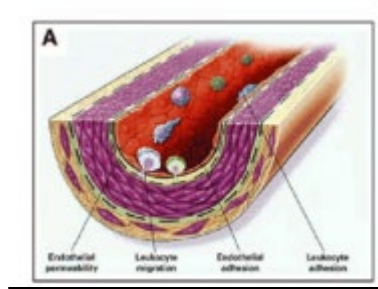
# Myriad Actions of 1,25-dihydroxyvitamin D



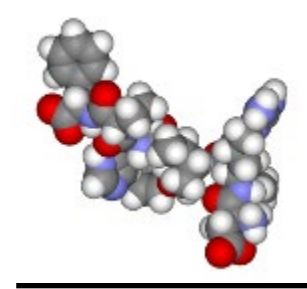
Immune cell  
function



Glucose  
homeostasis



VSMC



↓RAAS



Cardio-  
myocytes

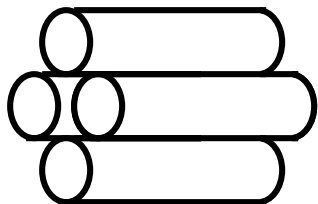
Atherosclerosis  
prevention

↓LVH

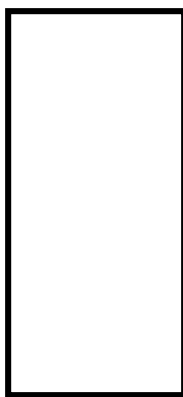
Ian de Boer

# Selected Reaction Monitoring

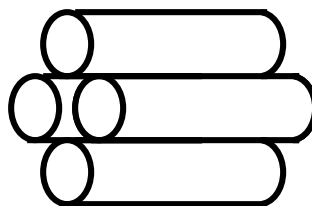
*Measuring Four Things At Once*



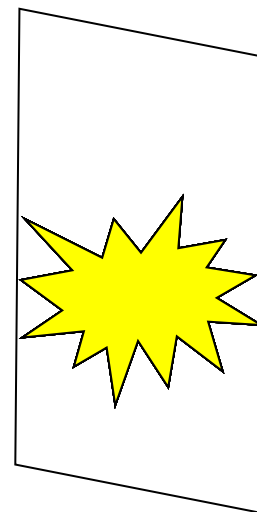
First  
Quadrupole



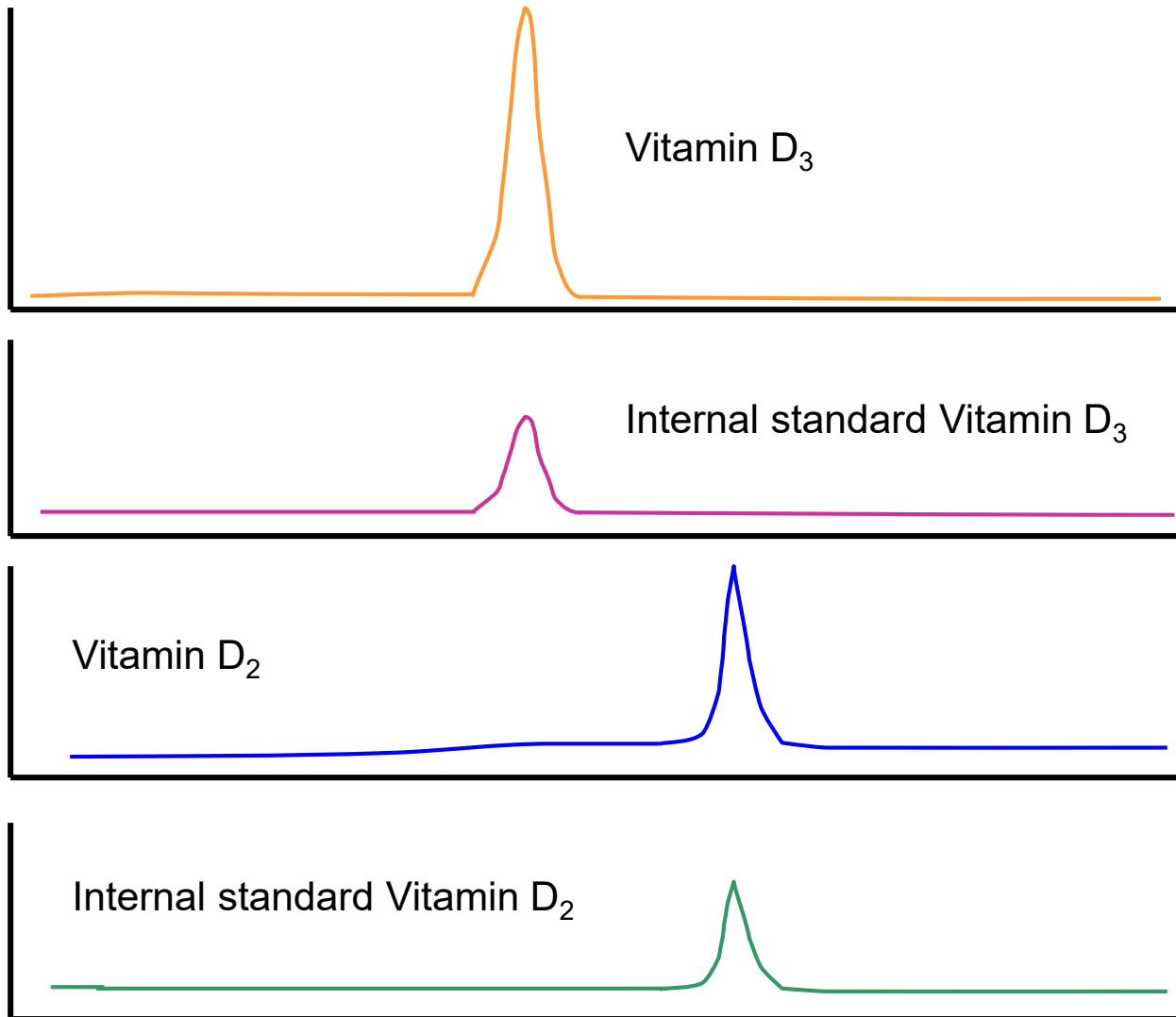
Collision  
Cell



Second  
Quadrupole

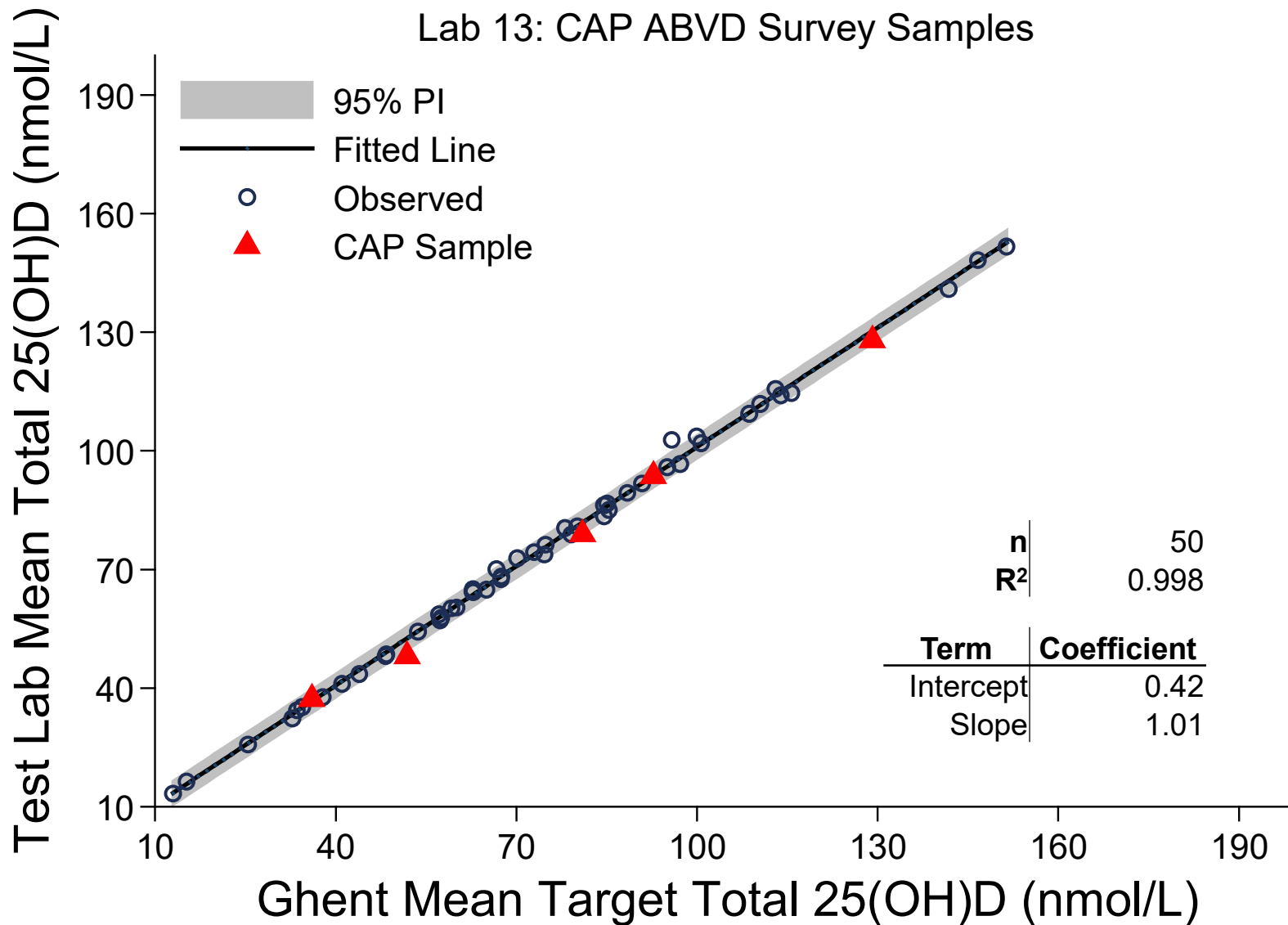


# Measuring Many Things at Once



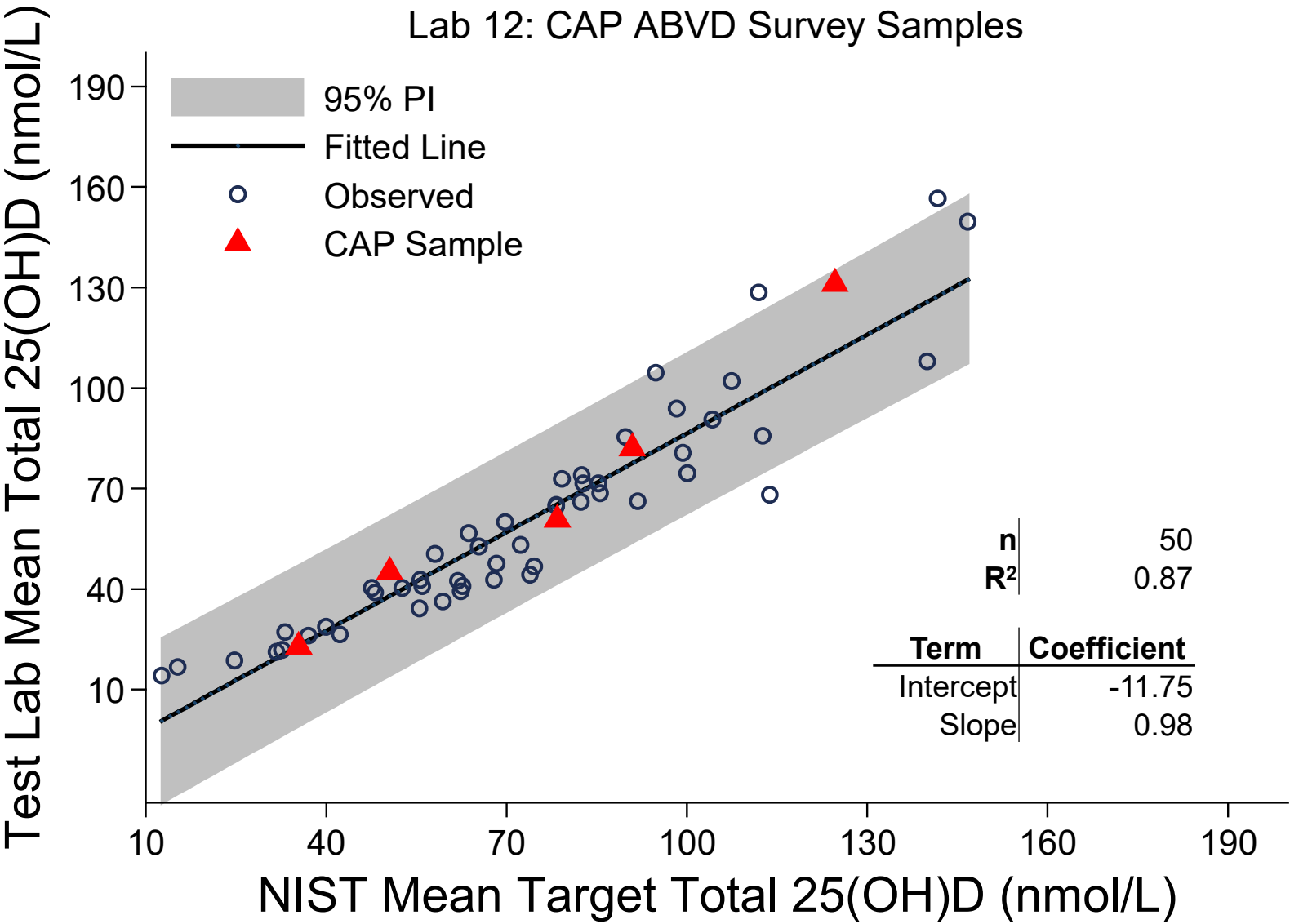
