Nitric Oxide Deficiency: potential cause and therapeutic target for SLEs in MELAS

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Professor, Department of Molecular and Human Genetics
Background

- **MELAS:**
  Mitochondrial Encephalomyopathy, Lactic Acidosis, with Stroke-like episodes

- Prevalence: 2:1,000,000 in Japan

- Most common pathogenic variant: m.3243A>G in *MTTL1* that encodes tRNA\(^{\text{Leu(UUR)}}\) and prevalence: 16:100,000 in Finland

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Manifestations</th>
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</thead>
</table>
| ≥90%      | Stroke-like episodes  
Dementia  
Epilepsy  
Lactic acidemia  
Ragged red fibers  
Exercise intolerance |
| 75–89%    | Hemiparesis  
Cortical vision loss  
Recurrent headaches  
Hearing impairment  
Muscle weakness |
| 50–74%    | Peripheral neuropathy  
Learning disability  
Memory impairment  
Recurrent vomiting  
Short stature |
| 25–49%    | Basal ganglia calcification  
Myoclonus  
Ataxia  
Episodic altered consciousness  
Gait disturbance  
Depression  
Anxiety  
Psychotic disorders  
Diabetes |
| <25%      | Optic atrophy  
Pigmentary retinopathy  
Progressive external ophthalmoplegia  
Motor developmental delay  
Cardiomyopathy  
Cardiac conduction abnormalities  
Nephropathy  
Vitiligo |
Mitochondrial proliferation

Ragged blue fibers

COX positive ragged red fibers
Metabolic strokes

- Mitochondrial proliferation also found in smooth muscle and endothelial cells of small blood vessels, seen as vessels surrounded by intense blue on SDH histochemistry
- SSVs (strong SDH-reactive blood vessels)
Pathogenesis of stroke-like episodes

- Mitochondrial proliferation occurring in cells surrounding small blood vessels of brain may result in endothelial dysfunction and decreased nitric oxide (NO) availability

- NO plays an important role regulating tone of smooth muscle surrounding blood vessels

- Altered metabolism of NO may lead to changes in the lining of blood vessels, decreased perfusion and stroke-like episodes (Koga et al, 2005)

Toda and Okamura, 2003; Koga et al., 2005; Koga et al., 2007
Background

- Arginine and citrulline act as NO precursors

IV arginine supplementation has led to clinical improvement if given within 3 h of onset of symptoms

- Hypocitrullinemia reported in MELAS

Effect of arginine supplementation

- 24 subjects with MELAS and stroke-like episodes received intravenous L-arginine (0.5 g/kg/dose) vs. placebo beginning within 3 h of the onset of stroke-like symptoms

- Led to improvement of stroke-like episodes symptoms: headache, weakness, and teichopsia (sensation of luminous appearance before the eyes with a zig-zag, wall-like outline)
Plasma arginine levels in MELAS

- Lower in patients with MELAS when compared to controls

<table>
<thead>
<tr>
<th></th>
<th>L-arginine</th>
<th>Citrulline</th>
<th>NOx</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>108.1 ± 27.6</td>
<td>34.6 ± 8.8</td>
<td>45.4 ± 30.1</td>
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<tr>
<td>MELAS</td>
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<tr>
<td>Interictal phase</td>
<td>83.6 ± 25.8</td>
<td>26.2 ± 9.6</td>
<td>91.4 ± 44.4</td>
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<tr>
<td>P &lt; 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Acute phase</td>
<td>46.6 ± 12.7</td>
<td>23.2 ± 10.2</td>
<td>24.0 ± 9.8</td>
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<td>P &lt; 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>P &lt; 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>P &lt; 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NS&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P &lt; 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
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NOx were lower in patients with MELAS syndrome during stroke-like episodes.
Oral arginine

Subjects with MELAS were treated with oral L-arginine with lack of uniformity in the dose in an unblinded and uncontrolled fashion.

Observation: decrease in frequency and severity of symptoms caused by the stroke

Koga et al., 2007
Citrulline: more effective NO precursor?

