



QUANTITATION OF ADRENOCORTICOTROPIC HORMONE (ACTH) USING A NOVEL REAGENT-FREE LCMS ASSAY

And Correlation Study to Clinical Immunoassay

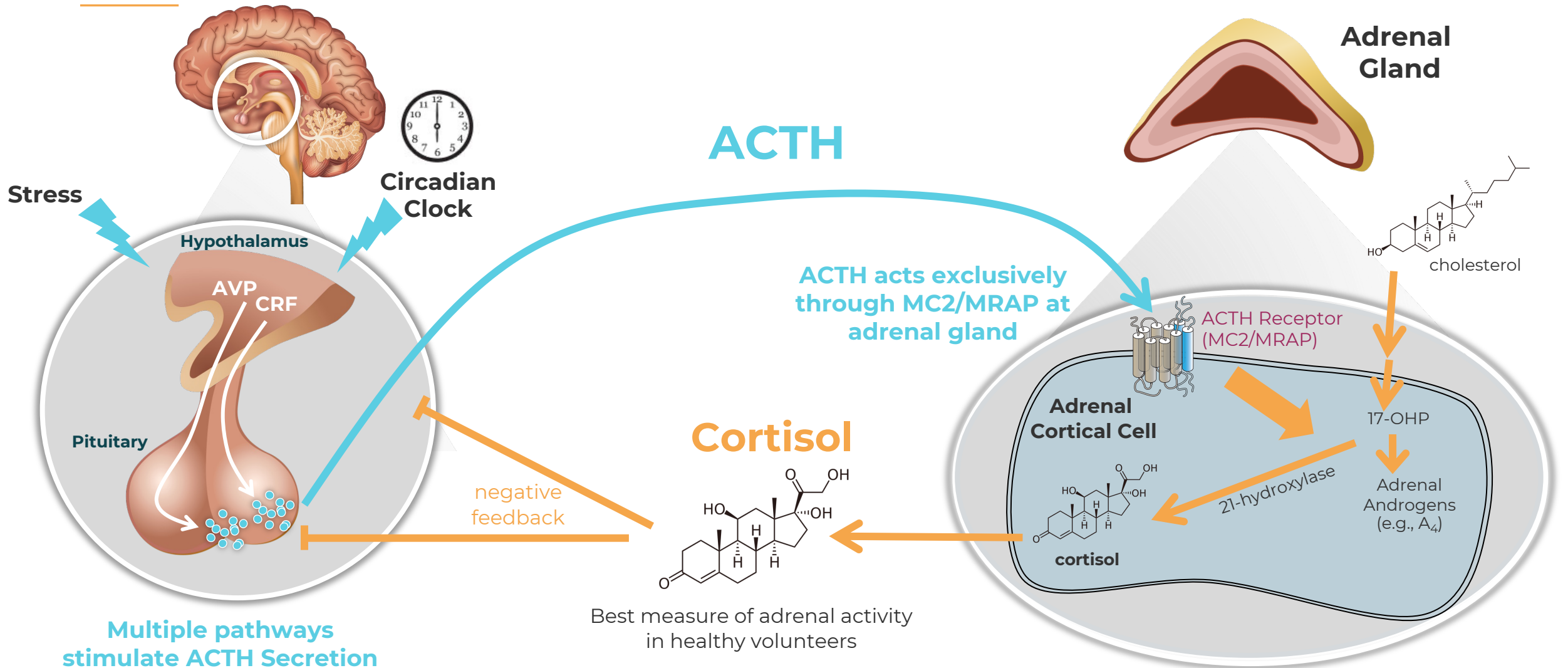
Barry Jones, Associate Director, Biomarker Bioanalysis

16th EBF Open Symposium, 15-17 November 2023

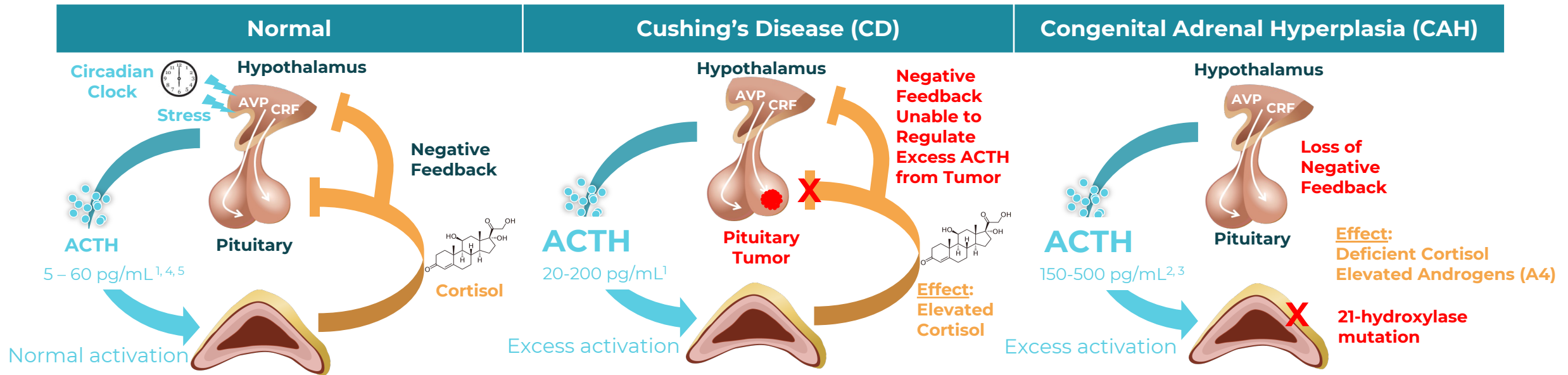
Objectives

- Improve measurement of peptide biomarkers (hormones) to support drug development
- Improve diagnostic tools for ACTH-dependent Cushing's Syndrome
 - Cushing's Disease
 - Ectopic ACTH Syndrome
- Investigate good correlation but poor agreement observed between two immunoassays for ACTH(1-39)
 - Development of a reagent-free multi-dimensional nanoLC-MS/HRMS assay
 - Comparison to a published hybrid IA-LC-MS/MS assay

The Hypothalamic-Pituitary-Adrenal (HPA) Axis: The ACTH Receptor Is Key for Adrenal Activation