# VDSP Commutability Study: LC-MS/MS

Lab 13: CAP ABVD Survey Samples



# VDSP Commutability Study: Immunoassay

Lab 12: CAP ABVD Survey Samples

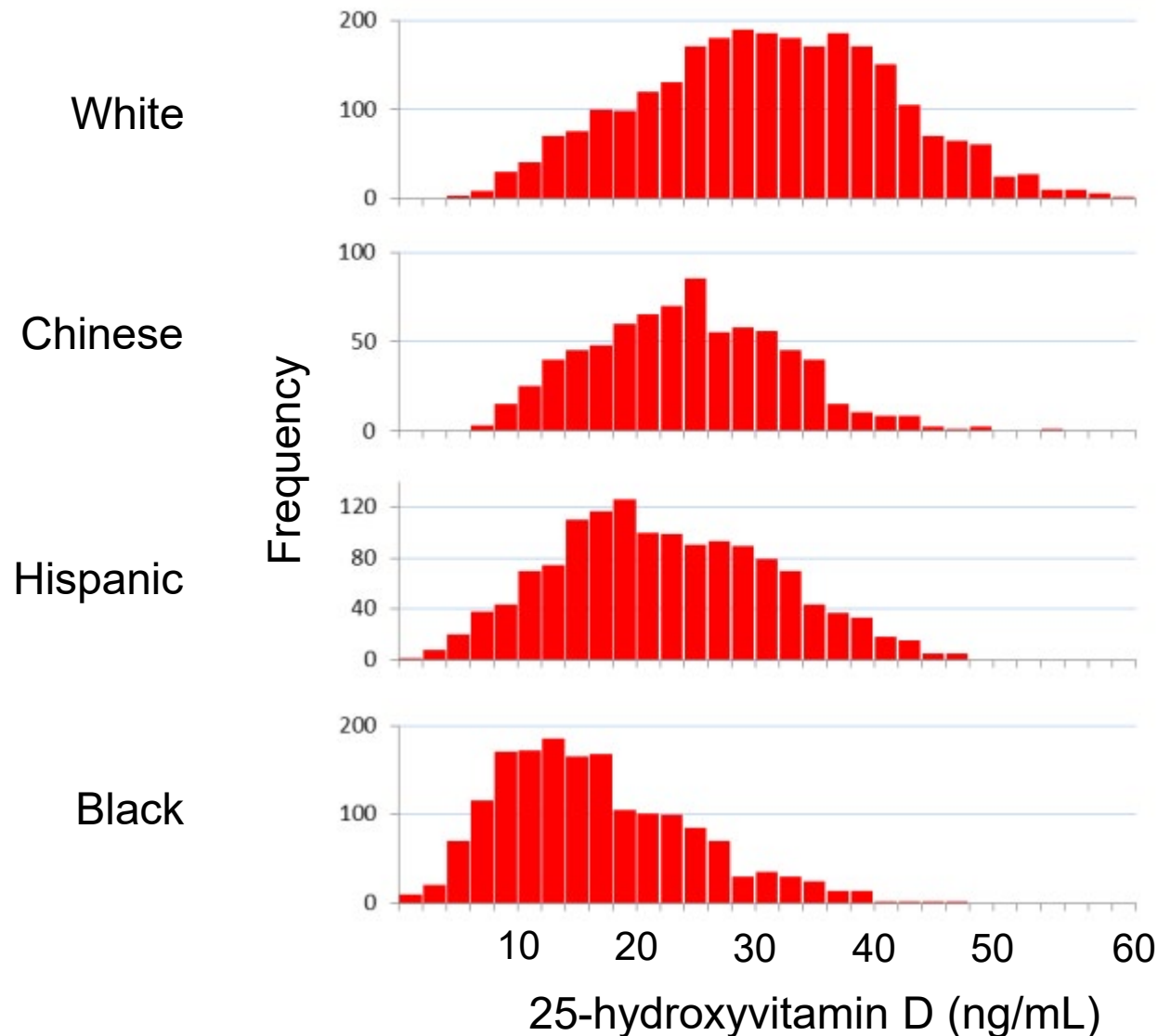


# Multi-Ethnic Study of Atherosclerosis (MESA)

**A large, representative group accrued throughout the US that includes four races and incredible follow-up**

- 6,814 people
- 45-84 years of age
- 53% female
- Sites: NC, NY, MD, MN, IL, CA
- African Americans, Chinese Americans, Whites, and Hispanics
- $\geq 8$  years follow-up

# Racial Differences in 25(OH)D Concentrations



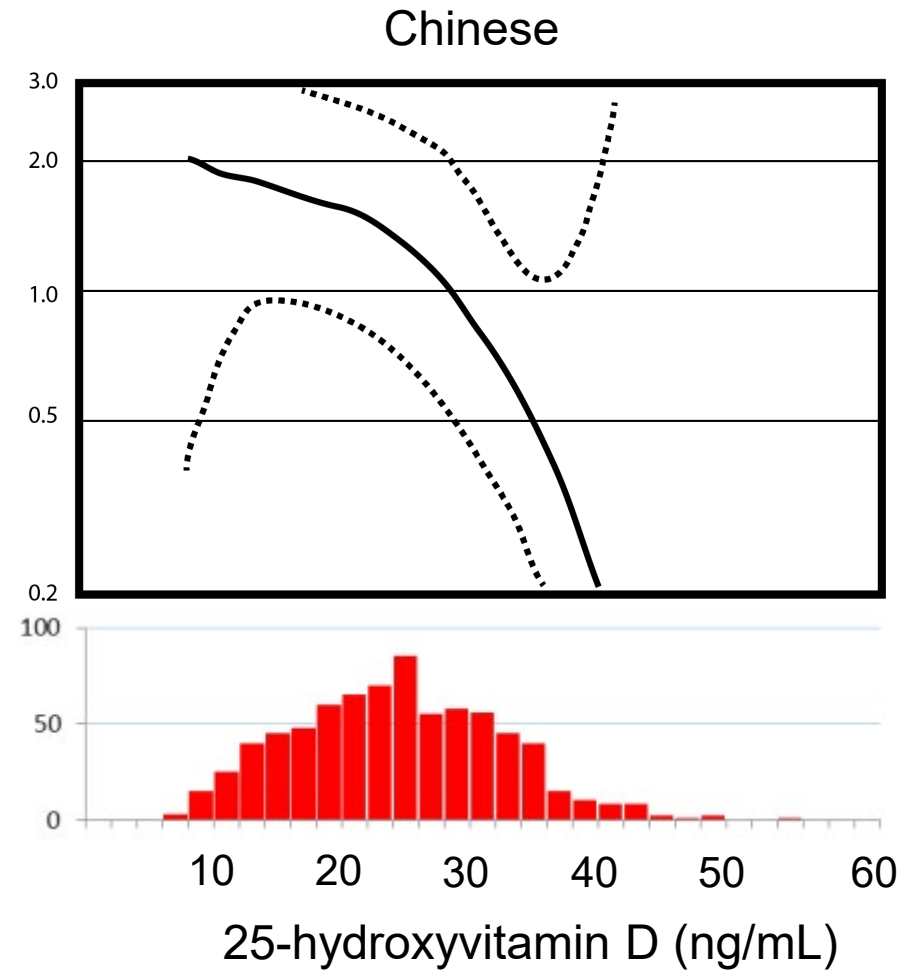
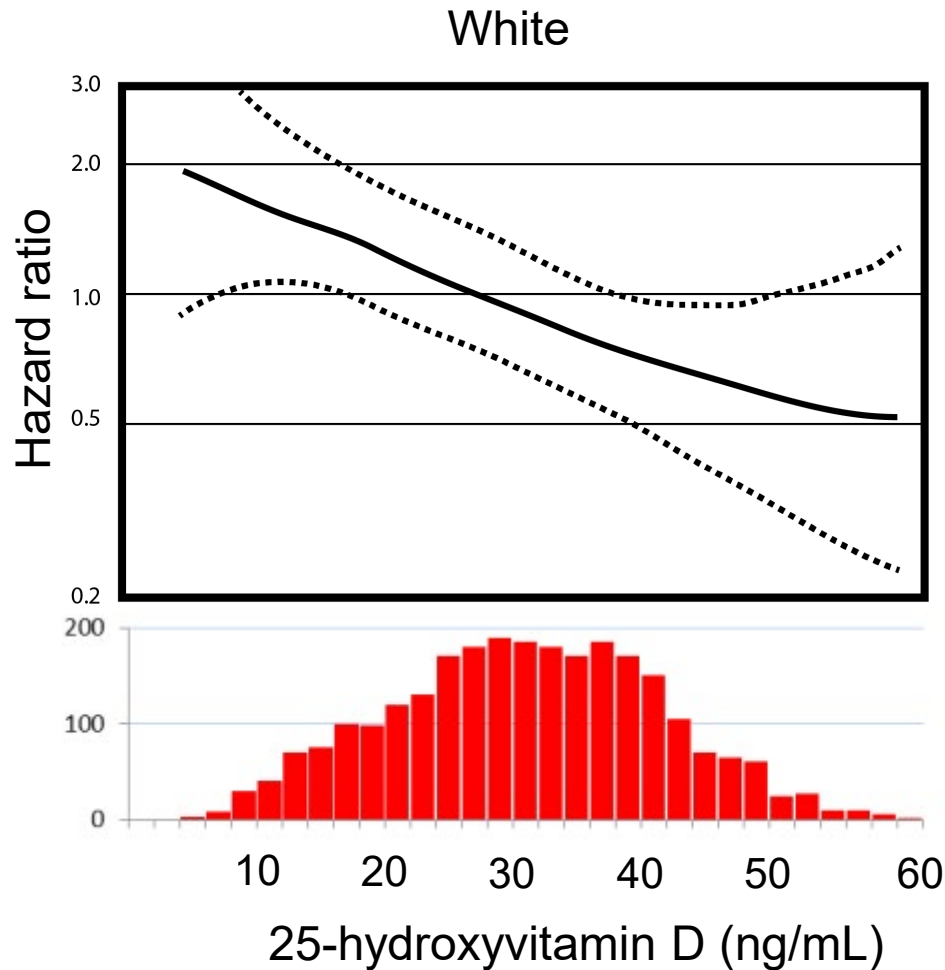
Adapted from Robinson-Cohen, 2013, JAMA

