CONTROVERSIES CONFERENCE
ON NEPHROPATHIC CYSTINOSIS:
DIAGNOSTICS AND BIOCHEMICAL FOLLOW-UP

Bruce A. Barshop, MD, PhD
University of California, San Diego
Disclosure of Interests
Bruce A. Barshop, MD, PhD

• Genzyme-Sanofi: Research Grant
• Pfizer: Honorarium/ Sponsored Education
• BioMarin: Sponsored Education

No other conflicts of interest to report.
why monitor? (Not controversial.)

Progression of disease dramatically affected by treatment with cysteamine.

“The target leukocyte cystine content is <1.0 nmol of half-cystine per mg protein.”
Predicted reciprocal serum creatinine at age 10 years as a measure of renal function in children with nephropathic cystinosis treated with oral cysteamine

William A. Gahl1, Jerry A. Schneider2, Joseph D. Schulman3, Jess G. Thoene4, and George F. Reed5

Table 3. Stratification according to leukocyte cystine levels

<table>
<thead>
<tr>
<th>Leukocyte cystine depletion groupsa</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>19</td>
<td>18</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Initial age (years)</td>
<td>3.27 ± 0.32(^b)</td>
<td>2.98 ± 0.37</td>
<td>2.90 ± 0.55</td>
<td>4.20 ± 0.43</td>
</tr>
<tr>
<td>Initial creatinine (mg/dl)</td>
<td>0.87 ± 0.07</td>
<td>0.91 ± 0.09</td>
<td>0.80 ± 0.10</td>
<td>1.29 ± 0.10</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min per 1.73 m(^2))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– initial</td>
<td>56.6 ± 4.4</td>
<td>57.8 ± 6.0</td>
<td>62.42± 5.5</td>
<td>43.0 ± 5.1</td>
</tr>
<tr>
<td>– final</td>
<td>64.6 ± 6.6</td>
<td>59.2 ± 6.4</td>
<td>63.0 ± 7.0</td>
<td>37.9 ± 6.9</td>
</tr>
<tr>
<td>– (final-initial)</td>
<td>8.0 ± 5.8</td>
<td>1.4 ± 4.2</td>
<td>0.5 ± 8.1</td>
<td>−5.0 ± 3.4</td>
</tr>
<tr>
<td>( P^* )</td>
<td>0.374</td>
<td>0.450</td>
<td>0.066</td>
<td></td>
</tr>
<tr>
<td>PRC(_{10})</td>
<td>0.96 ± 0.11</td>
<td>0.65 ± 0.12</td>
<td>0.68 ± 0.20</td>
<td>0.35 ± 0.18</td>
</tr>
<tr>
<td>( P^* )</td>
<td>0.064</td>
<td>0.203</td>
<td>0.006</td>
<td></td>
</tr>
</tbody>
</table>

a Groups 1–3: Two or more leukocyte cystine levels obtained per year
Group 1: Median level <1 nmol 1/2 cystine/mg protein
Group 2: Median level between 1 and 2 nmol 1/2 cystine/mg protein
Group 3: Median level over 2 nmol 1/2 cystine/mg protein
Group 4: Fewer than two levels obtained per year or could not tolerate full recommended dose of cysteamine
b SEM
* Two sided \( P \) value compared with group 1, using Student’s \( t \)-test
Cysteamine therapy delays the progression of nephropathic cystinosis in late adolescents and adults

Albane Brodin-Sartorius¹,², Marie-Josèphe Tête²,³, Patrick Niaudet²,³, Corinne Antignac²,⁴,⁵, Geneviève Guest²,³, Chris Ottolenghi²,⁶, Marina Charbit²,³, Dominique Moyse⁷, Christophe Legendre³,⁸, Philippe Lesavre¹,², Pierre Cochat⁹,¹⁰, Aude Servais¹,²

*Kidney International (2012) 81, 179–189*
pre-symptomatic diagnosis/ screening

• Screening = Population-based
  – e.g. newborn screening, Pap smear, mammograms

• Carrier testing
  – e.g. Dor Yeshorim for Tay Sachs carriers in orthodox Jewish communities

• Presymptomatic testing
  – e.g. pregnancy in progress in family at risk for cystinosis

• Preimplantation diagnosis
  – e.g. planned pregnancy in family at risk
pre-symptomatic diagnosis/ screening

• How soon to test post-natal?
  – No increased storage at birth, or at least non-diagnostic. Customary to wait... how long?