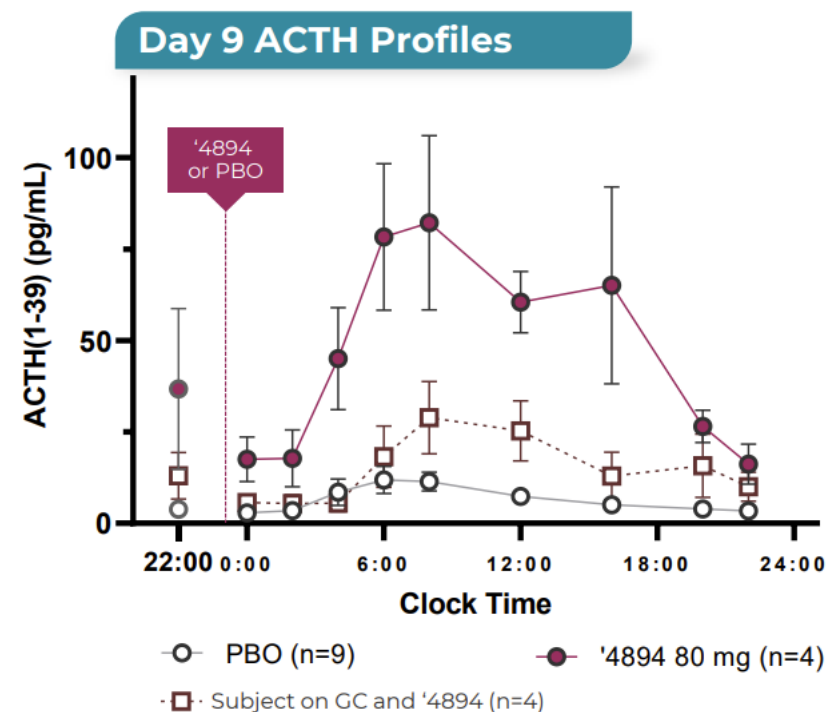
Disruptions in the HPA Axis Lead to Diseases of Excess ACTH and Excess Adrenal Activation



| | | |
|-----------------|--|---|
| Cause | ACTH-secreting pituitary tumor | Inability to produce cortisol leads to loss of negative feedback & excess ACTH |
| Symptoms | Central obesity and round face; Dorsal and supraclavicular fat pads; Hypertension; Stretch marks; Bone loss; Hyperglycemia; Psychiatric disturbances | Adrenal insufficiency; Infertility; Hirsutism; Short stature; Precocious puberty; Adrenal rest tumors |

CRN04894, ACTH Receptor Antagonist

- CRN04894 is an oral nonpeptide ACTH receptor antagonist
- Reverses ACTH-stimulated glucocorticoid secretion
- Loss of cortisol negative feedback results in healthy volunteer ACTH levels comparable to that seen in disease states
- Generally accepted reference range for ACTH is 10-50 pg/mL*
- In phase 1 study, ACTH was measured using Milliplex map Kit, Human Pituitary Magnetic Bead Panel 1 (“Luminex Assay”)

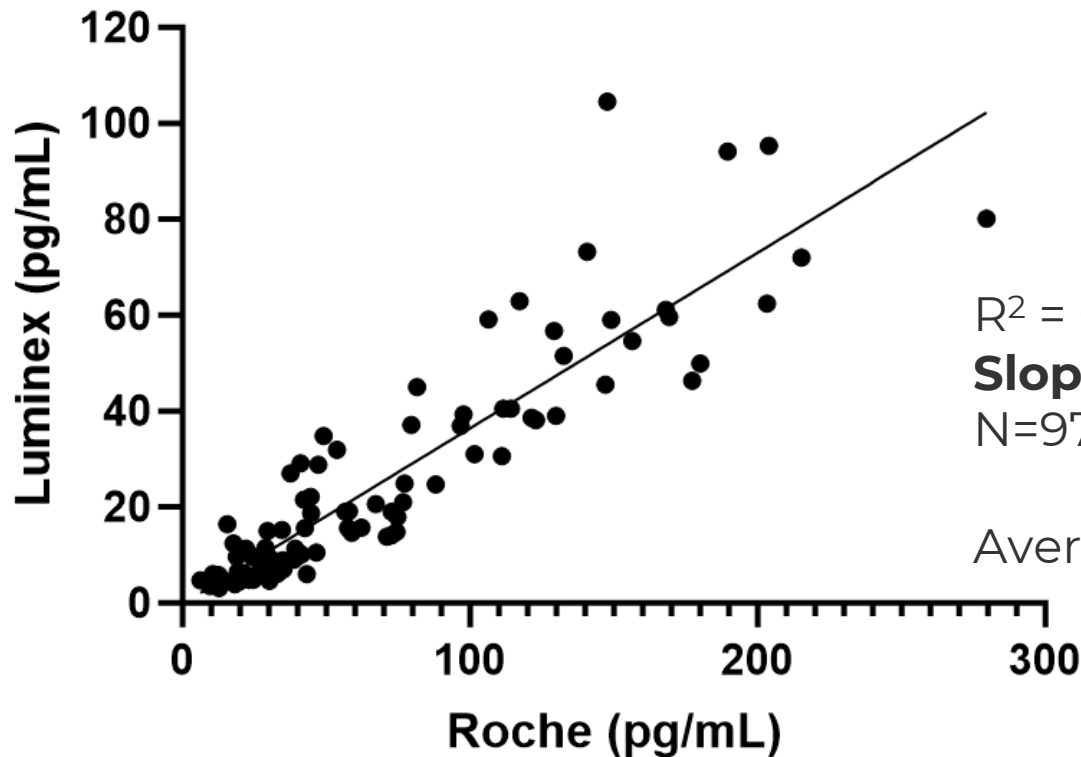


CRN04894 an oral, nonpeptide ACTH receptor antagonist reverses ACTH-stimulated glucocorticoid secretion in rodents and humans, Peter J. Trainer, Melissa Fowler, Alan Krasner, et al., Oral Presentation: International Congress of Neuroendocrinology (ICN); 8/10/22

*Cushing's Support and Research Foundation: "Normal Values of Cortisol and ACTH."

Roche Measures 3.2-Fold Higher than Luminex

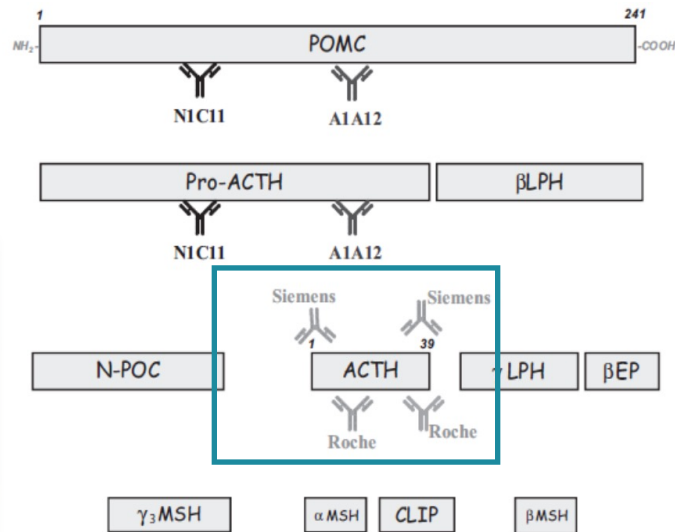
Luminex Kit vs Roche Elecsys Kit



- The ACTH results from the Luminex assay appeared low compared to the reference range established with the Roche Elecsys clinical diagnostic assay
- Pooled samples from phase 1 study were assayed using both immunoassay kits and compared