# National Health and Nutrition Examination Survey

Male	ng/mL	Female	ng/mL
<b>12–29 y</b>		<b>12–29 y</b>	
White	33.4	White	29.9
Black	20.0	Black	16.9
Mexican American	27.6	Mexican American	23.1
<b>30–59 y</b>		<b>30–59 y</b>	
White	30.0	White	26.4
Black	19.5	Black	16.7
Mexican American	25.4	Mexican American	21.4
<b>&gt;59 y</b>		<b>&gt;59 y</b>	
White	30.3	White	25.8
Black	21.3	Black	18.8
Mexican American	26.3	Mexican American	23.5

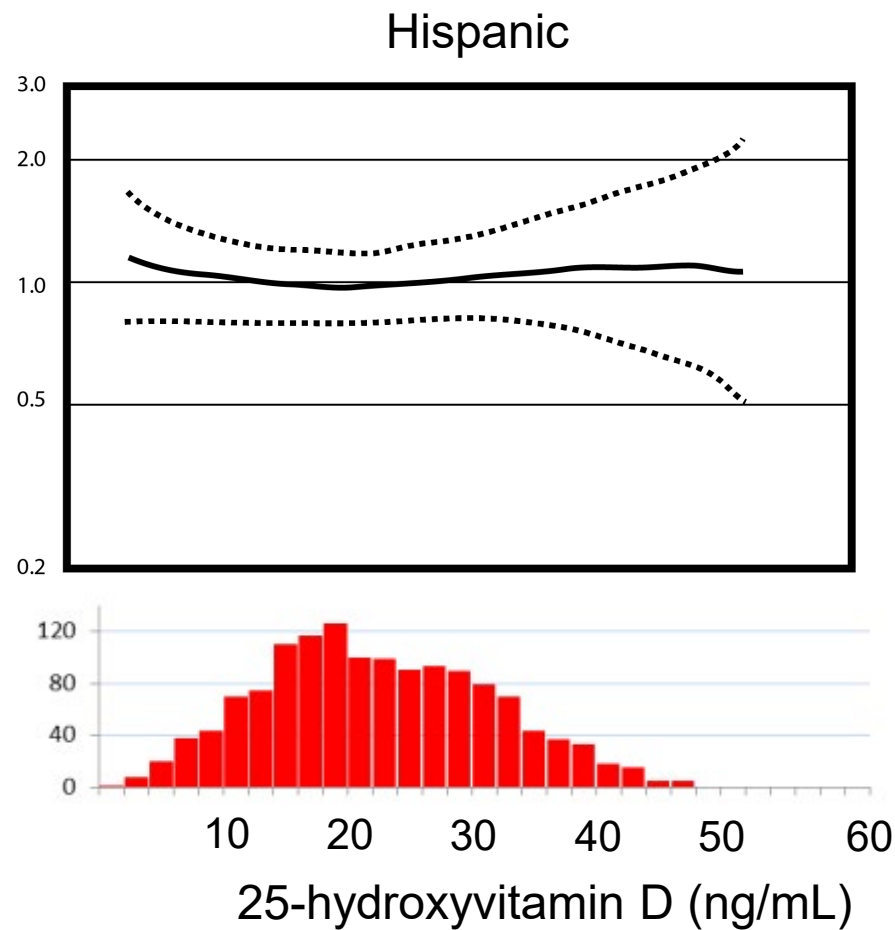
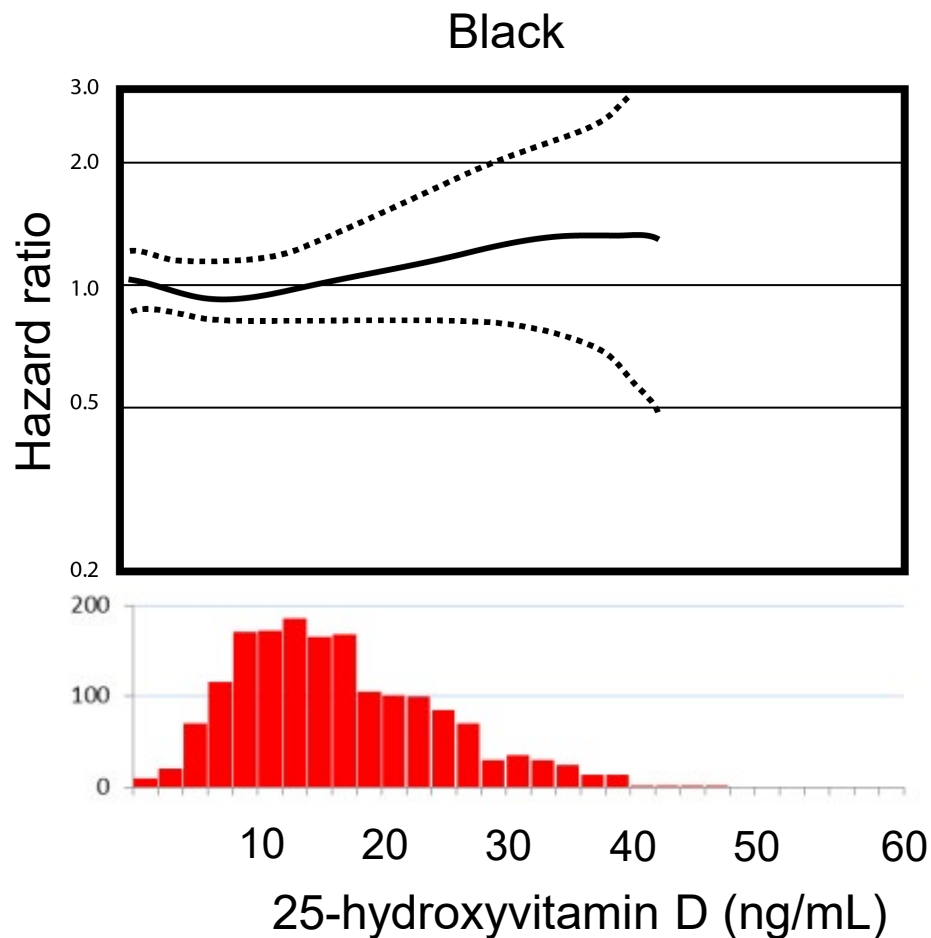
Data from Weaver and Fleet, AJCN, 2004

# Racial Differences in CVD Outcomes



Adapted from Robinson-Cohen, 2013, JAMA

# Racial Differences in CVD Outcomes



# The Vitamin D Paradox

**Blacks have lower 25(OH)D**

But...

No increased risk of CAD with low 25(OH)D

Better bone health

*What about other vitamin D metabolites?*

24,25(OH)<sub>2</sub>D

1,25(OH)<sub>2</sub>D

Free 25(OH)D

Bioavailable 25(OH)D →

Calculated from:

