Advances in molecular understanding of cystinosis: implications for therapy

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Diagnosis and management of inherited kidney diseases: What’s new?
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Cystinosis

- Rare autosomal recessive lysosomal storage disorder
- Defective lysosomal efflux of cystine
- Three clinical forms:
  - infantile or nephropathic (Fanconi syndrome)
  - juvenile
  - ocular non-nephropathic
- Multisystem disease

No genetic heterogeneity: complementation studies in somatic cell hybrids between fibroblasts from patients with different forms of cystinosis (Pellet, Smith et al. 1988)
### Treatment - cysteamine

**Cysteamine**

- **Oral administration:** improves growth & glomerular filtration rate; delays ESRD and the appearance of other clinical anomalies
- **Eye drops:** dissolve corneal cystine crystals
- **Side effects & need of regularly spaced doses:** (each 6h for oral cysteamine and each 1h for eye drops)
- **No effect on Fanconi syndrome**
- **New delayed-release form administrated twice a day** (Dohil, Gangoiti et al. 2010)
Cystinosin, the gene product of CTNS mutated in cystinosi

- Lysosomal membrane protein with two targeting motifs (Cherqui et al., JBC, 2001)
- Proton-cystine symporter active at low pH, allowing cystine export from lysosomes (Kalatzis et al., EMBO J, 2001)

- Component of the vATPase-Ragulator-Rag complex controlling the mTORC1 complex (Andrzejwska et al., JASN, 20017)

(R: Ragulator complex + Rag GTPases)
Cystinosis: Mutations in the CTNS gene encoding cystinosin

- ~120 different mutations in cystinosis patients all over the world
- 57kb “European” deletion (56 to 76% in Northern Europe)
- Several recurrent mutations in addition to the “European” deletion
- Maternal uniparental heterodisomy of chrom 17

Clear phenotype-genotype correlations:
- Two «severe» mutations in the infantile forms
- Two «mild» mutations or one «severe» and one «mild» mutation in the other forms
Functional studies of missense mutations

- Good genotype-phenotype correlation but some exceptions:
  - 2 mutants associated with infantile cystinosis are partially or fully active (additional, unidentified mutations in these patients? - less severe phenotype?)
  - 3 mutants associated with juvenile or atypical cystinosis do not transport cystine (additional role of cystinosin beyond cystine transport?)

![Diagram showing cystine transport as a percentage of cystinosin-ΔGYDQL activity for various mutations.]

- G110V
- V42I
- S298N
- D346N
- W182R
- N323K
- S139F
- K280R

**Mutation**

- Infantile
- Juvenile
- Atypical
- Ocular
Proposed cellular dysfunctions in cystinosis

- **Impact of cystine accumulation on glutathion synthesis and oxidative stress** (Chol et al., 2004; Laube et al., 2006; Mannuci Pastores et al., 2006; Bellomo, Corallini et al., 2010)
- **Increased apoptosis** (Thoene et al., Mol Genet Metab 2007; Sansanwal et al., Pediatric Nephrology 2010; Taub and Cutuli, BBRC 2012)
- **Increased ER stress** (Wei et al., HMG, 2007)
- **Reduced TFEB (master regulator of the autophagy–lysosomal pathway) expression and induced nuclear translocation** (Rega et al., KI, 2016)
- **Impaired lysosomal transport** (Johnson et al., MCB, 2013)
- **Involvement in the mTORC1 pathway** (Andrzejewska et al. JASN, 2015; Ivanova et al., JIMD 2016)

Direct impact of **cystine crystal** accumulation and/or the absence of **cystinosin**?
Proposed cellular dysfunctions in cystinosis

From Cherqui & Courtoy, *Nature Reviews | Nephrology* 2017
New potential therapeutic interventions

- Additional therapies to cysteamine
  - cysteine supplements
  - anti-oxydants
  - triggers of lysosome biogenesis,
  - CMA modulators
  - TFEB expression modulators (genistein)

- **Stem cell therapy**: How delivering a lysosomal transmembrane gene product to every tissue?
Hematopoietic stem cell (HSC) transplantation in Ctns−/− mice

Confocal Microscopy 4 months post-transplantation

Spleen

Kidney

Brain

Eye

Cystine content at 2 and 4 months post-transplant

GFP transgenic wild-type mouse

GFP-HSC Sca1+ cells

Ctns−/− mice

Long term significant reduction of cystine levels in all organs

Syres et al., Blood, 2009
Impact of HSC transplantation on the kidney pathology in $Ctns^{-/-}$ mice

