Baylor College of Medicine

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

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NAMDC
North American Mitochondrial Disease Consortium

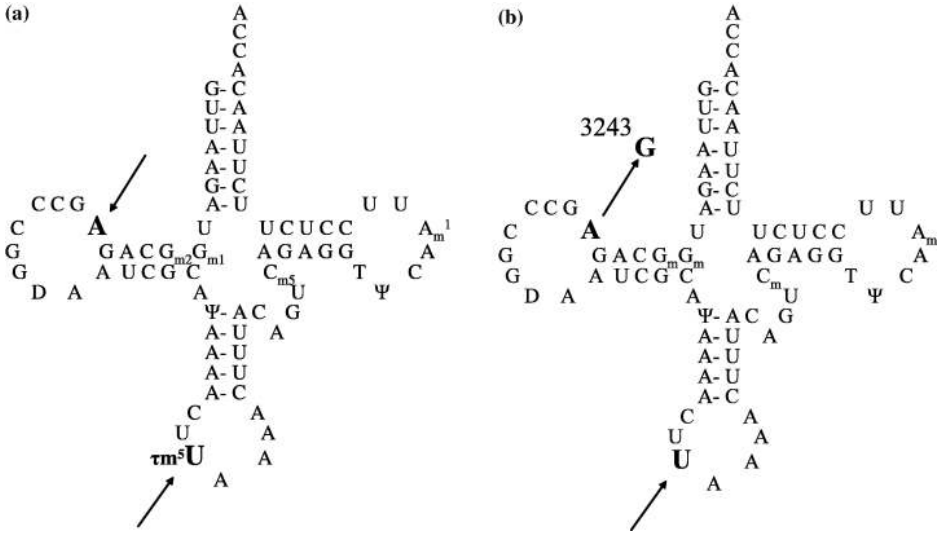
RARE DISEASES
CLINICAL RESEARCH NETWORK

Texas Children's Hospital

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 $tRNA^{Leu(UUR)}$ and prevalence: 16:100,000 in Finland



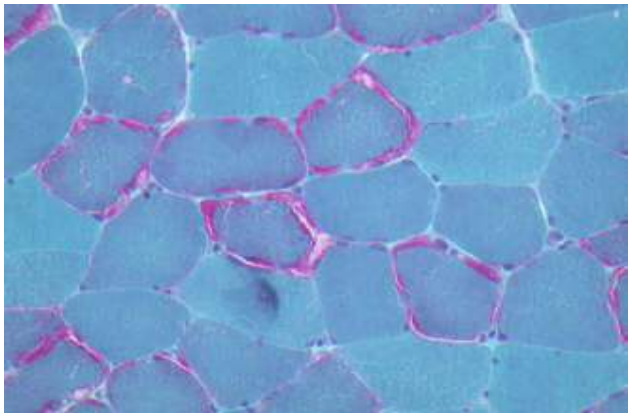
Pavlakis et al., Ann Neurol 1984; Hirano et al., J Child Neurol 1994; Majamaa et al., Neurology 1997; Chinnery et al., Am J Med Genet 2001

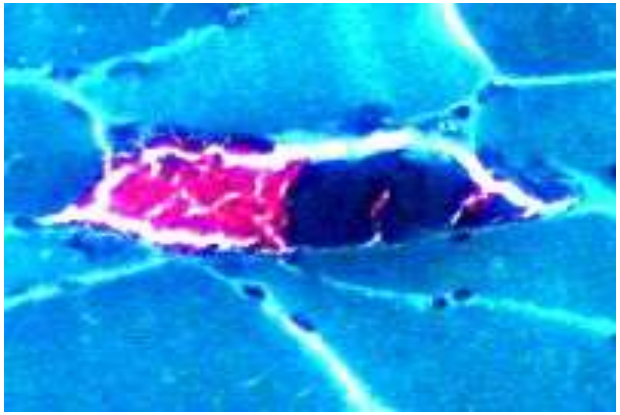
Overall manifestations of MELAS syndrome.