- Arginase II: found in small intestine
- Arginine undergoes first pass metabolism in the liver
- ADMA enters cells by CATs
- Arginase may compete with NOS for intracellular arginine
- ASS and ASL co-localize and co-induce with iNOS in various cell types resulting in substrate channeling of citrulline to more efficiently produce NO in certain sub-cellular compartments

Mori and Gotoh, 2000
Hypotheses

- Subjects (adults and children) with MELAS syndrome have impaired nitric oxide (NO) production rate

- Arginine or citrulline supplementation will increase NO production

- Citrulline supplementation will increase NO production to a higher degree than arginine supplementation
Design

- Study 10 adult subjects with MELAS syndrome (m.3243A>G in \textit{MTT1}) and 10 age-matched control subjects

- Age range for MELAS subjects: 18-57 years

- Age range for control subjects: 20-46 years

- Had at least one stroke-like episode

- Clinically stable at time of study
Design

- Measured:
  - m.3243 A>G heteroplasmy in blood/urine
  - Arginine, citrulline, lactate, ADMA, and nitric oxide metabolites (NO₂ and NO₃, NOx) concentrations
  - Arginine and citrulline flux and clearance, de novo arginine synthesis, and NO production rates via stable isotope infusion technique
Sample Analyses

- Plasma arginine and citrulline isotopic enrichments were measured by LC-MS/MS after conversion to their DANS derivatives.

- Isotopic enrichment of plasma NO metabolites (nitrite and nitrate; NOx) was determined by negative chemical ionization GC-MS.

- Using cadmium and acetic acid, nitrate was reduced to nitrite which was subsequently converted to its pentafluorobenzyl derivative and extracted with toluene.
At plateau of isotopic enrichments, the flux of a metabolite = (rate of infusion)/(isotopic enrichment)

Lanpher et al., Nat Rev Genet 2006
De novo arginine synthesis = Citrulline-to-arginine flux ($Q_{\text{Cit}} \rightarrow Q_{\text{arg}}$)
Nitric oxide synthesis = Arginine-to-citrulline flux (QArg→Qcit)
NO fractional synthesis rate (FSR)

FSR of nitric oxide (%/hr) = \( \frac{\text{IENO}_{x+6} - \text{IENO}_{+3}}{\text{IEArgPl}} \times \frac{100}{t6-t3} \)

ASR of nitric oxide (\( \mu \text{mol/L/h} \)) = FSR \times \text{NOx conc.}

Lanpher et al., Nat Rev Genet 2006
Design

Subjects with MELAS first admission

Day 1 | Day 2 | Day 3 | Day 4 | Day 5

Day 1 | 48 hours | 1st isotope infusion | 48 hours | Oral arginine supplementation | 2nd isotope infusion

Controlled diet

Subjects with MELAS second admission

Day 1 | Day 2 | Day 3 | Day 4 | Day 5

Day 1 | 48 hours | 1st isotope infusion | 48 hours | Oral citrulline supplementation | 2nd isotope infusion

Controlled diet

Control subjects

Day 1 | Day 2 | Day 3

Day 1 | 48 hours | 1st isotope infusion

Controlled diet
Design

Infusion protocol

Blood sampling

Isotope infusion

Loading
15N2-arginine
13C-, 2H4-citrulline
15N-citrulline

Continuous
15N2-arginine
13C-, 2H4-citrulline

0 hr 1 h 2 h 3 h 4 h 5 h 5.56 h
### Results

<table>
<thead>
<tr>
<th></th>
<th>Age (year)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>BMI</th>
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<tbody>
<tr>
<td><strong>Control</strong> (n=10)</td>
<td>29.7 ±9.6 (20 - 46)</td>
<td>68.0 ±11.9 (57.4 - 82.9)</td>
<td>172.8 ±9.0 (160.7 - 187.9)</td>
<td>22.6 ±1.9 (20.7 - 25.4)</td>
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<tr>
<td><strong>MELAS</strong> (n=10)</td>
<td>21.8 ±11.8 (18 - 57)</td>
<td>56.3 ±10.1 (44.5 - 72.1)</td>
<td>162.0 ±8.6 (149.7 - 170.9)</td>
<td>21.4 ±3 (18.0 - 27.4)</td>
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<tr>
<td>P value</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>NS</td>
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</table>
# Subjects with MELAS vs controls

<table>
<thead>
<tr>
<th>Plasma concentrations</th>
<th>Control subjects</th>
<th>Subjects with MELAS</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Arginine ($\mu$mol · L$^{-1}$)</td>
<td>77.8 ±4.4</td>
<td>57.2 ±3.2</td>
<td>&lt;0.005</td>
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<tr>
<td>Citrulline ($\mu$mol · L$^{-1}$)</td>
<td>28.1 ±1.1</td>
<td>22.6 ±1.7</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>NOx ($\mu$mol · L$^{-1}$)</td>
<td>17.3 ±1.4</td>
<td>15.3 ±1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>ADMA ($\mu$mol · L$^{-1}$)</td>
<td>0.38 ±0.02</td>
<td>0.54 ±0.04</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