Kidney histology in 15-17 month old mice after over 1 year post-transplantation

Wild-type

Treated $Ctns^{-/-}$

High level of donor-derived blood cell engraftment expressing $Ctns$ (>50%)

The higher the quantity of bone marrow cells expressing $Ctns$ the better the preservation of the kidney

Low level of donor-derived blood cell engraftment expressing $Ctns$ (<50%)
Impact of HSC transplant on cystine crystals in the kidney

Yeagy et al., *Kidney Int.*, 2011
Impact of HSC transplant on the eye defects in *Ctns*-/- mice

Eye study after over 1 year post-transplantation

**In Vivo Confocal Microscopy (IVCM)**

**Histology and central cornea thickness (CCT) measurement**

Rescue of corneal defects by HSC transplantation

Rocca *et al.*, *IOVS.*, 2016
Thyroid pathology in Ctns\(^{-/-}\) mice and impact of HSC transplantation

Most frequent and earliest endocrine complication of cystinosis

Cystine measurement in the thyroid

Mesure of Thyroid Stimulating Hormone (TSH) in serum

Drs X.H. Liao & S. Refetoff, UChicago

Gaide Chevrannay et al., Endocrinology, 2016
Clinical translation: autologous gene-modified HSC transplantation

- Safety
- Gene frequency
- Risk of integration mutagenesis

Adapted from Leboulch, Nature 2013
**Preclinical studies for genetically-modified HSC transplantation**

**Ctns<sup>-/-</sup> HSC**

- **Ctns<sup>-/-</sup> mice**
- Tail Vein Injection

**Ctns<sup>-/-</sup> mice**

- Decrease cystine levels in all tested tissues
- Long term transgene expression

**Kidney cystine content**

- 8 months post-transplant

**Renal function**

**Table 1. Serum and urine analyses for renal function**

<table>
<thead>
<tr>
<th></th>
<th>Wildtype (n=6)</th>
<th>Control Ctns&lt;sup&gt;-/-&lt;/sup&gt; (n=9)</th>
<th>pCCL-CTNS Treated Ctns&lt;sup&gt;-/-&lt;/sup&gt; (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Creatinine (mg/dL)</td>
<td>0.27 ± 0.03</td>
<td>0.31 ± 0.08</td>
<td>0.22 ± 0.11**</td>
</tr>
<tr>
<td>Serum Creatinine clearance (ml/min/kg)</td>
<td>4.44 ± 0.39</td>
<td>3.89 ± 1.42</td>
<td>4.86 ± 5.56</td>
</tr>
<tr>
<td>Serum Urea (mg/dL)</td>
<td>14.55 ± 1.67</td>
<td>28.29 ± 16.11</td>
<td>24.10 ± 7.32</td>
</tr>
<tr>
<td>Serum Phosphate (mg/dL)</td>
<td>12.25 ± 2.38</td>
<td>13.20 ± 2.90</td>
<td>13.10 ± 2.21</td>
</tr>
<tr>
<td>Urine Phosphate (mmol/24h)</td>
<td>6.82 ± 2.90</td>
<td>8.84 ± 4.60</td>
<td>4.78 ± 3.87**</td>
</tr>
<tr>
<td>Urine Volume (ml)</td>
<td>1.05 ± 0.51</td>
<td>1.26 ± 0.54</td>
<td>0.70 ± 0.60*</td>
</tr>
</tbody>
</table>

**<sup>*</sup>P<0.05 compared to wildtype mice**

**<sup>**P<0.05 compared to Ctns<sup>-/-</sup>**

**Cystine crystals quantification**

- Non-treated Ctns<sup>-/-</sup> mice
- pCCL-CTNS-treated mice

Harisson et al., *Mol. Ther.*, 2013
Characterization of the transplanted HSCs within the kidney

Differentiation, fusion or transdifferentiation?

Kidney

Eye

Thyroid

Transplanted HSCs differentiate into macrophages within tissues in Ctns−/− mice

How do transplanted HSCs mediate tissue repair in cystinosis?