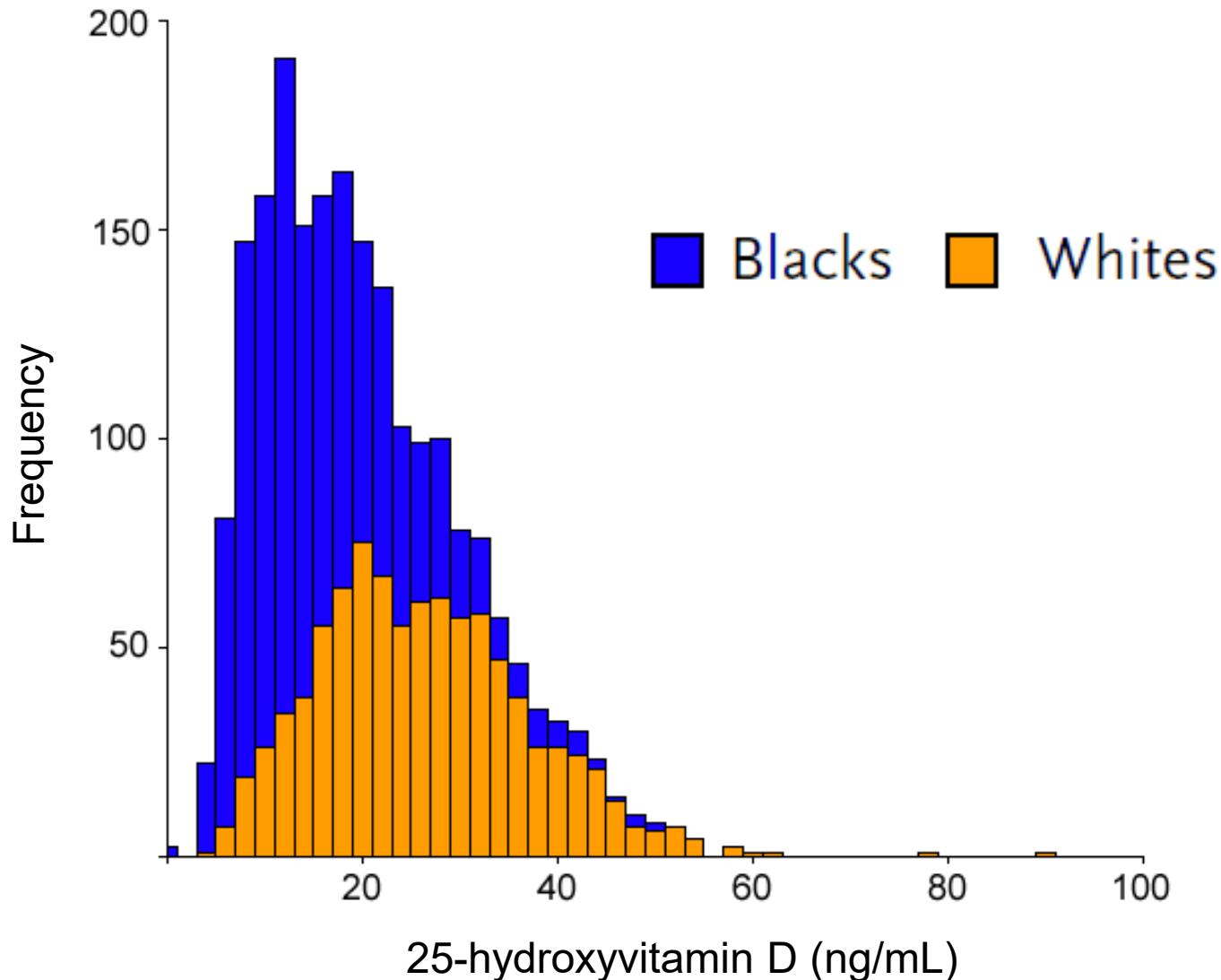
25(OH)D

Albumin

Vitamin D Binding Globulin

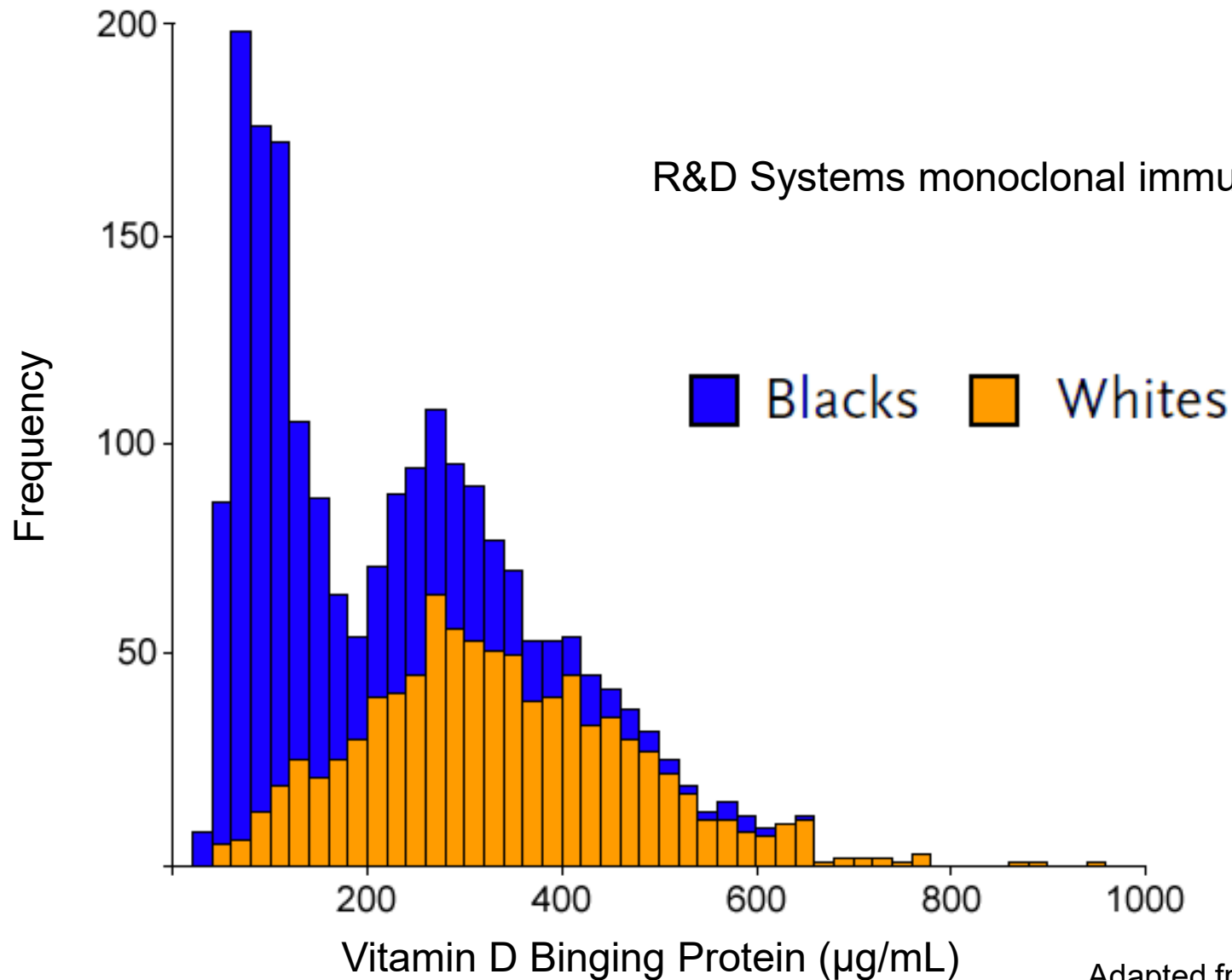
Equations determined long ago

# Blacks Have Lower 25-hydroxyvitmain D



Adapted from Powe, NEJM, 2013

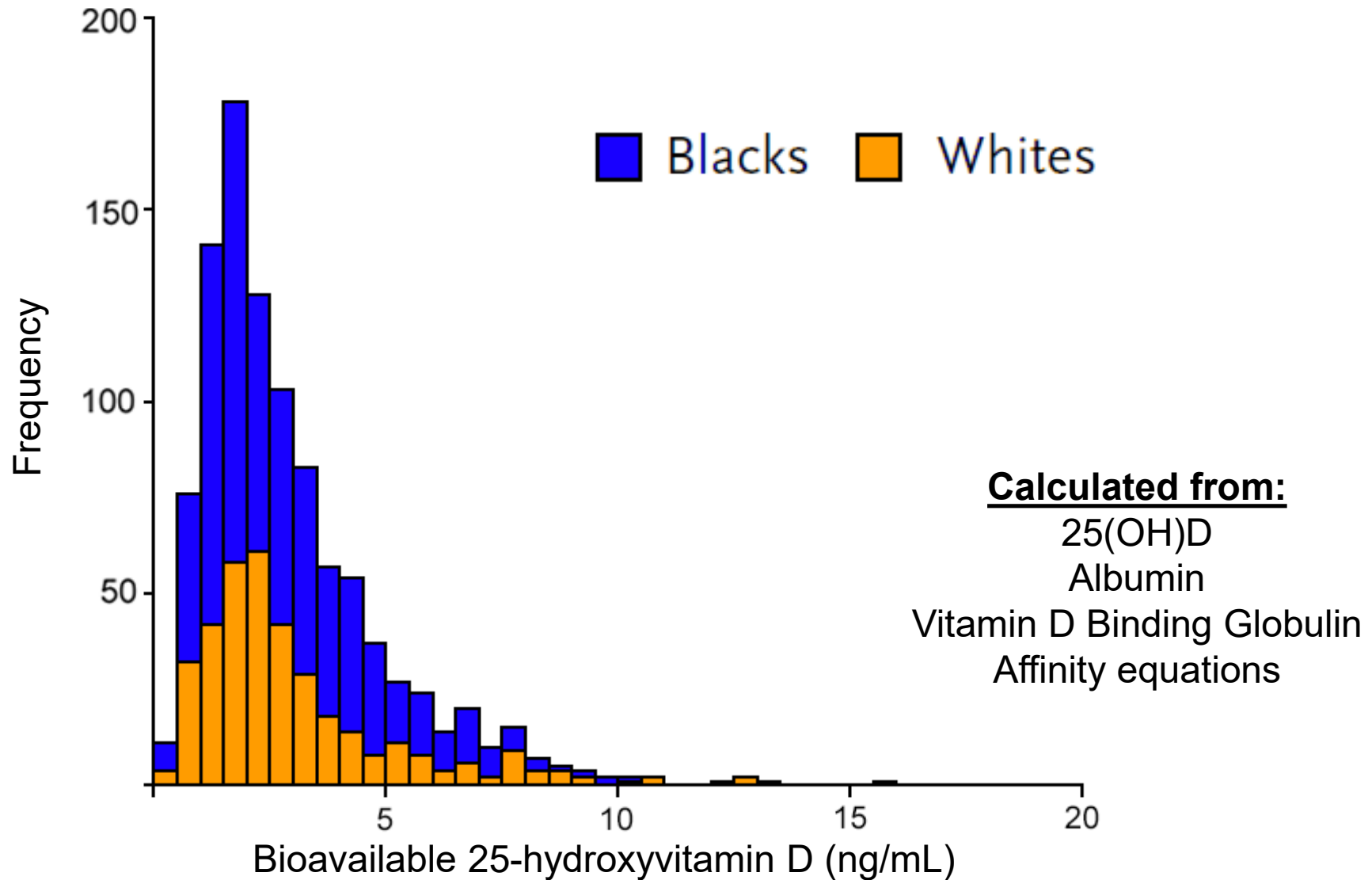
# Blacks Have Lower Vitamin D Binding Protein



Adapted from Powe, NEJM, 2013

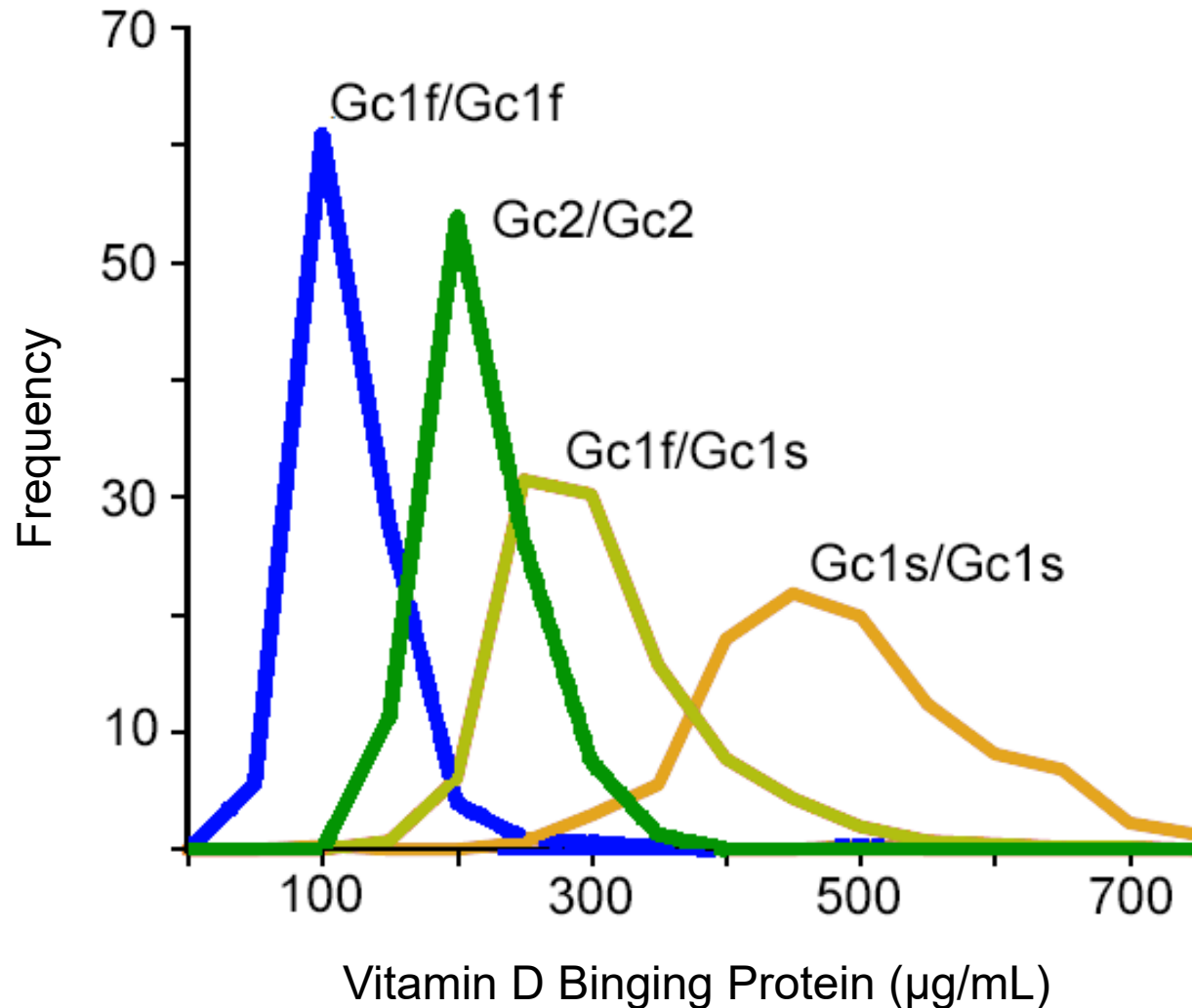
# Blacks and Whites Have The Same Bioavailable D

