Advances in Newborn Screening for Homocysteine Detection

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Outline

- Current analytical practices for homocystinuria (HCU) screening in newborns
- Towards a universal second-tier screening assay for biochemical NBS biomarkers
- Combination of first and second-tier screening biomarkers using separation before analysis by mass spectrometry (MS)
- Towards multiplexing homocysteine detection in primary flow injection analysis MS/MS (FIA-MS/MS) screening

Current analytical practices for HCU screening in newborns

- **Methionine (Met):** Biomarker used currently in primary newborn screening for Homocystinuria (HCU). Relatively poor sensitivity and specificity

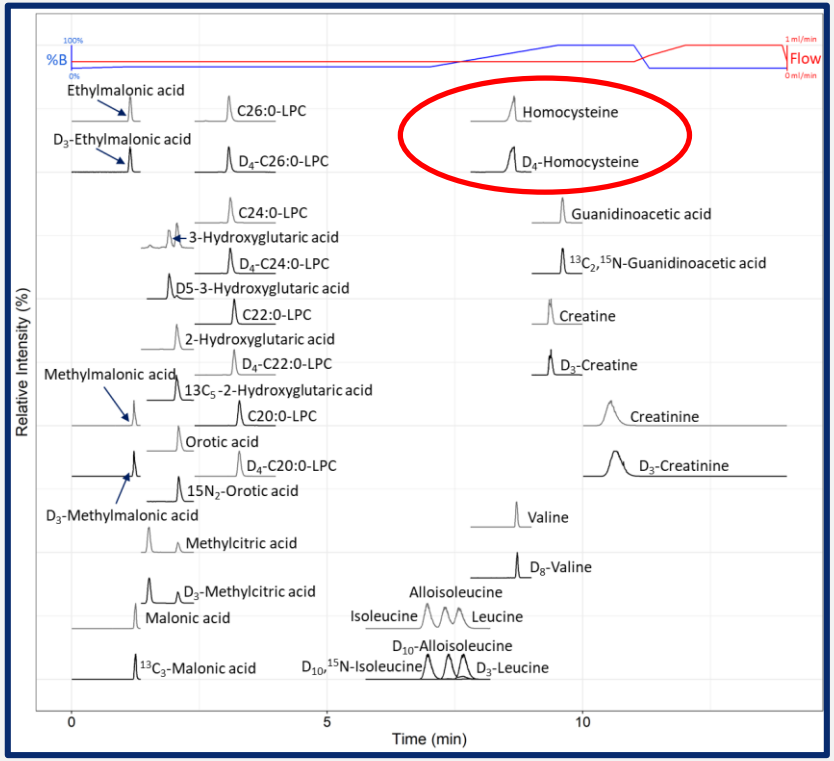
- **Total Homocysteine (tHcy):** Most specific marker for HCU, only used as a second-tier screening marker following a presumptive positive Met elevation in primary screening

- **Second-tier screening:** LC-MS/MS assays that measure only[1] tHcy or limited multiplexing[2,3] mainly with organic acids
  - Separate 2nd-tier assays for individual diseases
  - Low adoption for in-house 2nd-tier screening due to low reflex rates, many assays to maintain, need for separate MS instrument, delays in reporting etc
  - Regional 2nd-tier screening a possibility

1. Rose et al. Int. J. Neonatal Screen. (2017), 3, 32
2. Matern et al. J. Inherit Metab Dis (2007), 30, 585,
3. Turgeon et al. Clin. Chem. (2010), 56, 1686

Towards a universal second-tier screening assay for newborn screening biomarkers

- Screening for Adrenoleukodystrophy (ALD) by FIA-MS/MS requires 2nd-tier screening due to high false positives (up to 3%)
- Multiplexing (high reflex rate to 2nd-tier) ALD biomarkers with lower reflex rate biomarkers for other disorders may lead to higher 2nd-tier screening adoption rates (i.e., enough specimens to run 2nd-tier screening in-house daily)
- Hydrophilic Interaction Chromatography coupled to MS/MS. Assay was recently validated
- Multiplexes tHcy, organic acids, lysophosphatidylcholines (LPCs), Leu isomers, other analytes of interest