Issues with Immunoanalytical ACTH assay

- ❑ Biotin Interference
- ❑ Lack of standardization, variability
- ❑ Potential interference from ACTH precursors and other POMC-derived fragments



JOURNAL ARTICLE

Biotin Supplementation Creates the Misleading Diagnosis of Secondary Adrenal Insufficiency

Hooman Motahari, MD, Soumya Thumma, MD, Lakshmi Menon, MD

Journal of the Endocrine Society, Volume 5, Issue Supplement_1, April-May 2021, Pages A120–A121, <https://doi.org/10.1210/jendso/bvab048.242>

Published: 03 May 2021

European Journal of Endocrinology (2011) 164 505–512

ISSN 0804–4643

CLINICAL STUDY

Assessment of ACTH assay variability: a multicenter study

Francesca Pecori Giraldi, Andrea Sacconi, Francesco Cavagnini and The Study Group on the Hypothalamo-Pituitary–Adrenal Axis of the Italian Society of Endocrinology
 Chair of Endocrinology, University of Milan, Ospedale San Luca, Istituto Auxologico Italiano IRCCS, Piazzale Brescia 20, 20149 Milan, Italy
 (Correspondence should be addressed to F Cavagnini; Email: cavagnini@auxologico.it)

Clinical Chemistry 65:11
 1397–1404 (2019)

Endocrinology and Metabolism

An Intact ACTH LC-MS/MS Assay as an Arbiter of Clinically Discordant Immunoassay Results

Junyan Shi,^{1†} Pawan Dhaliwal,¹ Yu Zi Zheng,^{1†} Terry Wong,¹ Joely A. Straseski,² Mark A. Cervinski,³ Zahra Shajani-Yi,⁴ and Mari L. DeMarco^{1,5*}

Clinical Endocrinology (2016) 85, 569–574

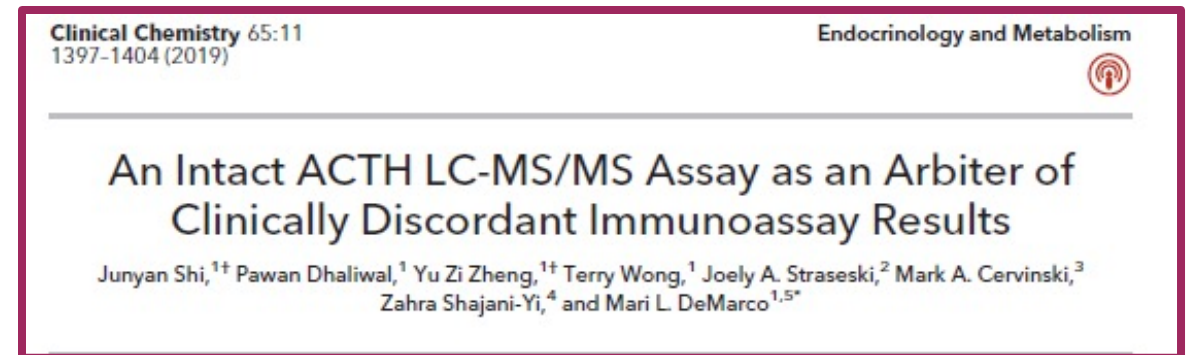
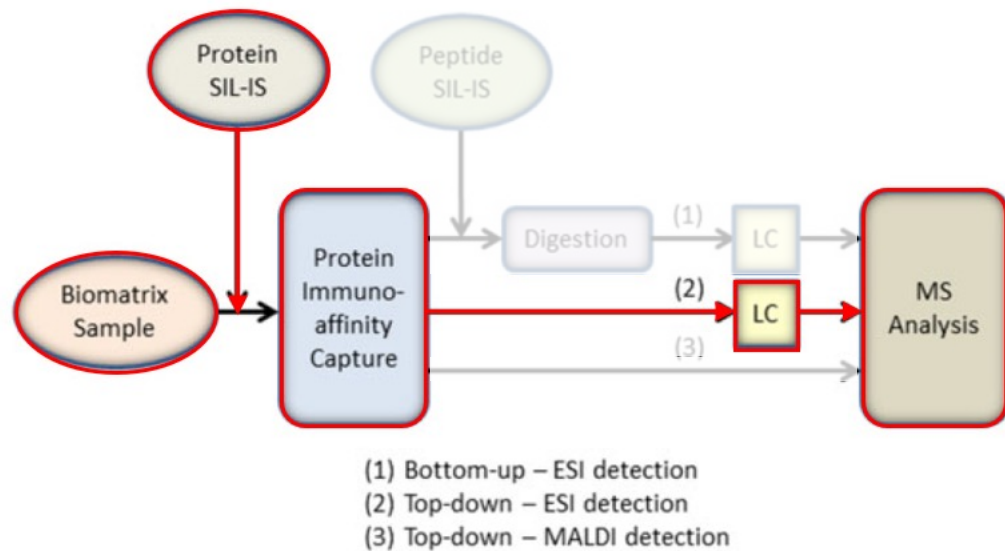
doi: 10.1111/ce.13118

ORIGINAL ARTICLE

Proopiomelanocortin interference in the measurement of adrenocorticotrophic hormone: a United Kingdom National External Quality Assessment Service study

P.J. Monaghan*, A. Kyriacout, C. Sturgeon†, A. Davies§, P.J. Trainer†¶, A. White§¶ and C.E. Higham†¶

Towards an LCMS-Based ACTH Assay



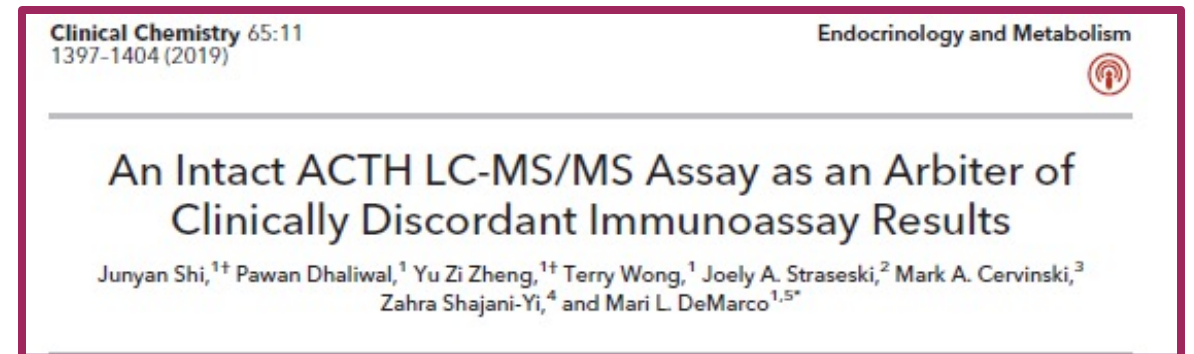
Neubert H, Shuford CM, Olah TV, Garofolo F, Schultz GA, Jones BR, Amaravadi L, Laterza OF, Xu K, Ackermann BL, Protein Biomarker Quantification by Immunoaffinity Liquid Chromatography-Tandem Mass Spectrometry: Current State and Future Vision (2020) *Clinical Chemistry*, 0:0, 1-20.