• Phagocytic functions
• Cross-correction i.e. transfer of cystinosin from the transplanted cells to the adjacent Ctns−/− cells
Cross-correction: *in vitro* studies

**Cystinosin transfer via cell-cell contact**

![Diagram showing Cystinosin transfer via cell-cell contact](image)

- **GFP**
  - macrophages
- DsRed Ctns
  - Fibroblasts

![Graph showing cystine content](image)

- Control
- Macrophages: 75%

![Image showing cystinosin-GFP fusion protein](image)

**Nanotubular Highways for Intercellular Organelle Transport**

Amin Rustom, Rainer Saffrich, Ivanka Markovic, Paul Walther, Hans-Hermann Gerdes

*Science, 2004*

![Image showing nanotubular highways](image)

**Cystinosin-GFP fusion protein**

- Ctns
  - Macrophages
- DsRed Ctns
  - Fibroblasts

Naphade et al., *Stem Cells, 2015*
Kidney

Cross-correction: *in vivo* studies

- Vesicular cross-correction in kidney
- Also demonstrated in cornea and thyroid

Naphade et al., *Stem Cells*, 2015
Rocca et al., *IOVS*, 2015
Gaide Chevironnay et al., *Endocrinology*, 2017
Conclusions

- Several key cellular dysfunctions are observed in cystinosis linked to the lysosomal cystine accumulation and/or additional roles of cystinosin beyond cystine transport.

- Several new lines of treatment are being developed:
  - In addition to cysteamine therapy, drugs targeting the various pathways altered in cystinosis.
  - **Stem cell therapy**
    - Long term significant reduction of cystine levels in all organs by hematopoietic stem cells in a *Ctns-/-* mouse model.
    - Differentiation of HSC in macrophages
      - Phagocytic function
      - Cross-correction through nanotubes
    - Clinical trials being set up in the US (autologous stem cell transplantation).
  - Novel additional eye treatments (corneal nanowafers).
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Cystinosin interacting partners
### Proteins interacting with cystinosin (by mass spectrometry)

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>CD63-GFP</th>
<th>Fibroblast cystinosin-GFP</th>
<th>MDCK cystinosin-GFP</th>
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<tr>
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<td>Human Cystinosin-GFP</td>
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<tr>
<td>GFP</td>
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<td>V-type proton ATPase catalytic subunit A</td>
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<td>V-type proton ATPase subunit B, brain isomorf</td>
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<td>V-type proton ATPase subunit H</td>
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<td>Ragulator complex protein LAMTOR1</td>
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<td>Ragulator complex protein LAMTOR3</td>
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<td>Ragulator complex protein LAMTOR5</td>
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<tr>
<td>Ras-related GTP-binding protein C</td>
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<td>4</td>
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<tr>
<td>Ras-related GTP-binding protein A</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

- **Additional control:** Lamp1-GFP
- **Ers1** (homolog of \textit{CTNS}) in yeast also involved in the TOR pathway

(Andrzejewska \textit{et al.}, JASN, 2015)
Cystinosin binding partners

v-ATPase

Rag GTPases
- RagA/B
- RagC/D

Ragulator
- p14
- MP1
- HBXIP
- C7orf59

IP GFP
- V0a1
- GFP
- RagC
- RagA
- p18

Lysates
- V0a1
- GFP
- RagC
- RagA
- p18

IP RagC
- V0a1
- GFP
- RagC
- RagA
- p18

Lysates
- V0a1
- GFP
- RagC
- RagA
- p18
Interaction networks for mutants of cystinosin

Mutation observed in infantile cystinosis
Mutations observed in juvenile, ocular or atypical cystinosis

Role of the 5th inter-TM loop +++

- N288K
- K280R
- N323K

Mutation observed in infantile cystinosis
Mutations observed in juvenile, ocular or atypical cystinosis
mTORC1 signaling complex

growth factors
- glucose
- oxygen levels
- energy levels
- amino acids

Growth promoting programs:
- eIF4E
- 4E-BP1
- S6K1
- mTOR
- DEPTOR
- RAPTOR
- RAPTOR
- PRAS40
- TTI1
- TEL2
- SREBP1/2
- ULK1
- ATG13
- FIP200
- TFEB
- eIF4E

Energy metabolism:
- ATP
- eIF4E

Lysosomal biogenesis:
- TFEB

Autophagy:
- ULK1
- ATG13
- FIP200

mTORC1 signaling complex is inhibited by Rapamycin.