*Problem Solved*



# Genotype Very Strongly Predicts VDBG

*But Wait*



CORRESPONDENCE



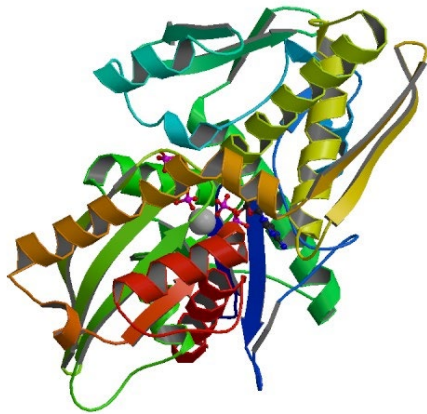
## Vitamin D–Binding Protein and Vitamin D in Blacks and Whites

...assay is based on a primary monoclonal antibody produced against a single peptide fragment of the human vitamin D–binding protein...the problem is that the monoclonal antibody assay gives lower values in blacks who have primarily the Gc1F variant of vitamin D–binding protein. The **monoclonal antibody discriminates between the Gc1F and Gc1S variants to provide artificially low values for total vitamin D–binding protein in blacks, thus bringing the conclusion of this study into question.**

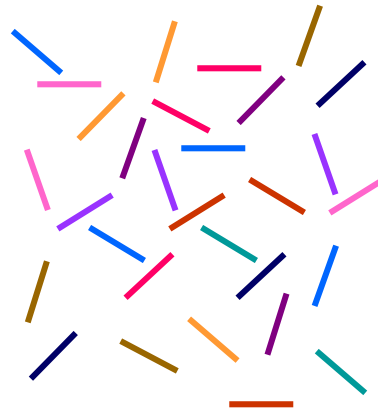
Hollis & Bikle, NEJM, 2014

# Targeted Proteomics Workflow

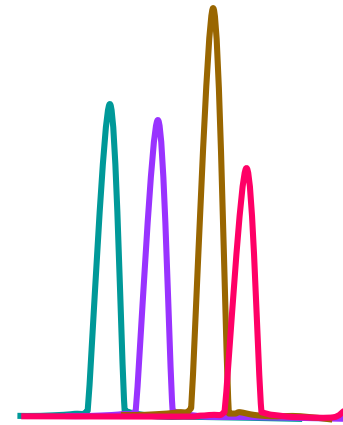
Complex sample



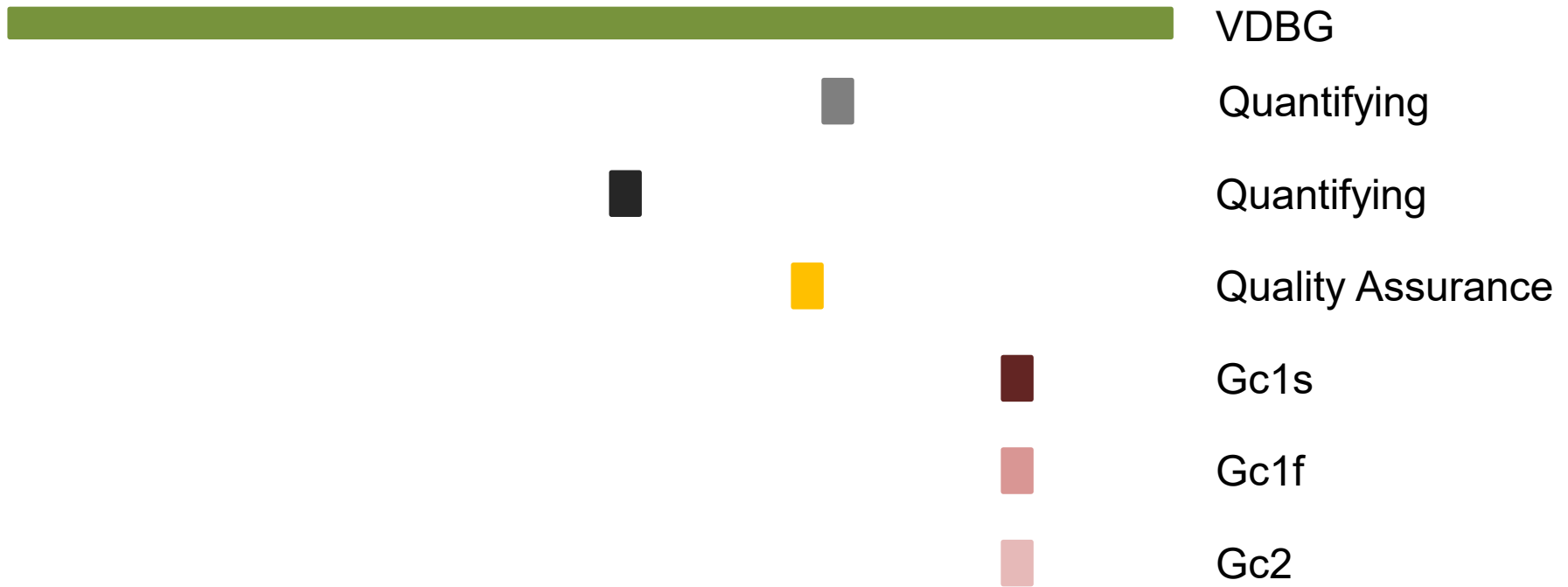
Proteolysis