• Feasible *in utero*?
  – Biochemical: CVS  ... but risk to fetus unwarranted unless info used to inform decision to terminate
  – Genetic: CVS or amino – or NIPT (non-invasive prenatal testing, cell-free DNA in maternal circulation)  ...with same proviso
  – Genetic: Preimplantation
pre-symptomatic diagnosis/ screening

- Common 57b kb deletion found to also delete CARKL (SHPK) (Touchman et al., 2000; Phornphutkul et al., 2001) and extend into TRPV1 (Freed et al., 2001).
Sedoheptulokinase

\[
\text{D-Sedoheptulose, Volemulose} \quad \xleftrightarrow{\text{SHPK, CARKL}} \quad \text{Sedoheptulose 7-phosphate} \quad \xleftrightarrow{\text{TALDO1}} \quad \text{D-Erythrose 4-phosphate}
\]

\[
\text{D-Ribose 5-phosphate} \quad \xleftrightarrow{\text{TKT}} \quad \text{D-Xylulose 5-phosphate} \quad \xleftrightarrow{\text{TALDO1}} \quad \text{D-Fructose 6-phosphate}
\]
Characterization of mammalian sedoheptulokinase and mechanism of formation of erythritol in sedoheptulokinase deficiency

Tamas Kardon\textsuperscript{a}, Vincent Stroobant\textsuperscript{b}, Maria Veiga-da-Cunha\textsuperscript{a}, Emile Van Schaftingen\textsuperscript{a,\ast}


Fig. 3. Metabolism of sedoheptulose and its alteration in sedoheptulokinase deficient patients. Sedoheptulokinase normally converts sedoheptulose to sedoheptulose 7-phosphate, which is metabolized by enzymes of the pentose phosphate pathway. Based on the properties of fructokinase and aldolase B, we propose that, in the absence of sedoheptulokinase, sedoheptulose would be phosphorylated by fructokinase to sedoheptulose 1-phosphate, which would then be cleaved by aldolase B to dihydroxyacetone-phosphate and erythrose. The latter would be reduced to erythritol by aldehyde reductase. Both sedoheptulose and erythritol are excreted in urine.
pre-symptomatic diagnosis/ screening

• Feasible screening? Biomarker for 57 kb deletion.

Elevated concentrations of sedoheptulose in bloodspots of patients with cystinosis caused by the 57-kb deletion: Implications for diagnostics and neonatal screening

M.M.C. Wamelink a,*, E.A. Struys a, E.E.W. Jansen a, H.J. Blom a, T. Viliboux b, W.A. Gahl b, M. Könhoff c, C. Jakobs a, E.N. Levchenko d

Molecular Genetics and Metabolism 102 (2011) 339–342

Fig. 2. Scatter plot of sedoheptulose concentrations in DBS measured via LC–MS/MS.
## ctnts mutations

<table>
<thead>
<tr>
<th>Type</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense/nonsense</td>
<td>50</td>
</tr>
<tr>
<td>Splicing</td>
<td>15</td>
</tr>
<tr>
<td>Regulatory</td>
<td>2</td>
</tr>
<tr>
<td>Small deletions</td>
<td>24</td>
</tr>
<tr>
<td>Small insertions</td>
<td>10</td>
</tr>
<tr>
<td>Small indels</td>
<td>4</td>
</tr>
<tr>
<td>Gross deletions</td>
<td>12</td>
</tr>
<tr>
<td>Gross insertions</td>
<td>0</td>
</tr>
<tr>
<td>Complex</td>
<td>0</td>
</tr>
<tr>
<td>Repeats</td>
<td>0</td>
</tr>
</tbody>
</table>

The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff 21-Nov-2014
Mutational Spectrum of the CTNS Gene in Egyptian Patients with Nephropathic Cystinosis

Neveen A. Soliman • Mohamed A. Elmonem • Lambertus van den Heuvel • Rehab H. Abdel Hamid • Mohamed Gamal • Inge Bongaers • Sandrine Marie • Elena Levchenko