Frequency	Manifestations
≥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

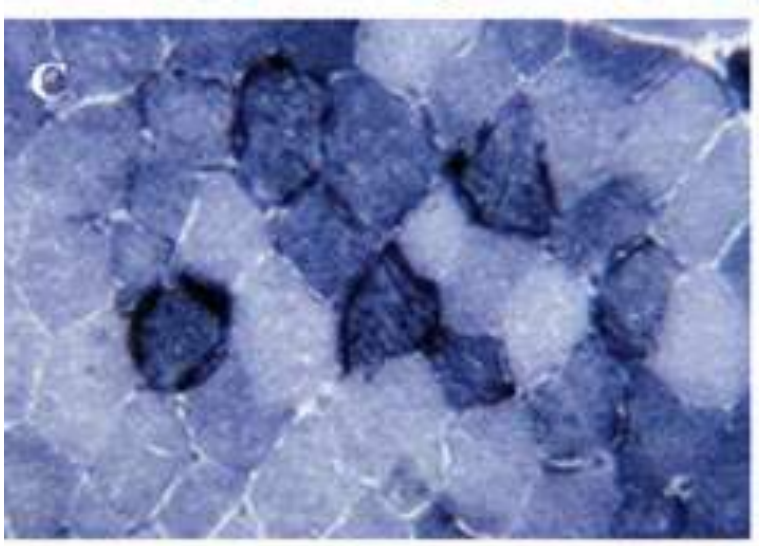
El-Hattab AW, Adesina AM, Jones J, Scaglia F. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. Mol Genet Metab. 2015 Sep-Oct;116(1-2):4-12.