Shi J, Dhaliwal P, Zi Zheng Y, Wong T, Straseski JA, Cervinski MA, Shajani-Yi Z, DeMarco ML (2019) *Clinical Chemistry*, 65, 1397-1404.

“Hybrid” IA/LC-MS(MS)

- Shi et.al. used a capture antibody from the Roche assay as a purification step to drive sensitivity
 - Is it fair to use Roche immunoassay capture antibody in an assay to compare Siemens and Roche kits?
- Assay was sensitivity-challenged
 - Authors conclude that nano-LC may be needed for enhanced sensitivity

“Although this LLMI was more than sufficient to answer the question faced, further assay optimization and/or change in instrumentation (e.g., microflow/nanoflow liquid chromatography) may be beneficial should greater analytical sensitivity be required.”



REAGENT FREE, MULTI-DIMENSIONAL NANO-LC-HRMS/MS FOR CIRCULATING PEPTIDES

Assay format previously described for Atrial Natriuretic Peptide (ANP) adapted for measurement of intact ACTH(1-39)

Atrial Natriuretic Peptide (ANP) example

Extraction:

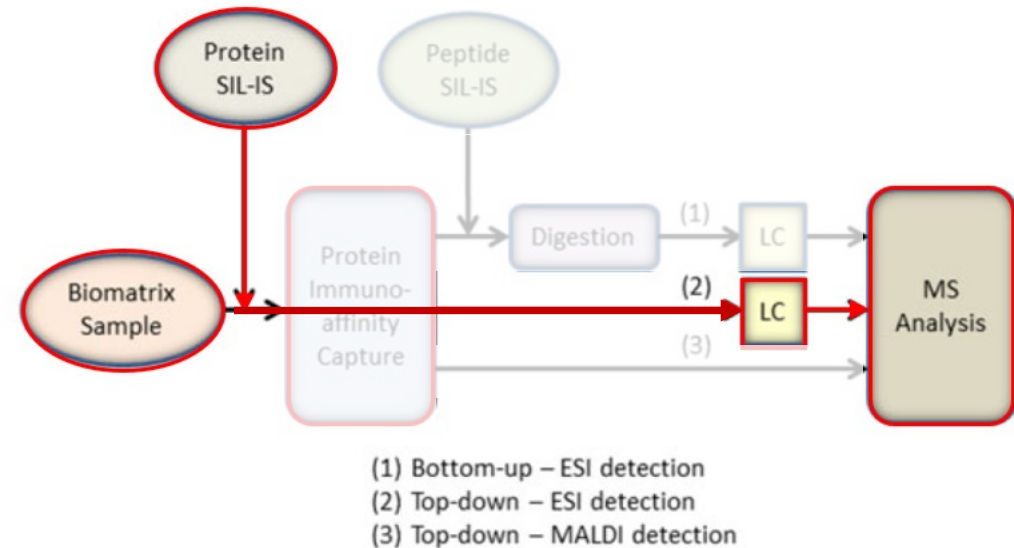
- SPE, 300 μ L plasma sample

LC:

- SEC column as first LC dimension
- Trap/elute to Easy Spray nano column
- 12.5 min LC time

MS:

- Resistant to fragmentation
- Quantitation on molecular ion using High Resolution Mass Spectrometry
- **3 pg/mL LLOQ**



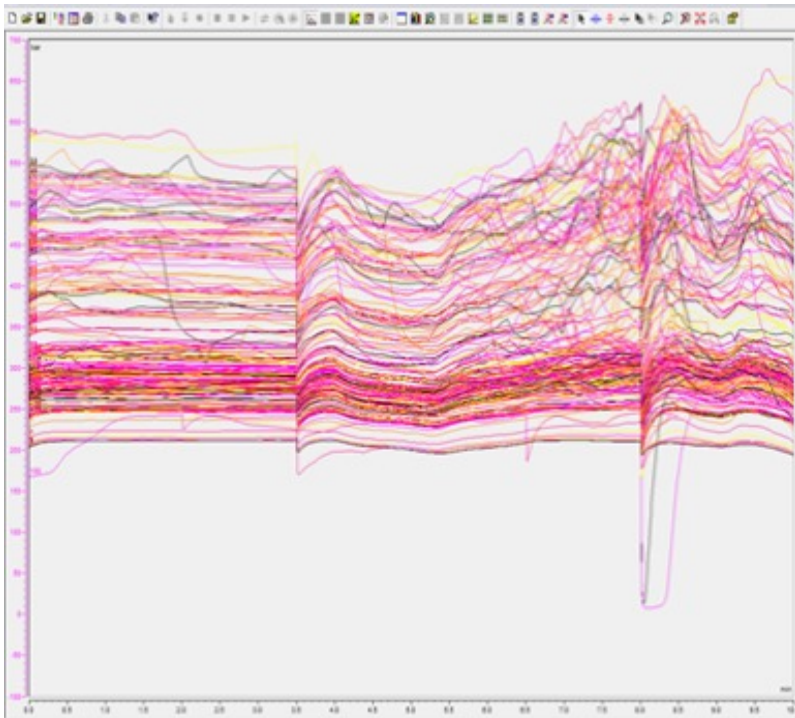
Lian Shan et. al., *Ultrasensitive quantitation of intact endogenous atrial natriuretic peptide in human plasma using multidimensional nano-LC and high-resolution mass spectrometry*, Poster Presentation: Workshop for Recent Issues in Bioanalysis, Atlanta, 2022.

Nano-LC Robustness Improvement

Pressure accumulates over course of 96 injections when using trap/elute approach, addressed with addition of third column (SEC)