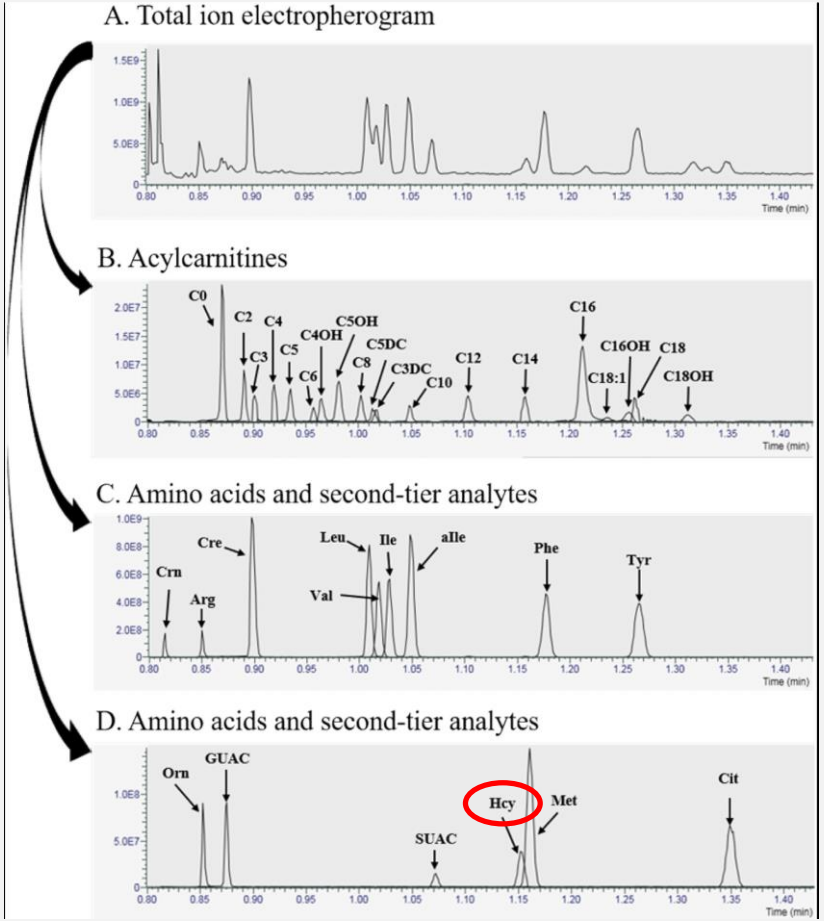


Highly multiplexed 2nd-tier screening

To be submitted to Anal. Chem.

Combining 1st and 2nd-tier screening using fast on-chip electrophoretic separations

- Fast separations before analysis by MS can allow the multiplexing of 1st-tier and 2nd-tier screening analytes
- Total separation time < 2 min
- tHcy, Leu isomers can be analyzed simultaneously with first tier markers
- Limitations: Inability to analyze organic acids, LPCs, cycle-time considerations



Pickens et al. Clin. Chem. (2021) 67, 1709 – CDC publication

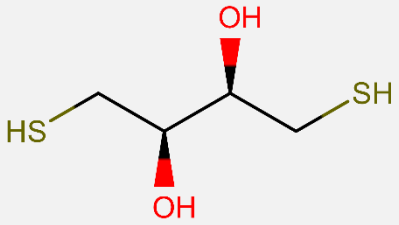
Towards multiplexing tHcy into primary FIA-MS/MS screening

- **A reducing step is required to be able to quantify tHcy**
  - >98% of Hcy either oxidized or bound to proteins
  - Reducing agents cleave the disulfide bond, making tHcy detection feasible

- **Considerations**
  - What are the challenges associated with tHcy multiplexing into a first-tier FIA-MS/MS method?
  - Are there interferences of tHcy during FIA-MS/MS analysis?
  - Impact of reducing agents on other biomarkers?
  - Solvent extraction issues or workflow considerations?