Mitochondrial proliferation



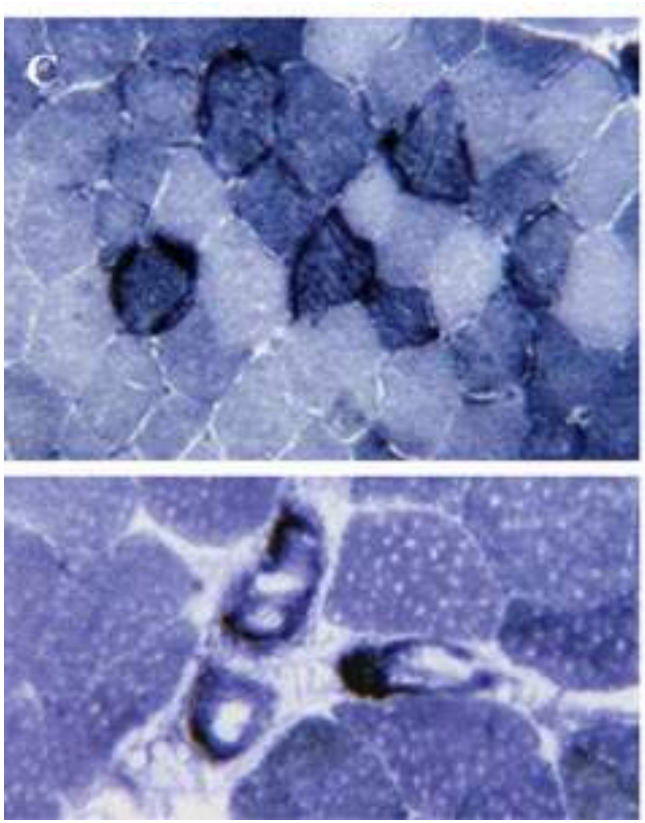


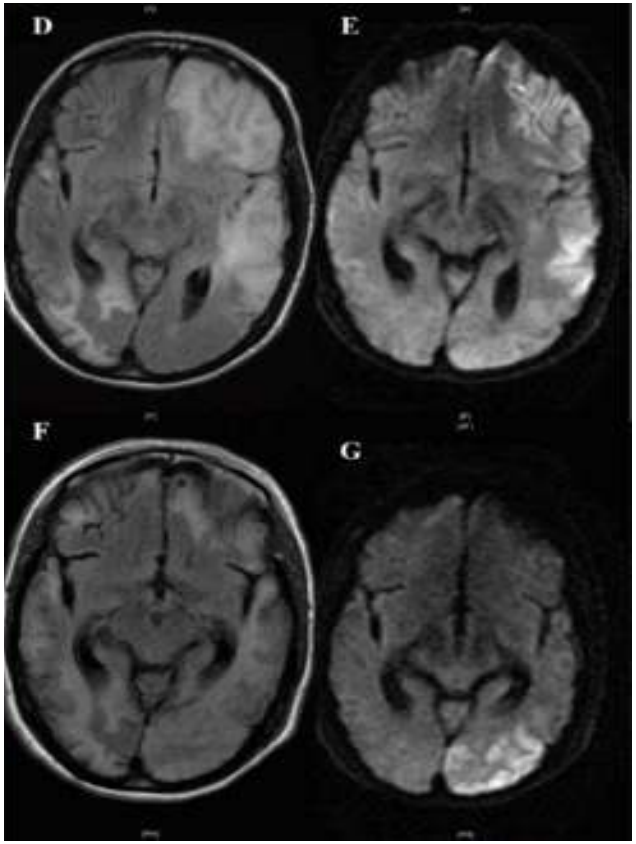
COX positive ragged red fibers



Ragged blue 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)

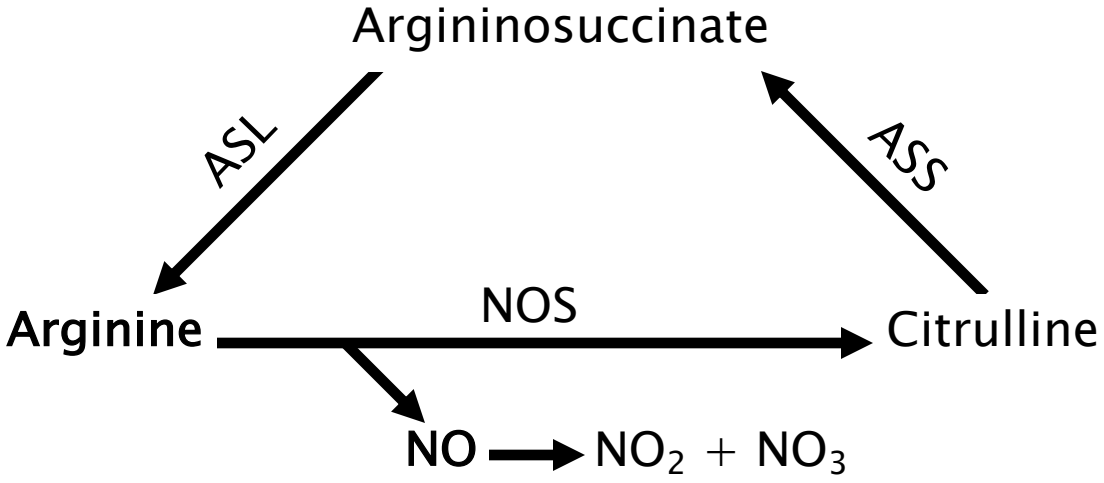
Pathogenesis of stroke-like episodes

- 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

Naini et al. J Neurol Sci, 2005 Koga et al., Neurology 2005; Koga et al., Mitochondrion 2007

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)