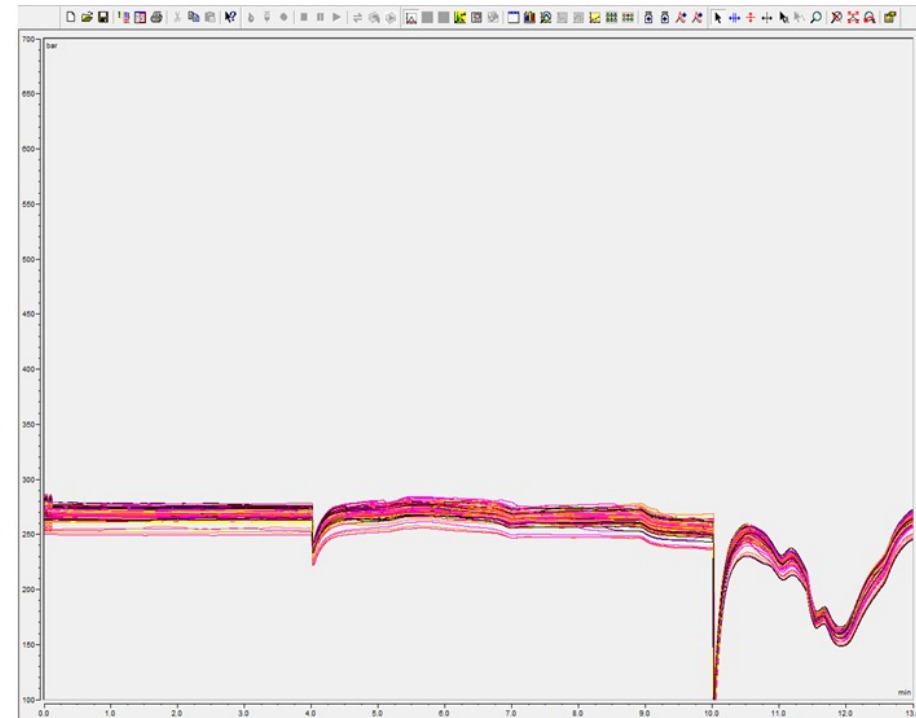
2-column LC

- 300 μm trap (300 $\mu\text{L}/\text{min}$)
- 75 μm analytical column (0.6 $\mu\text{L}/\text{min}$)

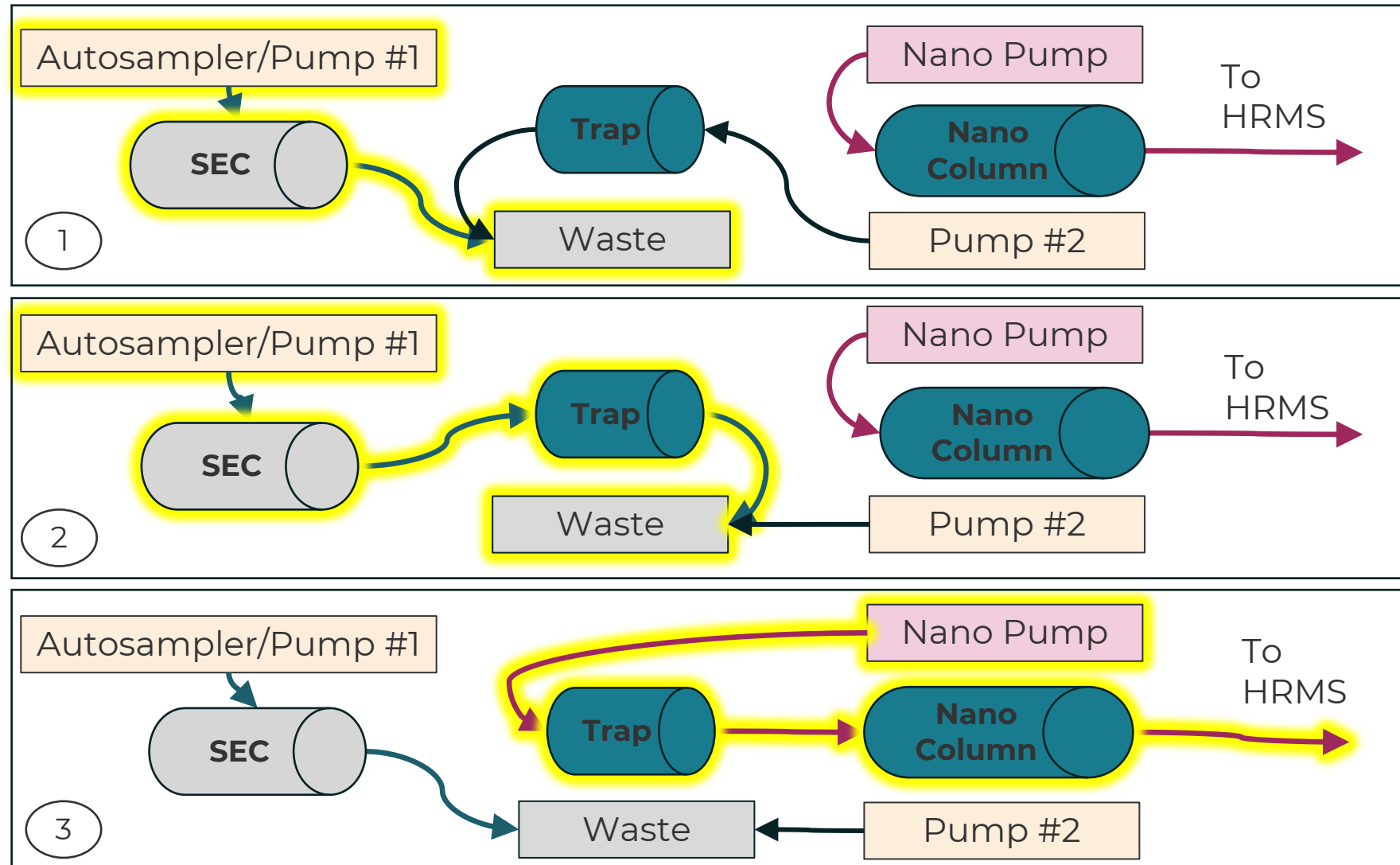


3-column LC

- 4.6 mm ID SEC column (300 $\mu\text{L}/\text{min}$)
- 300 μm ID trap column (300 $\mu\text{L}/\text{min}$)
- 75 μm ID analytical column (0.6 $\mu\text{L}/\text{min}$)