| Arginine and citrulline flux and clearance | | |
|-------------------------------------------|--------|-----------------|--------|
| Arginine flux ($\mu$mol · kg$^{-1}$ · h$^{-1}$) | 56.4 ±3.0 | 49.8 ±2.9 | 0.1 |
| Arginine clearance (ml · kg$^{-1}$ · min$^{-1}$) | 13.4 ±0.5 | 16.2 ±1.0 | <0.05 |
| Citrulline flux ($\mu$mol · kg$^{-1}$ · h$^{-1}$) | 8.2 ±0.6 | 5.7 ±0.4 | <0.005 |
| Citrulline clearance (ml · kg$^{-1}$ · min$^{-1}$) | 5.6 ±0.4 | 5.0 ±0.4 | 0.3 |

| De novo arginine | | |
|------------------|--------|-----------------|--------|
| De novo arginine synthesis rate ($\mu$mol · kg$^{-1}$ · h$^{-1}$) | 5.5 ±0.6 | 3.5 ±0.3 | <0.005 |
| Ratio of de novo arginine synthesis to arginine flux (%) | 9.9 ±1.0 | 7.1 ±0.6 | <0.05 |

| NO synthesis rates | | |
|--------------------|--------|-----------------|--------|
| Arginine-to-citrulline flux ($\mu$mol · kg$^{-1}$ · h$^{-1}$) | 0.100 ±0.013 | 0.067 ±0.008 | <0.05 |
| ASR of NOx ($\mu$mol · L plasma$^{-1}$ · h$^{-1}$) | 0.49 ±0.03 | 0.35 ±0.05 | <0.01 |
Discussion

- Lower NO production in subjects with MELAS syndrome may be due to:
  - Increased ADMA concentration (NOS inhibitor)
  - Increased arginine clearance results in lower plasma arginine
  - ↓ citrulline flux (de novo citrulline synthesis) → ↓ citrulline availability → ↓ de novo arginine synthesis → ↓ intracellular arginine availability.
Effect of arginine and citrulline supplementation

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<tr>
<th></th>
<th>L-arginine supplementation</th>
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<th>L-citrulline supplementation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>P value</td>
<td>Before</td>
</tr>
<tr>
<td>Arginine</td>
<td>61.1 ±3.3</td>
<td>143.8 ±8.1</td>
<td>&lt;0.001</td>
<td>57.5 ±2.5</td>
</tr>
<tr>
<td>Citrulline</td>
<td>20.3 ±1.3</td>
<td>25.4 ±2.4</td>
<td>&lt;0.05</td>
<td>21.9 ±2.0</td>
</tr>
<tr>
<td>NOx</td>
<td>16.8 ±1.7</td>
<td>18.8 ±1.9</td>
<td>0.07</td>
<td>17.2 ±2.1</td>
</tr>
<tr>
<td>ADMA</td>
<td>0.60 ±0.04</td>
<td>0.58 ±0.03</td>
<td>0.63</td>
<td>0.57 ±0.03</td>
</tr>
</tbody>
</table>
Arginine and to a greater extent citrulline supplementation increased fluxes of arginine and citrulline.
De novo arginine synthesis increased markedly with citrulline supplementation, superior efficacy of citrulline in increasing NO production.

De novo arginine synthesis: more important role in NO synthesis than plasma arginine.
Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation

Ayman W El-Hattab, Jean W Hsu, Lisa T Emrick, Lee-Jun C Wong, William J Craigen, Farook Jahooor, Fernando Scaglia

Affiliations  + expand

PMID: 22325939  PMCID: PMC4093801  DOI: 10.1016/j.ymgme.2012.01.016

Free PMC article
Effect of arginine and citrulline supplementation on plasma alanine concentration

<table>
<thead>
<tr>
<th></th>
<th>Mean ±SEM</th>
<th>P value</th>
<th>Reduction %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine before arginine (µmol/L)</td>
<td>532 ± 23</td>
<td>&lt;0.05</td>
<td>7%</td>
</tr>
<tr>
<td>Alanine after arginine (µmol/L)</td>
<td>496 ± 26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine before citrulline (µmol/L)</td>
<td>536 ± 19</td>
<td>&lt;0.001</td>
<td>19%</td>
</tr>
<tr>
<td>Alanine after citrulline (µmol/L)</td>
<td>434 ± 28</td>
<td></td>
<td></td>
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</tbody>
</table>
Pediatric study