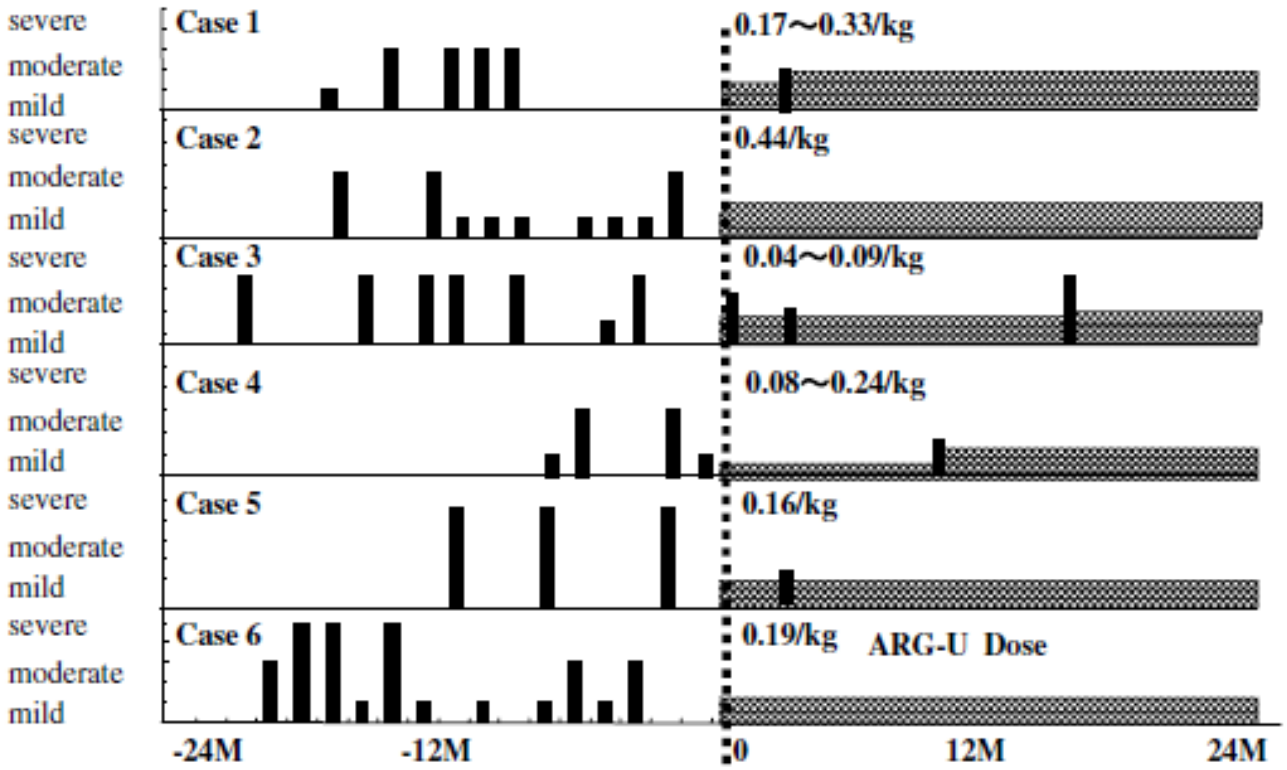
Plasma arginine levels in MELAS

- Lower in patients with MELAS when compared to controls

	L-arginine	Citrulline	NO_x
Control	108.1 ± 27.6	34.6 ± 8.8	45.4 ± 30.1
MELAS			
Interictal phase	83.6 ± 25.8	26.2 ± 9.6	91.4 ± 44.4
	$P < 0.01$[b]	$P < 0.01$[b]	$P < 0.01$[b]
Acute phase	46.6 ± 12.7	23.2 ± 10.2	24.0 ± 9.8
	$P < 0.01$[b]	$P < 0.01$[b]	$P < 0.01$[b]
	$P < 0.01$[c]	NS[c]	$P < 0.01$[c]