LC Configuration



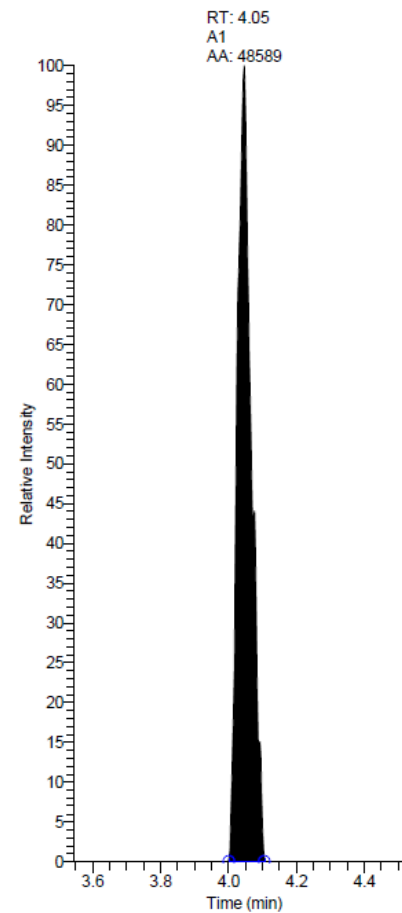
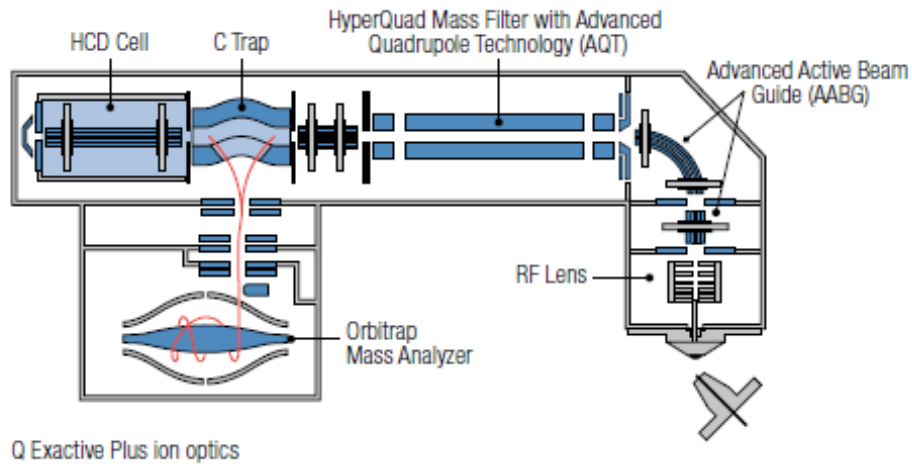
High Resolution Mass Spectrometry

For circulating peptide quantitation

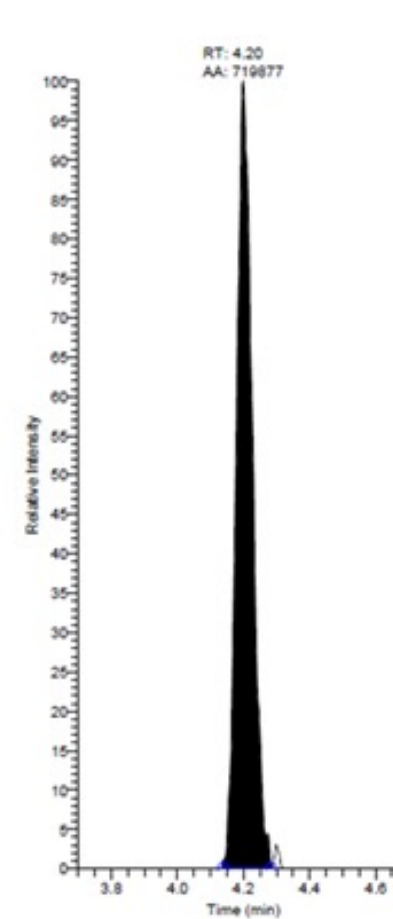
Multiple scan modes

Enhanced selectivity

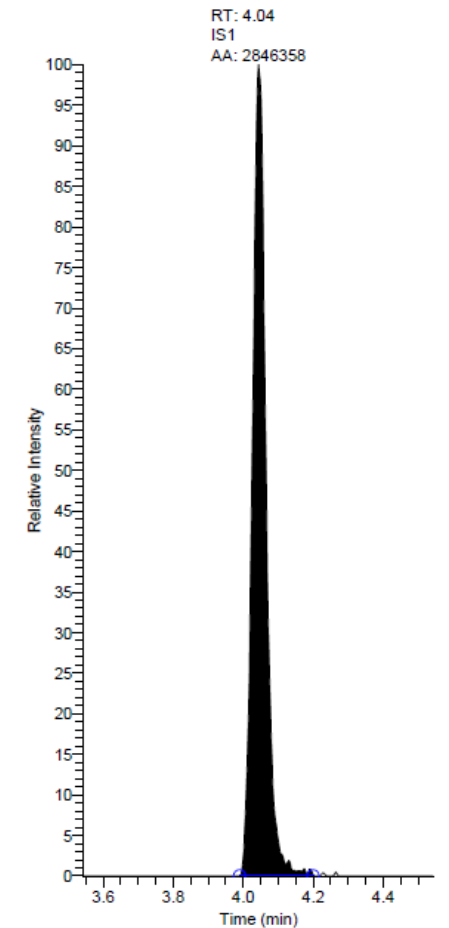
Enhanced selectivity can yield higher sensitivity



5 pg/mL
ACTH(1-39) in
surrogate matrix



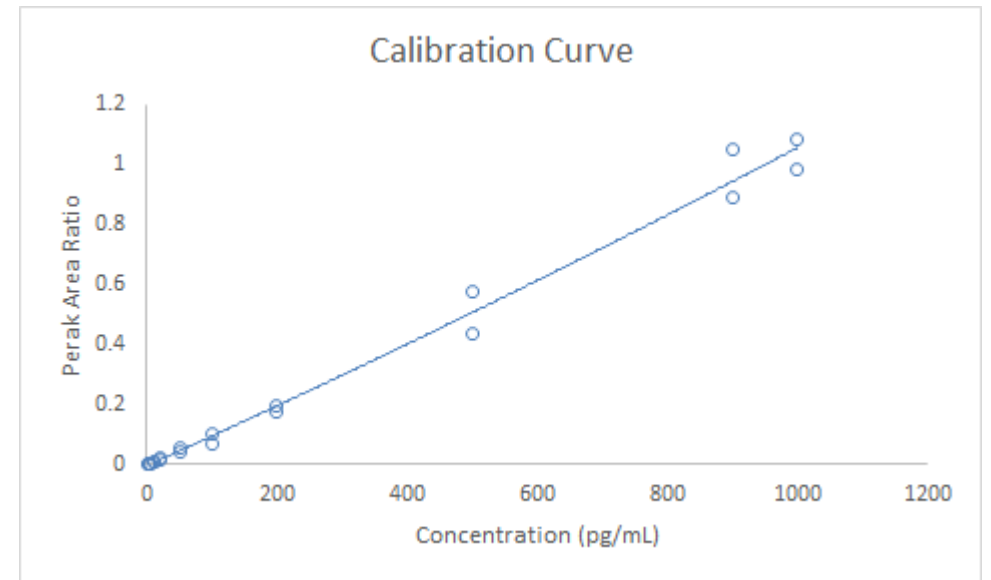
Endogenous
ACTH(1-39) in
plasma



Internal Standard
(Murine ACTH)

ACTH Assay Performance

- Surrogate matrix 5% BSA in PBS
- Sample aliquot = 300 μ L plasma
- Internal Standard: Murine ACTH(1-39)
- 3-5 pg/mL LLOQ