Upstream signals:
- TSC1
- TSC2

Rheb
- GTP
- TSC1
- TSC2
Amino acid-dependent activation of mTORC1 pathway

Zoncu et al. 2011
Bar-Peled et al. 2012
Characterization of mouse proximal tubular cell lines

Activity of brush border enzymes

γ-glutamyl transferase

33°C

39°C

alkaline phosphatase

Expression of markers of polarized epithelia

Ctns \(^{+/+}\)

Ctns \(^{-/-}\)

ZO-1

β-catenin
Cellular repartition of mTOR and Lamp-1 in response to nutrients

Defective mTOR relocalization in Ctns−/− cells correlates with impaired downstream signaling.

Lack of cystinosin alters mTOR localization.

Cystinosin essential for mTOR regulation by nutrients in MPT cells.

Defective mTOR relocalization in Ctns−/− cells correlates with impaired downstream signaling.
Rescue of mTOR signaling by RagA Q66L

Cystinosin acts upstream of Rags

RagA Q66L - dominant active mutant mimicking GTP-bound state of RagA
Dysregulation of mTOR signaling in Ctns<sup>−/−</sup> cells due to the absence of cystinosin and not the lysosomal cystine accumulation

No effect of cysteamine on mTOR signaling in Ctns<sup>−/−</sup> cells
Conclusions (I)

- Dual role of cystinosin
  - lysosomal cystine/proton symporter
  - part of the nutrient-sensing machinery involved in mTORC1 signaling – aminoacid sensor for the mTOR pathway?

- Mechanism for the development of Fanconi syndrome
  - mTOR-vATPase controls megalin expression in Drosophila epithelial cells and PTC in mouse (Gleixner et al., 2014)
  - Gradual loss of cubilin and megalin in Ctns-/ PT (Gaide Chevronnay et al., 2014)

- Low molecular weight proteinuria
Conclusions (II)

- Rationale to explain the apparent discrepancies between phenotype-genotype correlations in patients with juvenile phenotype and no cystine transport

- No effect of cysteamine on mTOR signaling: Need for developing new treatments besides lysosomal cystine depletion

- Other lysosomal amino acid transporters involved in the nutrient-sensing machinery [PAT1 (Ögmunsdóttir et al. 2012), SLC38A9 (Wang et al. 2015; Rebsamen et al 2015, Jung et al. 2015), PQLC2 / LAAT-1 (?)] – Is there a cumulative role of the defects?
What’s ongoing

- Analysis of mTORC1 activity and autophagy in cystinotic mice
- Characterization of the mTORC1 pathway in cell lines bearing the N288K vs. K280R, N323K mutations (CRISPR/Cas9 technology)
- Characterization of the strength of the interactions under aminoacid starvation
- Phenotype of the double KO Ctns/Tsc ?

- Search for modifier genes responsible for the absence of renal disease in the FVB background.
Development of an animal model
**Ctns**⁻/⁻ knock-out mice

- Sex ratio = 1
- No embryonic lethality
- Normal development and fertility
- No phenotype in the first months of life

- Widespread cystine accumulation increasing with age
- Ocular, muscular and bone abnormalities
  - Osteoporosis
    - Bone mineralization
    - Cortical width
    - Bone deformity
- Renal phenotype dependant upon the genetic background

(Cherqui et al., 2002; Nevo et al., 2010)
Ctns\(^{-/-}\) knock-out mice: renal phenotype dependent upon genetic background

- Proximal tubulopathy and progressive renal failure in C57BL/6 Ctns\(^{-/-}\) mice
  - Failure to thrive
  - Polyuria (from 2 months) with decreased urinary osmolarity
  - Marked increased CC16 excretion (LMW proteinuria)
  - Increased daily urinary excretion of glucose, phosphate and potassium
  - No hyper aminoaciduria
  - Chronic renal failure from 9 - 10 months
  - Great variability between mice even from the same litter

- No renal symptoms in FVB/N Ctns\(^{-/-}\) mice

(Nevo et al., 2009)
Proximal tubular lesions in kidneys of C57BL/6 Ctns -/- mice

- From 6 months, development of focal lesions of proximal tubules mainly in the superficial cortex
- Atrophy with complete disappearance of the epithelial cell layer and thickening of the BM leading to focal disappearance of proximal tubules
- More extensive lesions at 9-12 months
- No tubular lesions up to 18 months in FVB/N Ctns-/- mice
Multiple crystals followed by PTC atrophy

*, swan-neck atrophy; ➤, cystine crystals; ➣, vacuole bulging