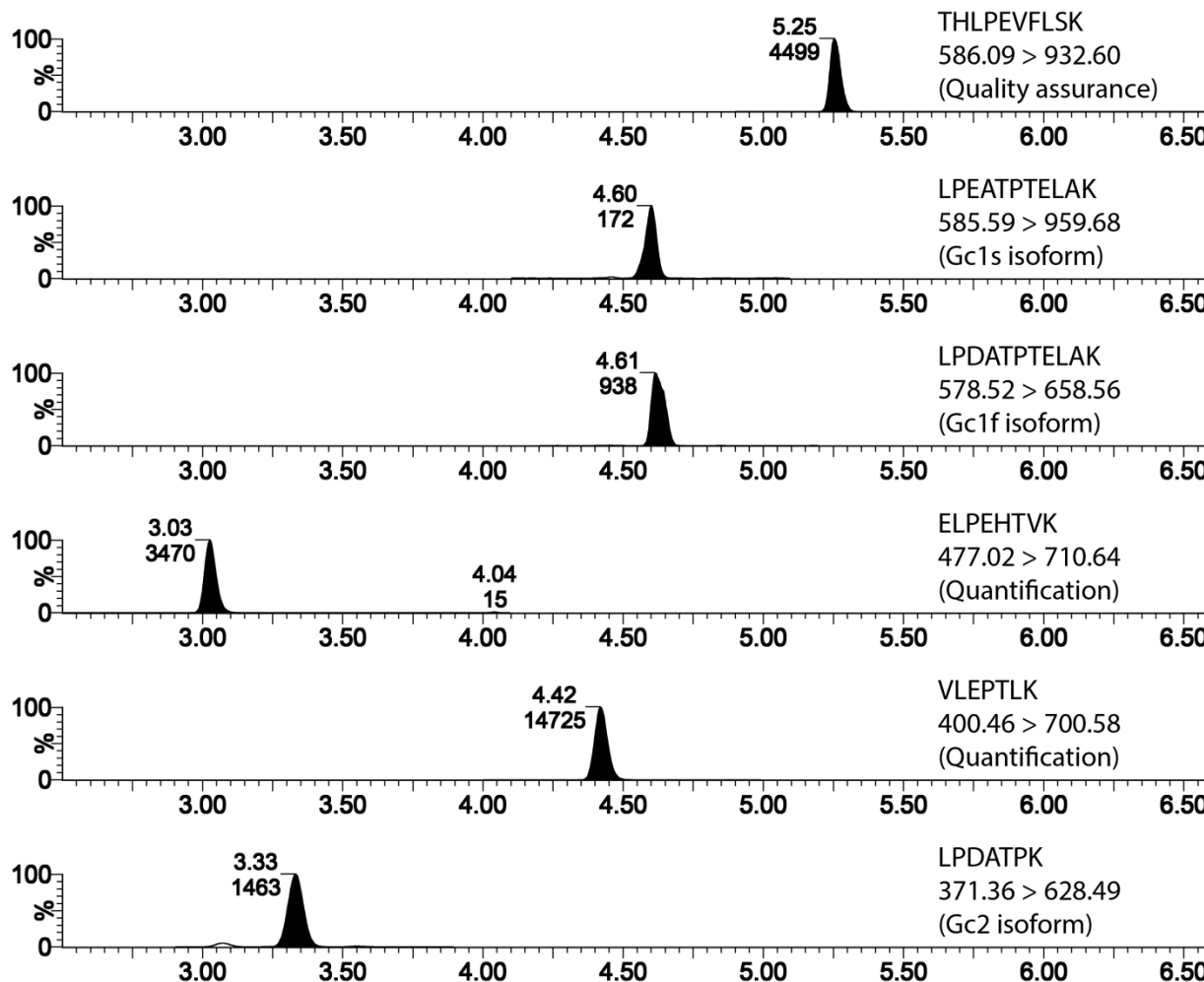
LC-MS/MS



# Selecting Peptides From VDBG



# Chromatography

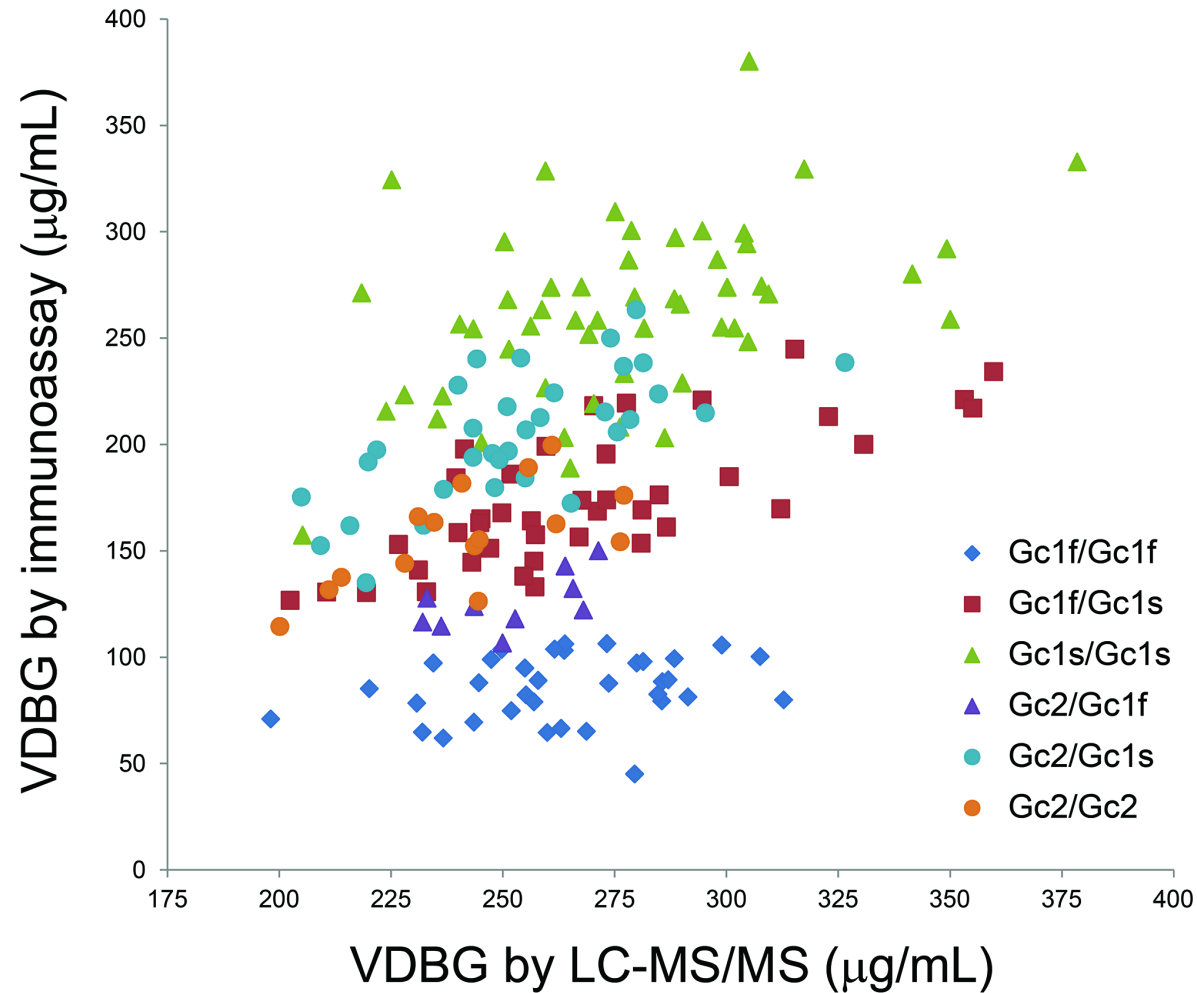


# Validating Protein Quantification For Publication

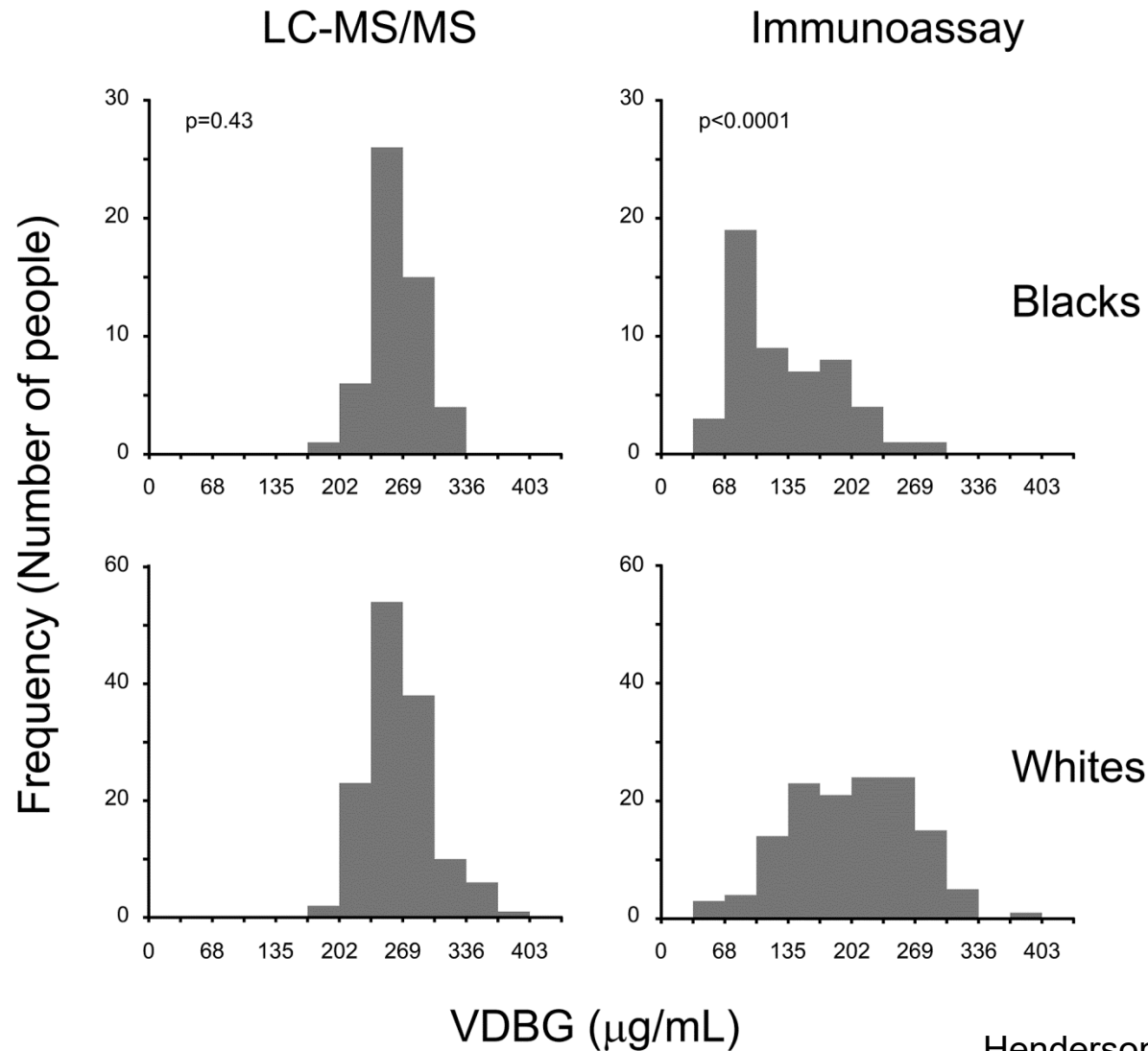
Validation		Experiment
Reproducibility		5x5 experiment
Peptide degradation	Spike IS peptides before/after digestion	
Linearity		Mix pools together
LLOQ	Dilution experiments of one or more pools	
Interferences	Add potential interferences to pools	
Stability	Stress pools before and after sample prep	
Transparency	Provide raw data from validation studies	

*Grant, ClinChem, 2014*

# R&D Immunoassay vs. LC-MS/MS

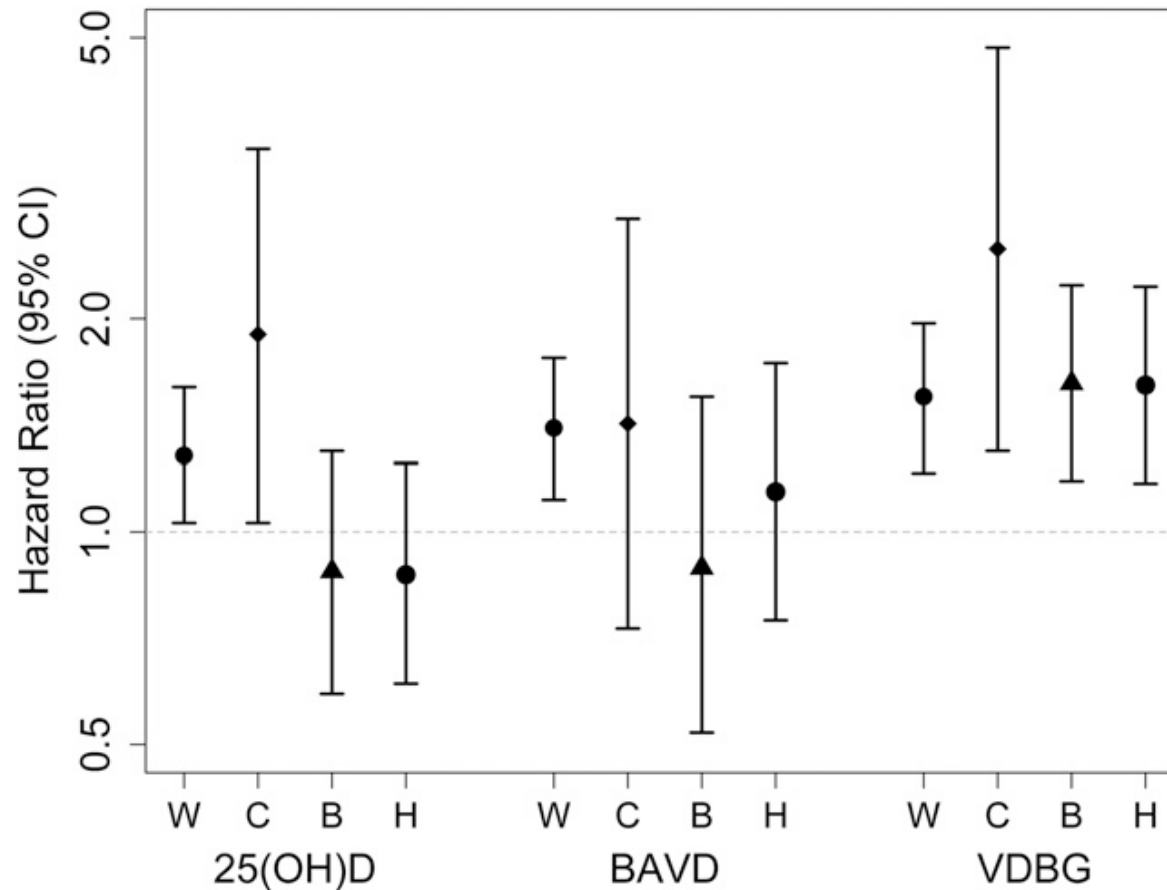


# Racial Distributions Are Not Different



# VDBG Predicts Cardiovascular Disease

*In MESA*



Robinson-Cohen, *JCEM*, 2017

*Higher VDBG is associated with increased risk of CVD more strongly and independent of CRP*