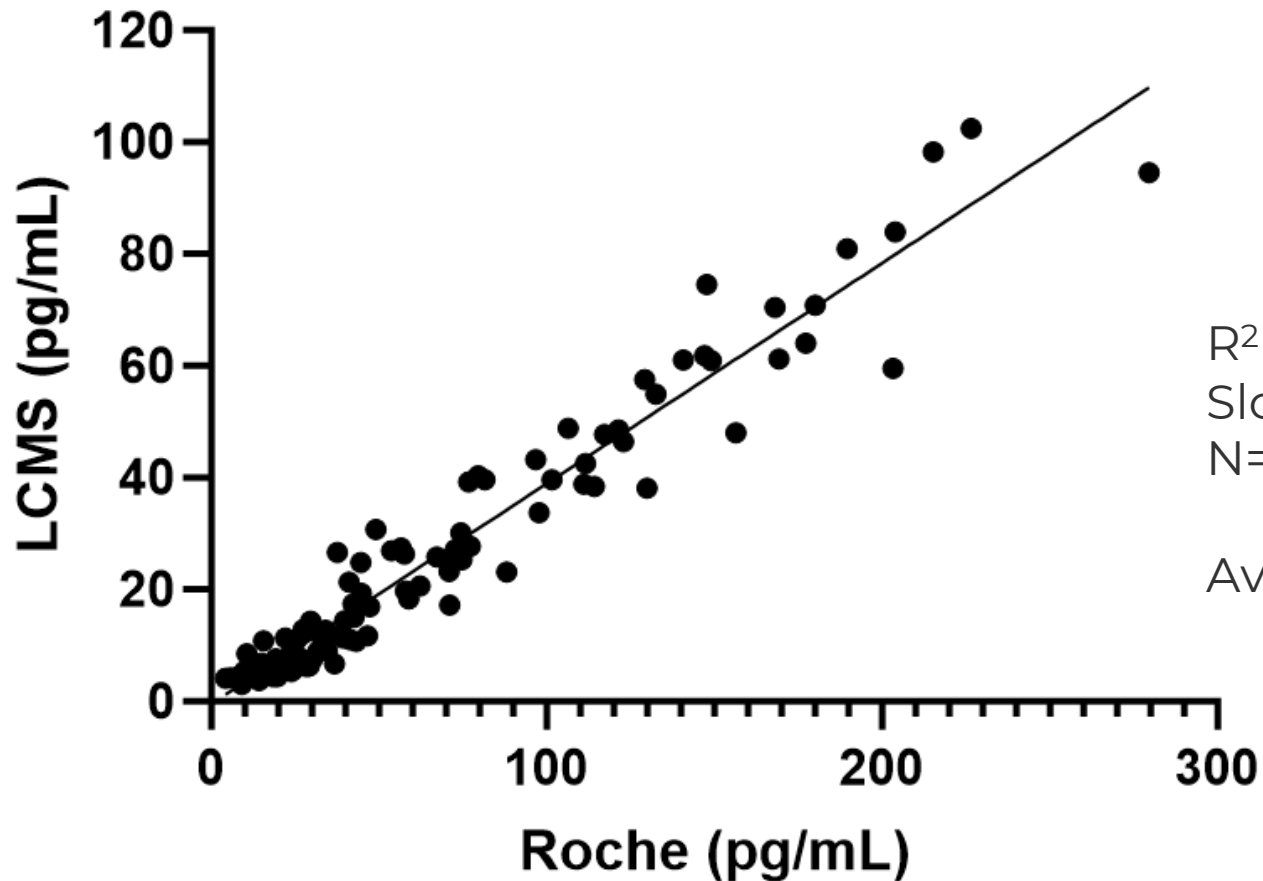


| | Surrogate Matrix | | | | | | | Plasma | |
|----------------------|------------------|----------|----------|-------|-------|-------|-------|--------|-------|
| | LLOQQC A | LLOQQC B | LLOQQC C | QC1 A | QC1 B | QC1 C | QC2 | PQC1 | PQC2 |
| Theor. Conc. (pg/mL) | 3 | 5 | 10 | 9 | 15 | 30 | 800 | 50 | 700 |
| Found Conc. | | | | | | | | | |
| #1 | 3.956 | 4.864 | 9.769 | 7.639 | 14.22 | 29.86 | 819.8 | 38.6 | 799 |
| #2 | 2.739 | 4.244 | 10.87 | 5.987 | 13.65 | 29.97 | 803.7 | 39.34 | 717.3 |
| #3 | 3.406 | 4.833 | 9.52 | 10.04 | 14.72 | 28.14 | 824.7 | 50.78 | 818.8 |
| #4 | 3.353 | 4.186 | 8.951 | 8.798 | 18.08 | 33.7 | 778.8 | 48.87 | 697.1 |
| #5 | 3.065 | 4.684 | 12.18 | 8.432 | 15.53 | 28.84 | 783 | 40.94 | 795.6 |
| #6 | 3.957 | 5.915 | 10.62 | 9.863 | 14.7 | 27.34 | 822 | 59.8 | 728.1 |
| Mean | 3.413 | 4.788 | 10.32 | 8.46 | 15.15 | 29.64 | 805.3 | 46.39 | 759.3 |
| S.D. | 0.4837 | 0.624 | 1.155 | 1.508 | 1.564 | 2.228 | 20.34 | 8.311 | 51.07 |
| %CV | 14.2 | 13.0 | 11.2 | 17.8 | 10.3 | 7.5 | 2.5 | 17.9 | 6.7 |
| %Theoretical | 113.8 | 95.8 | 103.2 | 94.0 | 101.0 | 98.8 | 100.7 | 92.8 | 108.5 |
| n | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