JIMD Reports 2014

Fig. 1 Worldwide geographical distribution of 57-kb deletion and previously reported CTNS mutations detected in the Egyptian population. Oval: geographical distribution of 57-kb deletion. Circle: geographical distribution of the Middle Eastern mutation c.681G>A
common mutations/ uncommon mutations

  - 0/25 pts had the 57 kb deletion (het or homo). 1/25 hom novel mut, c.153-155insCT, 1/25 hom and 1/25 cpd het with c.923G>A. Also three known muts: c.18-21delGACT, c.1017G>A, and c.681G>A in 11/25. No mut detected in 11/25 pts.
  - 0/12 patients had the 57-kb deletion. 4 known variations (c.140+1 G>T, c.1015 G>A (p.G339R), c.18_21del GACT (p.T7FX7), c.681 G>A (p.E227E)), 5 new variants: a 10-kb deletion (c.62-1083_551del10217bp), 3 missense variants (c.518A>G (p.Y173C), c.451A>G (p.R151G), c.470 G>A (p.G157D)), and a nucleotide substitution in a potential branch point site of intron 4 (c.141-22a>g).
  - 0/15 patients had 57-kb deletion; 27 mutant alleles and 12 pathogenic mutations detected, incl. 6 novel mutations.
  - 57-kb deletion only in 17% of 84 chromosomes. Several splice site mutations.
Biomarker for 57 kb deletion: Ethnically targeted?

Ethical implications and practical considerations of ethnically targeted screening for genetic disorders: the case of hemoglobinopathy screening
Cynthia F. Hinton*, Althea M. Grant and Scott D. Grosse

Ethnicity & Health
Vol. 16, Nos. 4–5, August–October 2011, 377–388

1. Categories of race/ethnicity are social constructs, therefore, observed or self-identified broad racial/ethnic categories are not necessarily reliable indicators of geographic ancestry or genetic risk.
2. Targeting based on ethnicity poses serious issues of logistics and equity for public health programs and clinical services.
**America’s Churning Races:**

*Race and Ethnic Response Changes between Census 2000 and the 2010 Census*

www.census.gov

Table 4. Non-Hispanic black, American Indian, and/or white response stability and change

<table>
<thead>
<tr>
<th>Race response in 2000 Census linked data</th>
<th>Race response in 2010 Census linked data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
</tr>
<tr>
<td>black (B)</td>
<td>14,881,514</td>
</tr>
<tr>
<td>AIAN</td>
<td>16,307</td>
</tr>
<tr>
<td>white (W)</td>
<td>102,464</td>
</tr>
<tr>
<td>B &amp; AIAN</td>
<td>50,000</td>
</tr>
<tr>
<td>B &amp; W</td>
<td>90,086</td>
</tr>
<tr>
<td>AIAN &amp; W</td>
<td>87,809</td>
</tr>
</tbody>
</table>
pre-symptomatic diagnosis/ screening

- Can apply a “universal”, “comprehensive” panel of mutations, but a) always changing, b) inherently discriminatory and disenfranchising for rare minorities.
- Better at present is to first use a (less specific) biomarker, and then use such a mutation panel to confirm findings, e.g. immunoreactive trypsinogen for cystic fibrosis.
- Presently no such biomarker exists for cystinosis....
- Things are changing quickly with regard to comprehensiveness of genetic/genomic screening.
optimal technique for testing

- Amino acid analyzer - soon proved insufficiently sensitive.
- Cystine binding protein - very demanding, slow throughput limited supply.
- Tandem mass spectrometry (LC-MS/MS) - the methodology of choice presently.
ERNDIMQA - ANALYTE IN DETAIL

Cystine in White Blood Cells

Scale
Standard Deviations

-3SD  < 0.404
-2SD   0.404 - 1.78
-1SD   1.78 - 2.47
-0.5SD  2.47 - 3.16
0.0SD  3.16 - 3.85
0.5SD   3.85 - 4.54
1.0SD   4.54 - 5.23
1.5SD   5.23 - 5.92
2-3SD   5.92 - 6.61
1.5 - 2.0SD  6.61 - 7.30
2-3SD   7.30 - 8.68
>3SD   > 8.68
optimal technique for testing

• Stabilization of -SH: inhibition of disulfide exchange
  – N-Ethylmaleimide: method of choice
  – Acidic storage (e.g. sulfosalicylic acid): may suffice and may be favored when cell isolation is done at a remote location.