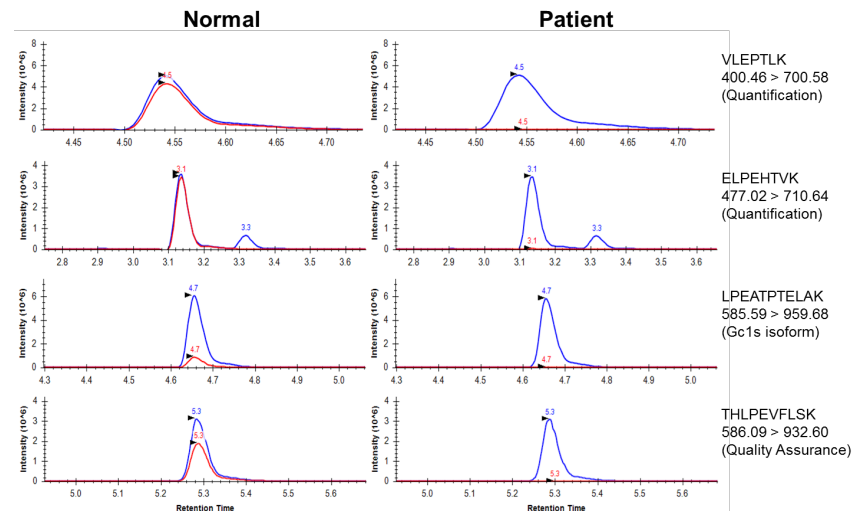
# Vitamin D Binding Protein Deficiency Is Not Lethal Severe Autoimmune Disease

The NEW ENGLAND JOURNAL of MEDICINE

## BRIEF REPORT

### Vitamin D–Binding Protein Deficiency and Homozygous Deletion of the GC Gene

Clark M. Henderson, Ph.D., Susan L. Fink, M.D., Ph.D., Hanan Bassyouni, M.D.,  
Bob Argiropoulos, Ph.D., Lindsay Brown, Ph.D., Thomas J. Laha, M.T. (A.S.C.P.),  
Konner J. Jackson, B.S., Raymond Lewkonja, M.B., Ch.B., Patrick Ferreira, M.B., B.S.,  
Andrew N. Hoofnagle, M.D., Ph.D., and Julien L. Marcadier, M.D.



# Summary

## *Vitamin D Binding Globulin*

### **Targeted proteomics**

- Outperforms immunoassays

- Meets the needs of epidemiologists

- Is completely transferable

### **Validation is key**

- Transparency is too

# Low Abundance Proteins

*Thyroglobulin*

# Thyroglobulin as a Tumor Marker

## *After Treatment*

*After surgery and radioablation of thyroid tissue*

### **Monitor serum thyroglobulin**

Residual tumor

Metastatic disease

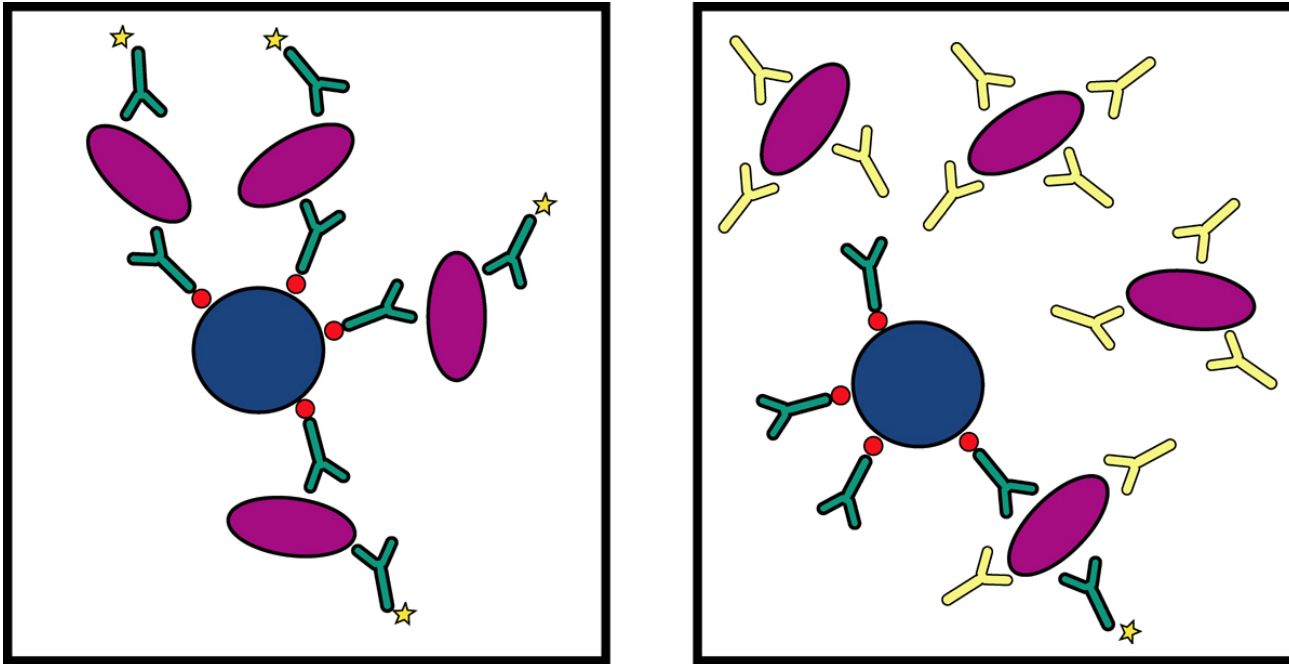
Recurrence of carcinoma

*Thyroglobulin leaks into lymphatics from any tissue that makes it*

**Up to 25-30% of cancer patients make autoantibodies**

# Autoantibody Interference

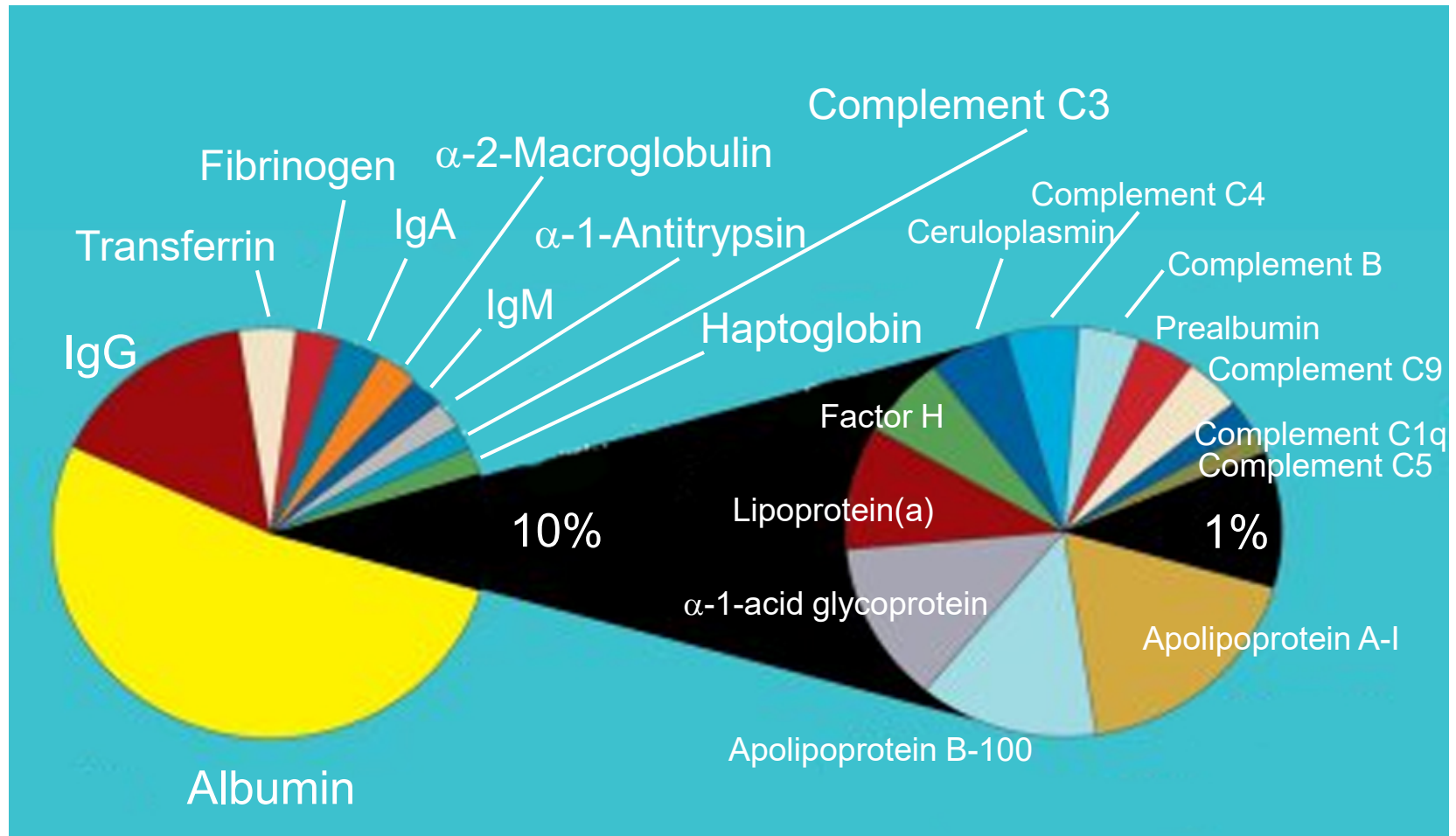
Autoantibodies can sequester antigen away from detectable complexes



Falsely negative results with sandwich assays

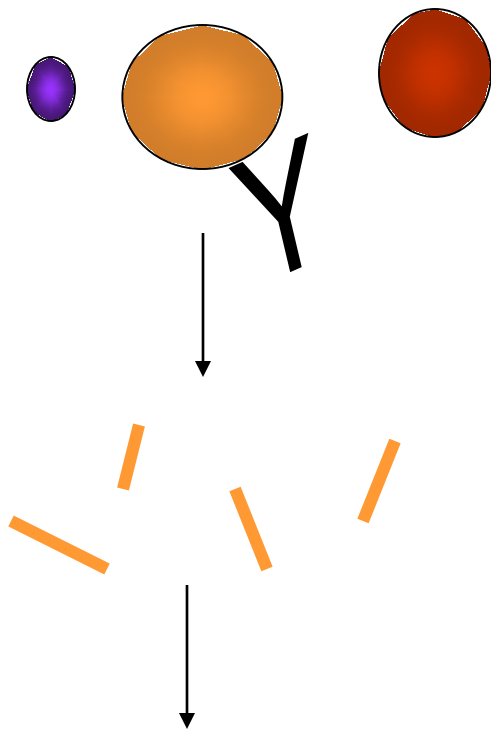
# Could This Really Work?