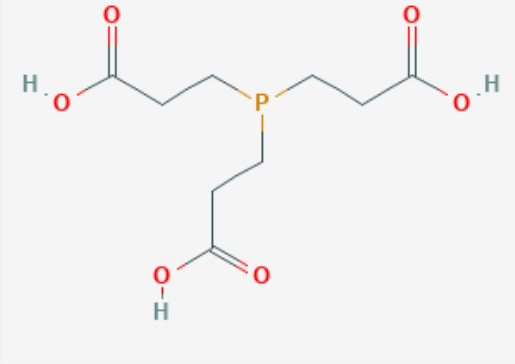
Common disulfide bonds reducing agents

Dithiothreitol (DTT)



- Commonly used in NBS papers
- Reversible reaction
- Does not ionize (+) mode

Tris(2-carboxyethyl)Phosphine (TCEP)

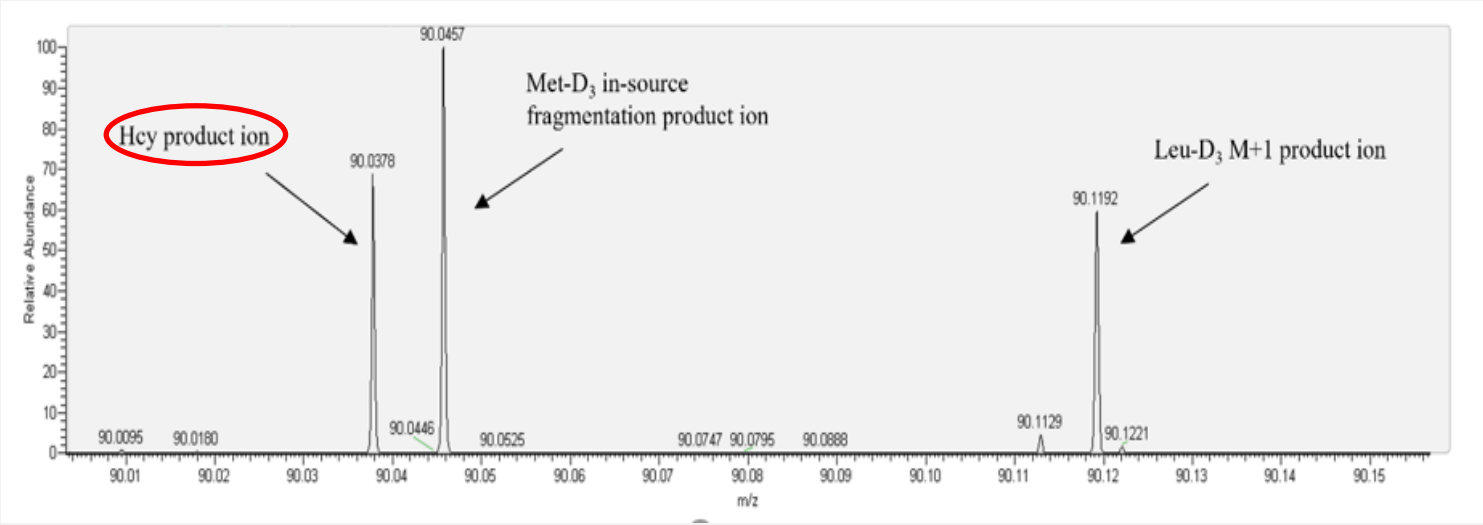


- Stronger reducing agent, better stability [1]
- Byproducts may form with heating [2]
- Ionizes in (+) mode
- Potential for post-reaction removal with magnetic nanoparticles [3]

1. Peiran Liu. J Am Soc Mass Spectrom. 2010 May;21(5):837-44;
2. Wang et al. Rapid Commun Mass Spectrom. 2010 Feb;24(3):267-75;
3. Zwyssig et al. Chemistry. 2017 Jun 27;23(36):8585-8589.