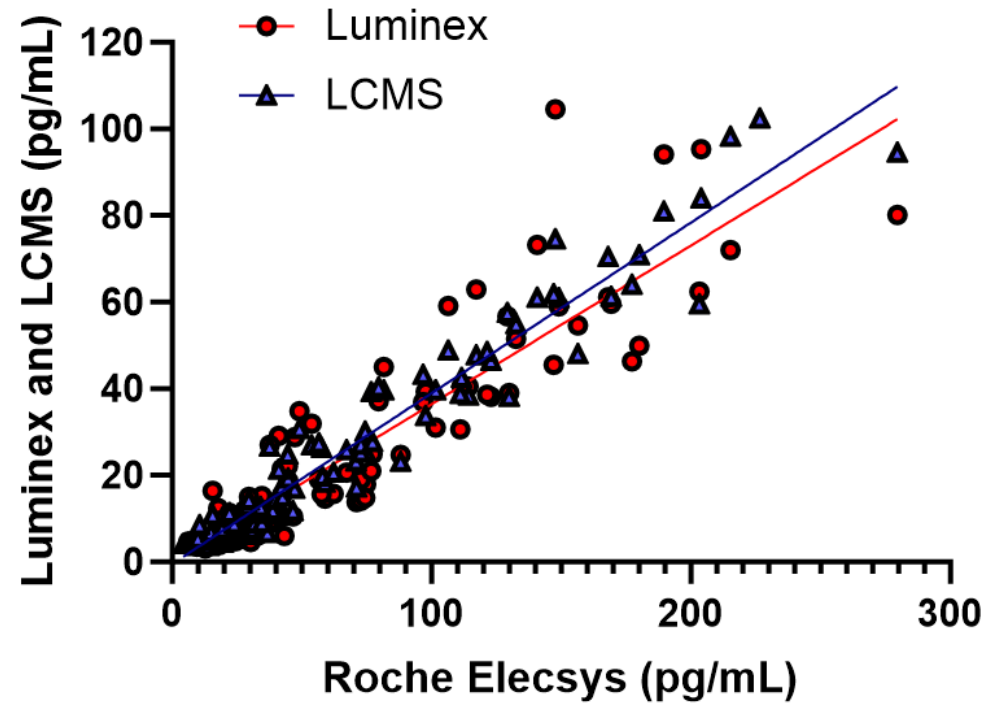
LCMS CORRELATION TO IMMUNOASSAY

Roche Measures 2.8-Fold Higher than LCMS

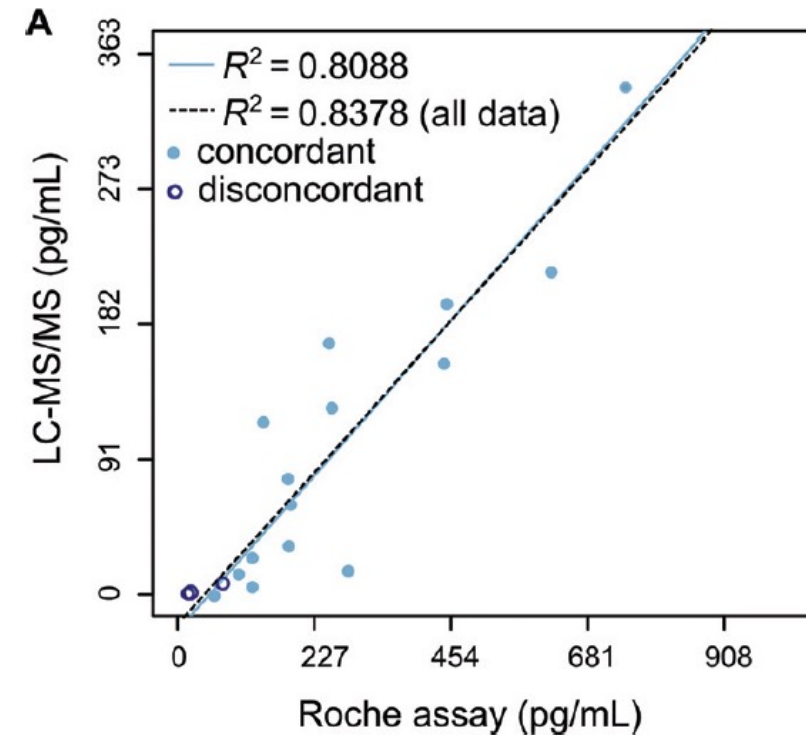
Reagent-Free LC-MS vs Roche Elecsys Kit



Further ACTH Assay Comparison



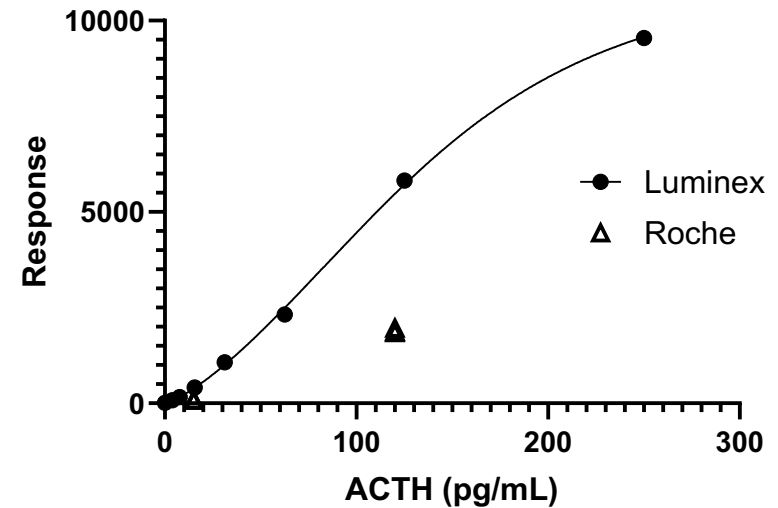
Hybrid IA-LC-MS/MS* vs Roche





* from:
Shi J, Dhaliwal P, Zi Zheng Y, Wong T, Straseski JA, Cervinski MA, Shajani-Yi Z, DeMarco ML (2019) Clinical Chemistry, 65, 1397-1404.

ACTH Calibrator Comparison

- Roche Elecsys calibrator was measured against Luminex curve at two concentrations
- On average, measured concentration of Roche ACTH calibrator material was 36.3% compared to nominal



| Analytical Antibodies | Roche versus: | Slope | R ² | n |
|---|-------------------------|--------|----------------|-----|
|  | Luminex | 0.3663 | 0.8135 | 97 |
|  | Hybrid IA/LC-MS/MS* | ~0.4 | 0.8378 | 18 |
| | Reagent Free LC-MS/HRMS | 0.3942 | 0.9423 | 113 |

*from:
Shi J, Dhaliwal P, Zi Zheng Y, Wong T, Straseski JA, Cervinski MA, Shajani-Yi Z, DeMarco ML (2019) Clinical Chemistry, 65, 1397-1404.

Conclusions

- Good correlation is observed between Roche, Luminex, and reagent-free LC-MS
- ACTH(1-39) results from Roche Elecsys are approximately ~3X of those from Luminex and reagent-free LC-MS/HRMS
 - Similar disparity can be inferred for hybrid IA-LC-MS/MS from literature
 - Lack of agreement caused by differences in reference materials
- Reagent-free LC-MS and hybrid IA-LC-MS/MS appear to show comparable correlation and agreement with Roche Elecsys
- Low-flow ionization permits sensitive, robust measurement of low-abundance circulating peptides such as ACTH(1-39)
 - Robustness afforded with orthogonal purification, not necessarily affinity-based
- Reduced method development burden and improved ability to multiplex without relying on cross-reactivity of the capture antibody

Acknowledgements

Crinetics Pharmaceuticals

Bioanalytical

Jian Wang

Joanna Zheng

George Stavrakis

Stephanie Dunchock

Clinical Pharmacology

Rosa Luo

Carl LaCerte

Medical Sciences

Alejandro Ayala

Peter Trainer

Alan Krasner

Chris Ferrara-Cook

Clinical Operations

Stephanie Miller

Sohail Ahmed

Sonic Oun

Q² Solutions

Lian Shan

Adriane Spytko

Steve Lowes

Pharmaron-US

Mitesh Sanghvi

Junlong Shao

Accurant Biotech Inc.

Justin Senerchia

Dongbei Li

Eccolab Group Co.

Noel Carrion

University of Manchester

Anne White

Leanne Walker



THANK YOU

Barry Jones

bjones@crinetics.com

1023-CUSH-0016