*22 proteins make up 99% of plasma*



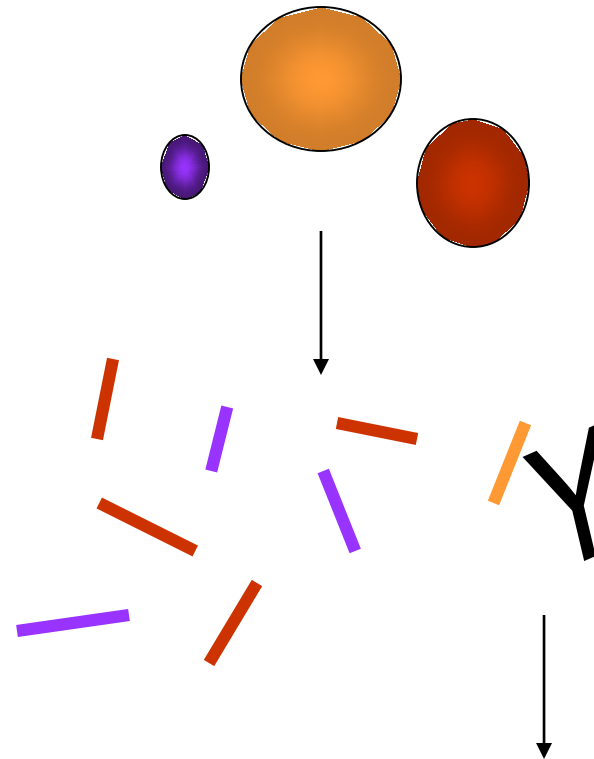
# Immunoaffinity Enrichment

Anti-protein antibody



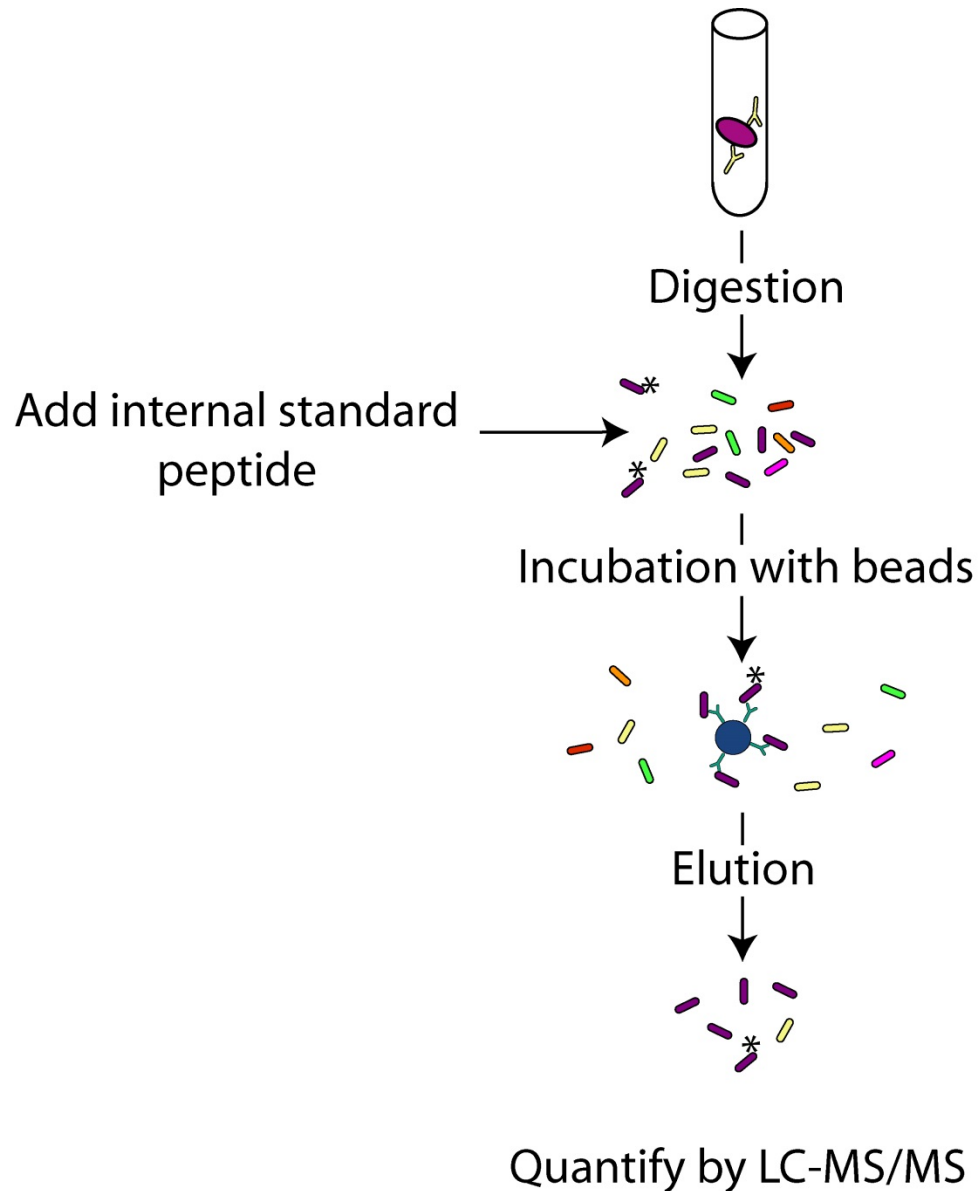
LC-MS/MS

Anti-peptide antibody



LC-MS/MS

# Immunoaffinity Enrichment After Trypsin Digestion

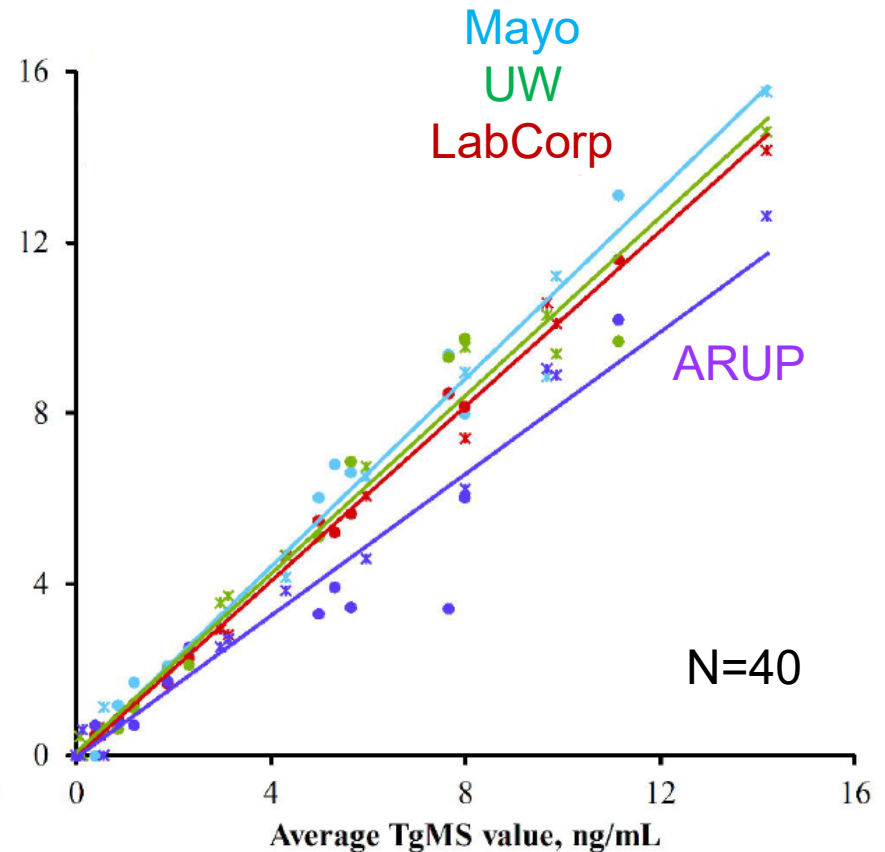
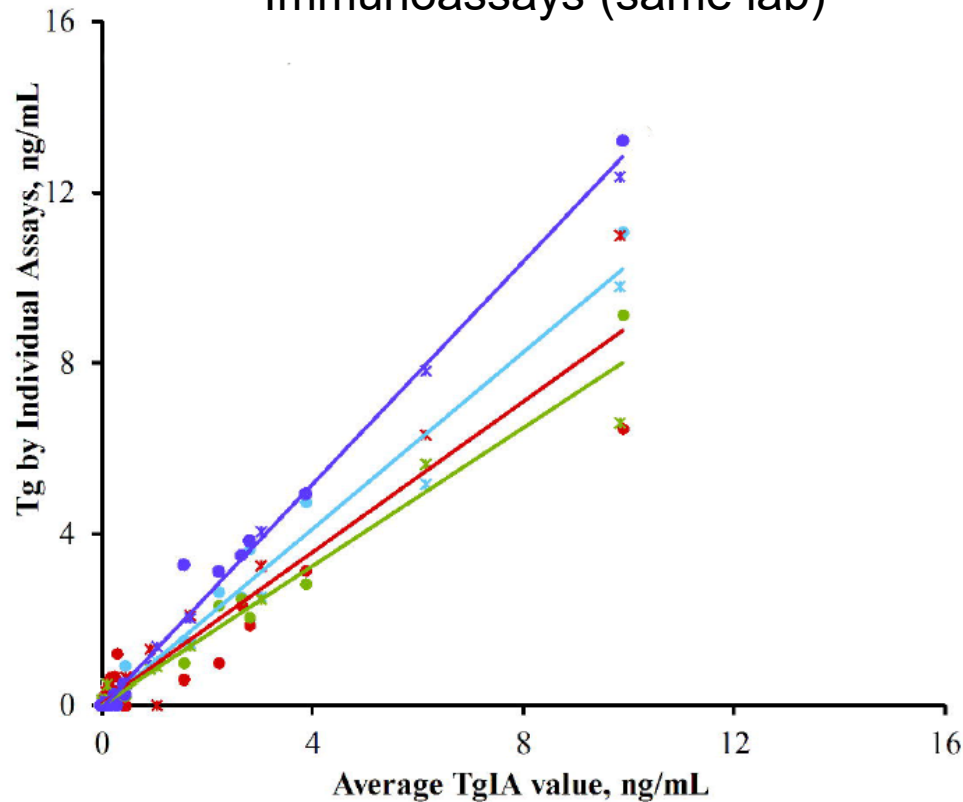


# Inter-laboratory Concordance

Lab	Pre-digestion	Ab	Calibration
UW	None	CPTAC FSP	Native Human
Mayo	Ammon sulfate	CPTAC FSP	BCR457
LabCorp	None	CPTAC FSP	Beckman
ARUP	Rabbit polyclonal & Ammon sulf	In-house polyclonal VIF	Beckman

# Inter-laboratory Concordance

Immunoassays (same lab)



# Acknowledgments

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Bryan Kestenbaum

Ian de Boer

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Alicia Algeciras-Schimmich

Ravinder Singh

Stefan Grebe

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