• Stable isotope dilution: preferable with LC-MS/MS

• Quantification of protein may be the bigger issue in the analytical phase.
Comparison of BCA and Lowry total protein assay values, using a bovine serum albumin standard, from 106 clinical samples from patients with cystinosis, revealed a significant and consistent difference in values from identical samples. The mean and standard deviation of the ratio between BCA and Lowry results over all samples was 0.65±0.07. Discovery of the discrepancy in total protein values allowed values to be normalized and the study to proceed. We suspect that the discrepancies observed are based on variable assay sensitivity to different protein types, as has been noted…

Though Lowry method and bicinchoninic acid (BCA) method agreed perfectly well on BSA standards and on ERNDIM protein unknowns (which were simply BSA), there were differential responses of the two methods when used on leukocyte lysates.
“Because we observed a clear difference between cystine content in ML preparations and PMN cells, we suggest that each laboratory produces its own reference values based on the upper cystine values found in heterozygotes.”
optimal prep/storage

- Problem is in pre-analytical phase, i.e. cell prep
  - Erratic results with prolonged storage of whole blood
  - Some anticoagulants are better than others - ACD
- Differences in protein recovery depending upon anticoagulant

![Graph showing changes in leukocyte cystine content](image)

Changes in leukocyte cystine content after 24 hours storage at room temperature and 4°C

- baseline
- 24h room temp
- 24h 4°C
- 48h 4°C

0.0  50.0  100.0  150.0  200.0  250.0

- Li Hep
- Na Hep
- ACD
- EDTA

Fidler et al., 2011, unpublished
optimal prep/storage

- Problem is in pre-analytical phase, i.e. cell prep
  - Erratic results with prolonged storage of whole blood
  - Some anticoagulants are better than others - ACD
    - Differences in protein recovery depending upon anticoagulant

![Graph showing cystine vs. protein content in samples prepared from 3 mL of blood at baseline]

Fidler et al., 2011, unpublished
optimal prep/storage

- Problem is in pre-analytical phase, i.e. cell prep
  - Erratic results with prolonged storage of whole blood
  - Some anticoagulants are better than others - ACD
- Differences in protein recovery depending upon anticoagulant

Fidler et al., 2011, unpublished
new denominators: alternative normalization

Discovery of peptides from lysosomal proteins endogenous in leukocytes

Whole blood → Mixed leukocyte extraction

White blood cell lysis / protein precipitation / resolubilization

<table>
<thead>
<tr>
<th>N</th>
<th>% protein Coverage</th>
<th>Protein Name</th>
<th>Peptides(95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51.2</td>
<td>fibronectin 1 isoform 5 preproprotein [Homo sapiens]</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>56.7</td>
<td>ftan 1 [Homo sapiens]</td>
<td>62</td>
</tr>
<tr>
<td>30</td>
<td>37.5</td>
<td>elastase 3, neutrophil preproprotein [Homo sapiens]</td>
<td>9</td>
</tr>
<tr>
<td>40</td>
<td>41.4</td>
<td>azurocidin 1 preproprotein [Homo sapiens]</td>
<td>5</td>
</tr>
<tr>
<td>91</td>
<td>14.8</td>
<td>proteinase 3 (serine proteinase, neutrophil, Wegener granulomatosis autoantigen) [Homo sapiens]</td>
<td>2</td>
</tr>
<tr>
<td>181</td>
<td>3.3</td>
<td>transaldolase 1 [Homo sapiens]</td>
<td>1</td>
</tr>
</tbody>
</table>

Peptides for nearly 180 proteins were matched with over 95% confidence using Protein Pilot (AB Sciex). Peptides from 3 lysosome-localized proteins in neutrophils were found: **Elastase 2**, **Azurocidin 1**, and **Proteinase 3**.

MS/MS scans were performed on several thousand peptides from a single LC gradient run on a C18 capillary column coupled to a Q-TOF.