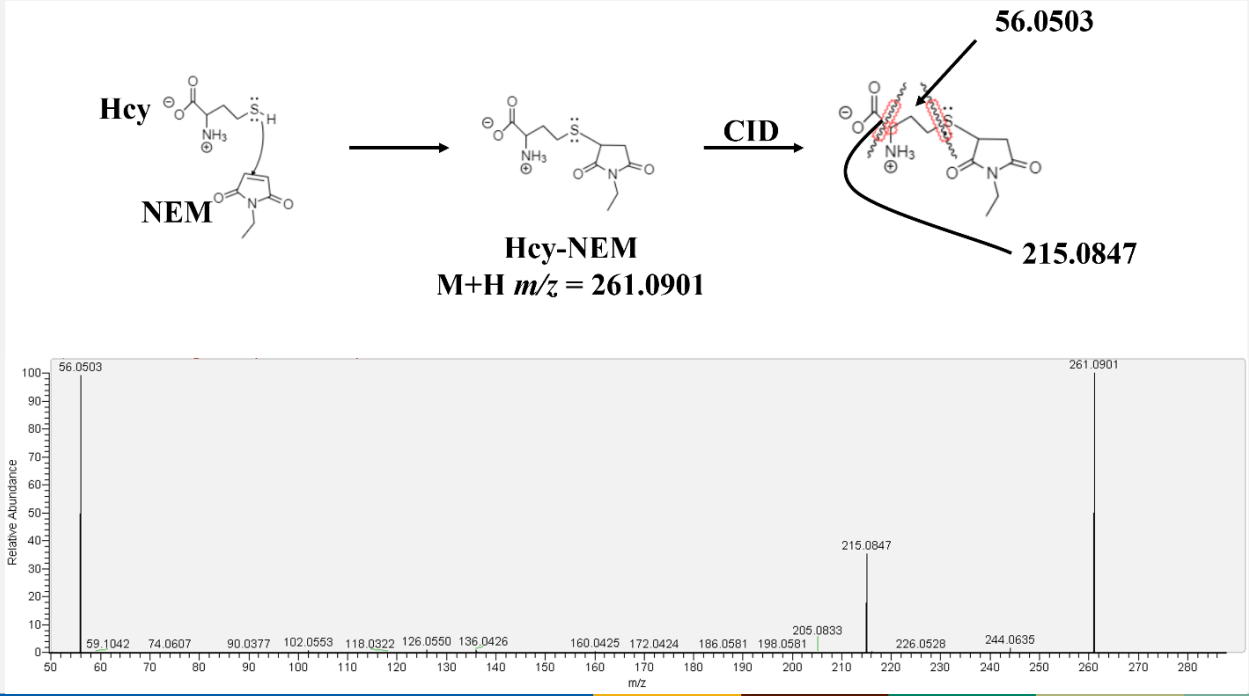
Identification of Hcy interferences

- **Interferences from commonly used internal standards used in all NBS assays**
  - Hcy dissociates to 136 > 90
  - Met-D3 153 > 136 in source fragmentation, 136 > 90
  - Leu-D3 M+1 is 136 > 90
  - Investigated thiol derivatization to shift the Hcy transition