- Study 5 children with MELAS syndrome (m.3243A>G in MTTL1, with at least one metabolic stroke and clinically stable) and 5 age matched pediatric subjects to assess NO production rate and arginine and citrulline fluxes.
- Children with MELAS admitted twice:
  - First admission, stable isotope infusion was performed at baseline and after 48 hours of oral arginine supplementation.
  - Second admission, study was performed at baseline then after 48 hours of citrulline supplementation.
Stable isotopes

• Isotope infusion:
  - A bolus containing $^{15}$N$_2$-arginine (5 µmol/kg), $^{13}$C,$^2$H$_4$-citrulline (1 µmol/kg), and $^{15}$N-citrulline (0.16 µmol/kg)
  - Followed by a continuous infusion of $^{15}$N$_2$-arginine (5 µmol/kg/h) and $^{13}$C,$^2$H$_4$-citrulline (1 µmol/kg/h) for 6 h

• Blood samples were drawn before and hourly during the stable isotope infusion
# Results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control children ($n = 5$)</th>
<th>Children with MELAS ($n = 5$)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>12.1 ± 3.6</td>
<td>9.4 ± 4.8</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>2/3</td>
<td>4/1</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>44.8 ± 16.0</td>
<td>20.5 ± 7.3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>153.5 ± 22.3</td>
<td>123.9 ± 23.5</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>18.3 ± 2.0</td>
<td>13.1 ± 1.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

NS: not statistically significant.

El-Hattab et al, 2016
Age differences in flux values

- Higher flux values in children are associated with higher metabolism rate during childhood comparing to adulthood. Therefore, it is extremely important not to use adults as control when studying metabolic flux in children.

<table>
<thead>
<tr>
<th></th>
<th>Adult Controls</th>
<th>Pediatric Controls</th>
<th>Adults with MELAS</th>
<th>Children with MELAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO production µmol/kg/h</td>
<td>0.10 ± 0.01</td>
<td>0.19 ± 0.03</td>
<td>0.07 ± 0.01</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>Arginine flux µmol/kg/h</td>
<td>56 ± 3</td>
<td>159 ± 12</td>
<td>50 ± 3</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>Citrulline flux µmol/kg/h</td>
<td>8.2± 0.6</td>
<td>9.1 ± 0.4</td>
<td>5.7 ± 0.4</td>
<td>8.0 ± 0.3</td>
</tr>
</tbody>
</table>

Lower NO production rate in children with MELAS

El-Hattab et al, 2016
Lower NO production rate in children with MELAS was associated with lower fluxes of arginine and citrulline

El-Hattab et al, 2016
Both citrulline and arginine supplementation resulted in increased NO production rate in pediatric subjects

El-Hattab et al, 2016
Accompanied with increments in both arginine and citrulline flux with arginine and citrulline supplementation.

El-Hattab et al, 2016
Plasma arginine concentration

![Bar graph showing arginine concentration in control and MELAS groups with a statistically significant difference (P<0.001).](image)

El-Hattab et al, 2016
Greater ability of citrulline supplementation to increase arginine and citrulline concentration

Greater ability of citrulline to increase intracellular arginine pool by increasing *de novo* arginine synthesis rate
Increased NO production is driven by increased availability of arginine

El Hattab et al, 2016
Impaired nitric oxide production in children with MELAS syndrome and the effect of arginine and citrulline supplementation

Ayman W El-Hattab, Lisa T Emrick, Jean W Hsu, Sirisak Chanprasert, Mohammed Almannai, William J Craigen, Farook Jahoor, Fernando Scaglia

Affiliations † expand

PMID: 26851065  PMCID: PMC4818739  DOI: 10.1016/j.ymgme.2016.01.010
NIH funded phase 1 clinical trial

- Inclusion criteria:
  - 1. Clinical diagnosis of MELAS (neurological or muscular symptoms: stroke-like events, seizures, exercise intolerance, fatigue, or any combination of these)
  - 2. Subject must be aged 18 to 65 years and with m.3243A>G pathogenic variant in MT-TL1 gene
  - 3. Elevated plasma lactate (>2.2 mmol/L)
  - 4. Negative urine pregnancy test, if applicable
  - 5. Score of 26 or higher on the Montreal Cognitive Assessment
  - 6. Cessation of arginine, citrulline or compounds affect NO one week prior to study
NIH funded phase 1 clinical trial

- Primary aim: establish MTD of L-citrulline in 14 adult subjects with MELAS at one of following doses: 10 g/day, 20 g/day, 30 g/day, or 40 g/day (four times a day)

- Primary safety outcomes/dose limiting toxicities associated with NO production: orthostatic hypotension, syncope, hypoglycemia, headaches
Phase 1 clinical trial