Liquid chromatography coupled to MS/MS (Qstar Elite, AB Sciex)
Non-hematologic cell types

Prospect: Renal tubular epithelial cells isolated from urine:
Advantages: May better reflect long-term medication exposure
Readily accessible cell type; May reflect therapeutic effect at key site;
?Volume req’d, ?effect of treatment PK/urine [cysteamine]
Approach: immuno-purification.

Prospect: Buccal epithelium by cheek swab:
Advantages: Readily accessible cell type; May reflect therapeutic effect
at other tissues; could be valid post transplant. Could have home collection.
Approach: Demonstrate feasibility in heterozygotes and treated homozygotes
post rinse, 10 swipes with buccal swab, placed in SSA solution, extracted.
optimal timing of testing

Population pharmacokinetics and pharmacodynamics of cysteamine in nephropathic cystinosis patients

Naïm Bouazza¹,²*, Jean-Marc Tréluyer¹,²,³,⁴, Chris Ottolenghi⁵, Saik Urien¹,²,⁴, Georges Deschenes⁷, Daniel Ricquier⁵, Patrick Nlaudet⁶ and Bernadette Chadeaux-Vekemans⁵

Orphanet Journal of Rare Diseases 2011, 6:86

Proposed daily dose:
- 80 mg/kg/d (QID): 10-17 kg,
- 70 mg/kg/d (QID): 17-25 kg,
- 60 mg/kg/d (QID): 25-40 kg,
- 50 mg/kg/d (QID): 40-70 kg.

Generally would presume best testing time would be trough level of drug/ highest level of cystine. But drug level most variable in terminal phase.
optimal timing of testing

A Randomized Controlled Crossover Trial with Delayed-Release Cysteamine Bitartrate in Nephropathic Cystinosis: Effectiveness on White Blood Cell Cystine Levels and Comparison of Safety

Terminal elimination phase
residual cysteamine as surrogate marker

Quality of Life is Improved and Kidney Function Preserved in Patients with Nephropathic Cystinosis Treated for 2 Years with Delayed-Release Cysteamine Bitartrate

Langman et al.
J Pediatr. 2014 September; 165(3): 528–533

Figure 2.
WBC (cystine) vs plasma (cysteamine) for all study patients who had a WBC (cystine) ≤1 nmol/1/2 cystine/mg protein; 94.5% of measured plasma (cysteamine) values were >0.1 mg/dL when the WBC (cystine) was ≤1 nmol ½ cystine/mg protein.
residual cysteamine as surrogate marker

Quality of Life is Improved and Kidney Function Preserved in Patients with Nephropathic Cystinosis Treated for 2 Years with Delayed-Release Cysteamine Bitartrate


Percent with WBC ½-cystine <1 nmol/mg:

- >0: 81.8%
- >0.1: 94.5%
- >0.2: 95.2%
- >0.3: 93.75%
controversies conference

- why monitor?
- target range
- earliest treatment/earliest measurement
- pre-symptomatic diagnosis/screening
- sedoheptulokinase
- ctns mutations
- common mutations/uncommon mutations
- optimal technique for testing
- denominator effects
- optimal prep
- optimal storage
- alternative normalization
- alternative cell types for cystine assay
- optimal timing of testing
- residual cysteamine as surrogate marker
other questions

• Is it feasible to perform pre-symptomatic screening of cystinosis? In utero and in newborns?
• What is the optimal technique for white blood cell (WBC) isolation and storage?
• What is the optimal technique for WBC cystine measurement, including timing of the measurement?
• Are there alternatives to WBC cystine measurements to monitor cysteamine treatment (plasma cysteamine, others)?
• What is the role of cystine as a biomarker and cysteamine blood levels as a surrogate?
• Can we measure crystal loads?
• Is genetic diagnosis mandatory?
• Is urine analysis helpful to raise the suspicion or make the diagnosis?
• What other biochemical monitoring should be undertaken in treated patients?
• What are the major clinical hints, providing high index of suspicion to diagnose cystinosis as early as possible?
• What is the final decision regarding carnitine supplementation for patients post transplant? Is therapy worth the cardiovascular risk?
• To discuss controversies of newborn screening, molecular diagnosis availability
• To discuss controversies of the time post Procysbi dose to evaluate WBC cystine reduction: 11.5 versus 12.5 hours