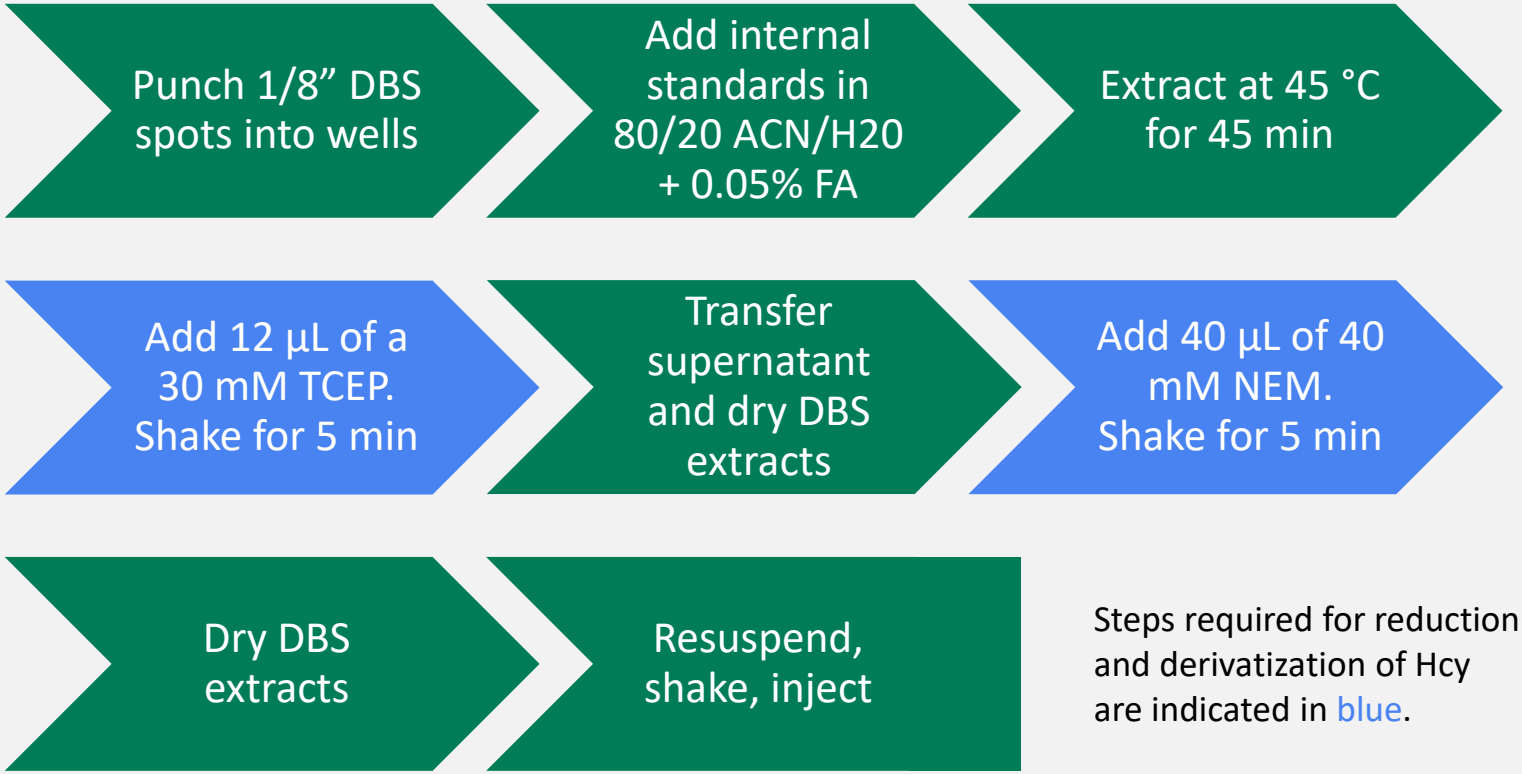


How does N-ethylmaleimide (NEM) work?

- **NEM reacts with any free thiol group**
  - Hcy, DTT, etc.
- **NEM shifts Hcy into a new transition (261>56). No interferences were observed**
- **DTT reacts with:**
  - NEM
  - Acylcarnitines
- **Used TCEP instead**



Sample Preparation of the TCEP-NEM protocol



Steps required for reduction and derivatization of Hcy are indicated in blue.

FA: Formic Acid, NEM: N-ethylmaleimide, TCEP: Tris(2-carboxyethyl)Phosphine

Method validation in progress – Preliminary findings

- Selective derivatization with NEM increases tHcy signal by 3-4 times
- **Linearity** for tHCY: R^2=0.99 from ≈2-120 µmole/L
- **Precision** (N=20) for tHCY: %RSD < 11.3%
- **LOQ** for tHCY: ≈ 2.8 µmole/L
- **No interferences** detected for tHCY
- Effect on other analytes:
  - TCEP+NEM increase the ion-suppression overly but still enough sensitivity for all analytes to be detected
  - C5:1 the only analyte that was highly affected by the new protocol. Uses C5 as a surrogated internal standard (IS). Synthesizing C5:1 as IS to try to mitigate the problem