- Secondary aims of study are:
  - Determine cerebral blood flow and cerebral blood vessel reactivity using arterial spin-labeling (ASL) MRI
  - Measure plasma amino acids at baseline, week one and week four to determine citrulline, arginine, alanine, and ornithine levels
  - Measure plasma lactate at baseline, week one and week four
  - Measure plasma guanidino compounds by untargeted metabolomics analysis at baseline and week 1 to determine potential arginine toxicity
ASL-MRI

- ASL brain MRI at CAMRI (BCM) with breath holding task following visual cues will be performed to measure end tidal CO2 (measured with a MR-compatible capnography system: Biopac system)

- An echo-planar functional MRI scan is used to measure hemodynamic response to hypercapnia-induced cerebral vascular reactivity (CVR)

- CVR will be assessed by comparing hemodynamic (BOLD = blood oxygen level dependent) response to CO2

- Maps for cerebrovascular reactivity have been calculated
Study design

- Time-To-Event Continual Reassessment Method design to establish MTD of L-citrulline in 14 adult subjects with MELAS
- Received dosage will be determined by TITE-CRM algorithm
- Study subjects will be enrolled one at a time serially.
- First subject will receive L-citrulline dosage of 10 g/day for up to one month or until a safety outcome is reached.
Study design

- Dosage for each subsequent subject enrolled will either be increased or decreased based on determination of TITE-CRM algorithm.

- TITE-CRM algorithm takes into account rate of safety outcomes encountered by previously enrolled subjects to determine dosage for subsequent subjects.

- After a subject has received study medication for 1 month, or has encountered a safety outcome, administration of drug will cease.

- Enrollment and administration of L-citrulline will continue in this manner until a MTD is established.
## Follow-up and assessments

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4 (Final)</th>
<th>Follow-up*</th>
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</thead>
<tbody>
<tr>
<td>Blood pressure measurement</td>
<td>X</td>
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<td>ASL MRI to measure cerebral</td>
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<td>blood flow</td>
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<td>Plasma and urine guanidino</td>
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<td>compounds</td>
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<td>plasma alanine a</td>
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<td>Plasma lactate</td>
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<td>Blood glucose</td>
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<td>Comprehensive metabolic panel</td>
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<td>Complete blood count with</td>
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<td>Renal function tests (blood</td>
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<td>Seizure diary collection</td>
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<td>Blood sugar diary collection</td>
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<td>Blood pressure diary collection</td>
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<td>Headache calendar collection</td>
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<td>GI diary collection</td>
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<td>Medication pill count</td>
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<td>(compliance measure)</td>
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<td>Neurologic assessment</td>
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<tr>
<td>Monitoring Follow Up Call</td>
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</table>

*Follow-up visits to take place at 2 weeks and at 1 month post cessation of study medication (Week 6 and week 8)

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* Following a baseline plasma amino acids sample collection (Time 0), study subject will be given the indicated dose of L-citrulline and then repeated plasma amino acid samples will be drawn at 0.5, 1, 2, and 6 hours.

b eGFR will be calculated based on the MDRD equation (GFR (mL/min/1.73 m²) = 175 × (Scr)^{-1.154} × (Age)^{-0.203} × (0.742 if female) × (1.212 if African American)
Regulatory Process

- FDA: IND 132117
- NIH approval
- Protocol initially approved by BCM IRB
- Protocol approved by Office of Clinical Research at Baylor (feasibility study)
- Protocol approved by CRC Committee at BSLMC
- Trial is listed in ClinicalTrials.gov with an NCT number 03952234
- Data Monitoring Coordinating Center: eCRFs
- Data Safety Monitoring Board in place and Independent Medical Monitor
Interim Report

- Trial is proceeding under NIH and IRB approved protocol, with full engagement and watchfulness from clinical and statistical teams and the independent medical monitor, and above all under the guidance of DSMB

- Weekly meetings with DMCC, NAMDC statistical team, and BCM team

- Last DSMB meeting was held on March 9th, allowed to continue

- Annual review to FDA was submitted on April 4th and FDA review team completed the review of protocol amendment on May 31st with no comments

- Ten participants have been screened, enrolled, and finished the study

- Process of screening additional participants
Conclusions

- First study that provided an *in vivo* whole-body assessment of NO metabolism in adult and pediatric subjects with MELAS

- Use of well-established stable isotope infusion protocols determined lower NO production in MELAS and citrulline’s superior efficacy in restoring NO production

- Citrulline may have a better therapeutic effect than arginine leading to an ongoing phase 1 clinical trial to assess safety of citrulline (MTD)
Acknowledgements

Patients and their families

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NINDS/NICHD: 5U54NS078059-08
Thanks

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