Effect on other analytes: Comparing analyte concentrations of TCEP-NEM method against the control method

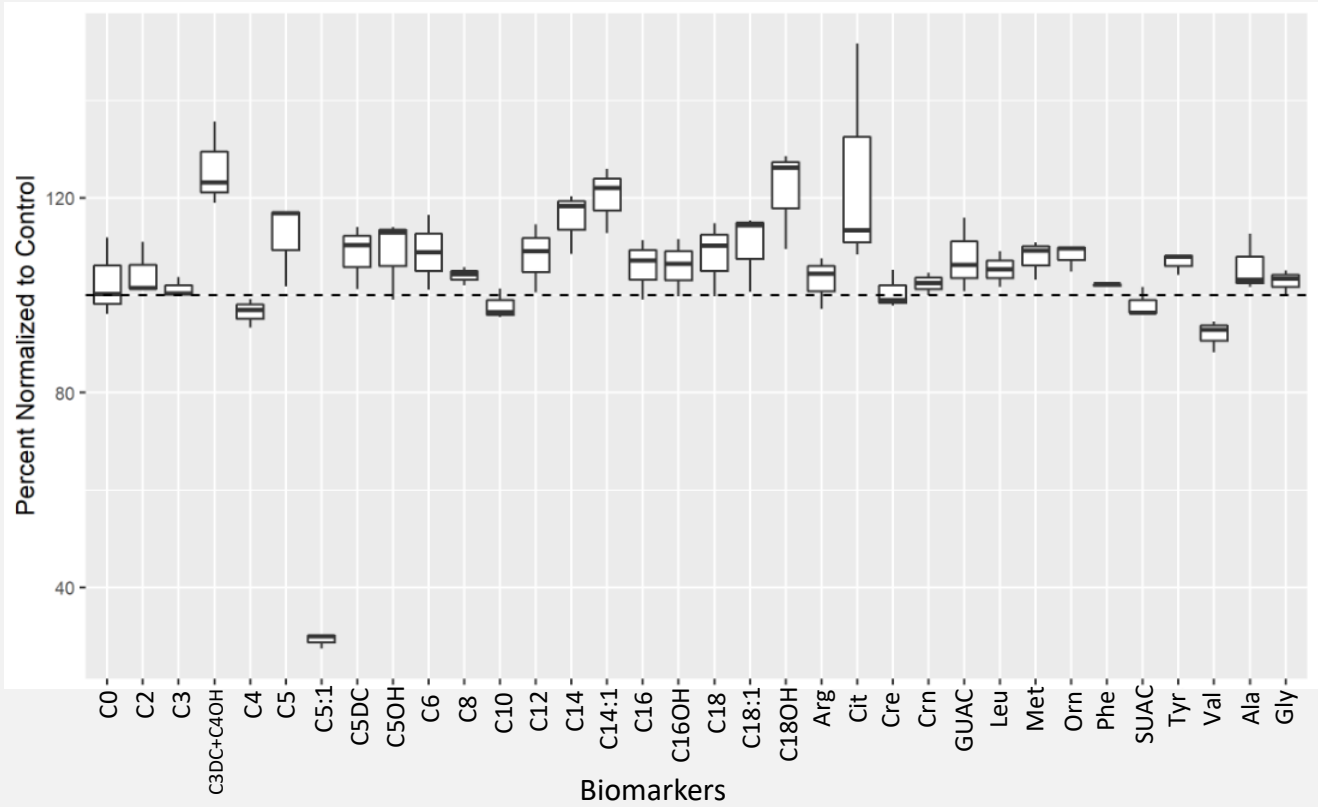
No change →

Side-by-side comparison

N=3

Specimen QC D2015

Most biomarkers within 0-20% from the control method (non-derivatized LDT)



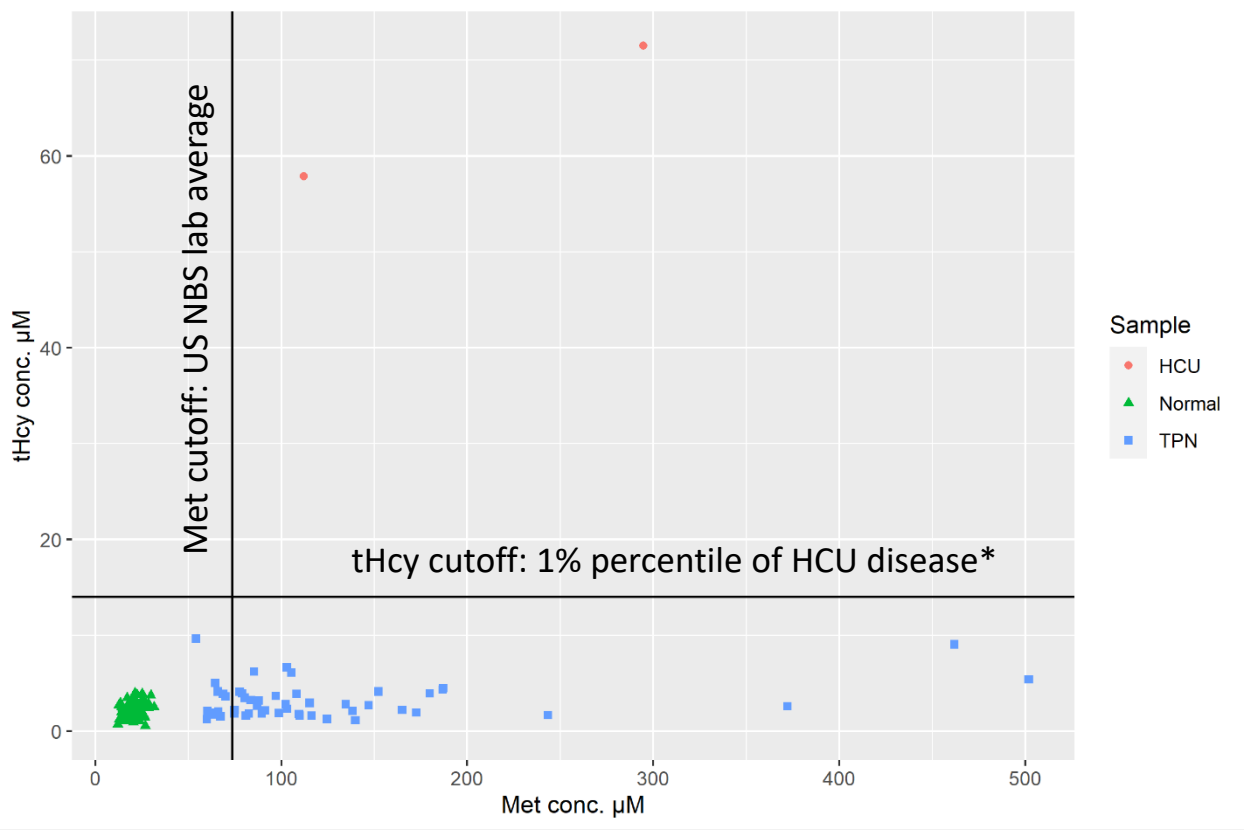
Analysis of residual NBS specimens: Normal, Total Parenteral Nutrition (TPN) and confirmed HCU

- Residual Specimens from TX
- HCU missed on first screen, presumptive positive on second based on Met measurements

First screen results

Age (h)	Met	Met/Phe
24	64.93	0.87
36	53.9	0.63

- First screen residual specimens were not available
- HCU specimens from second screen shown here



*Source: CLIR database

Summary

- Hcy is a more clinically relevant screening biomarker for HCU than Met and should be included into HCU screening algorithms
- Multiplexing C26:0-LPC with organic acids and amino acids in 2nd-tier screening generate enough specimens for daily, in-house use
- Proof of concept shows that high-throughput separations before analysis by MS/MS could play a significant role in the future
- Multiplexing Hcy into primary FIA-MS/MS screening could streamline the use of Hcy as a screening marker for HCU similar to succinylacetone multiplexing for Tyrosinemia type I

Acknowledgments

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Thank you!

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