NOx were lower in patients with MELAS syndrome during stroke-like episodes

Oral arginine

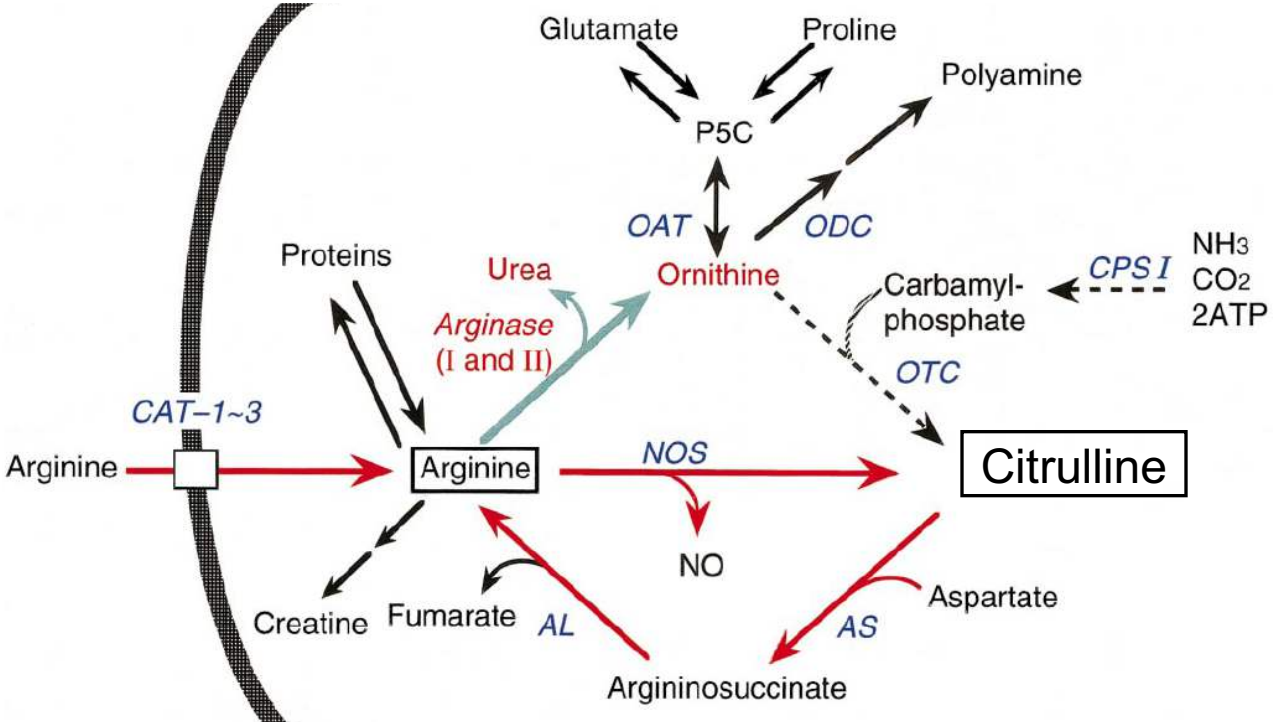


Subjects with MELAS were treated with oral L-arginine with lack of uniformity in the dose in an unblinded and uncontrolled fashion

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

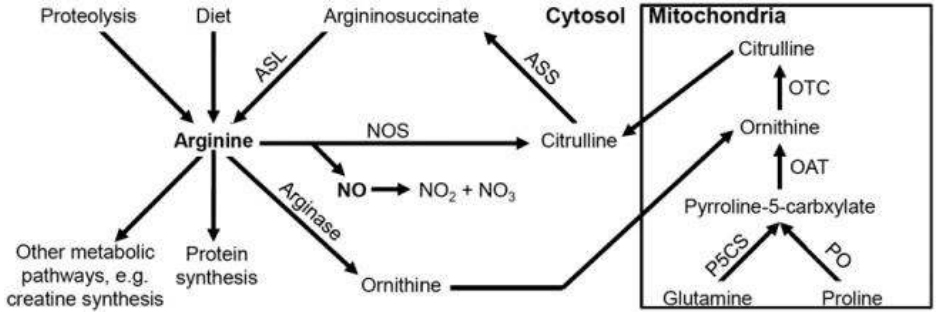
Mori and Gotoh, 2000

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 *MTTL1*) 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 (NO_2 and NO_3, NOx) concentrations
  - Arginine and citrulline flux and clearance, *de novo* arginine synthesis, and NO production rates via stable isotope infusion technique

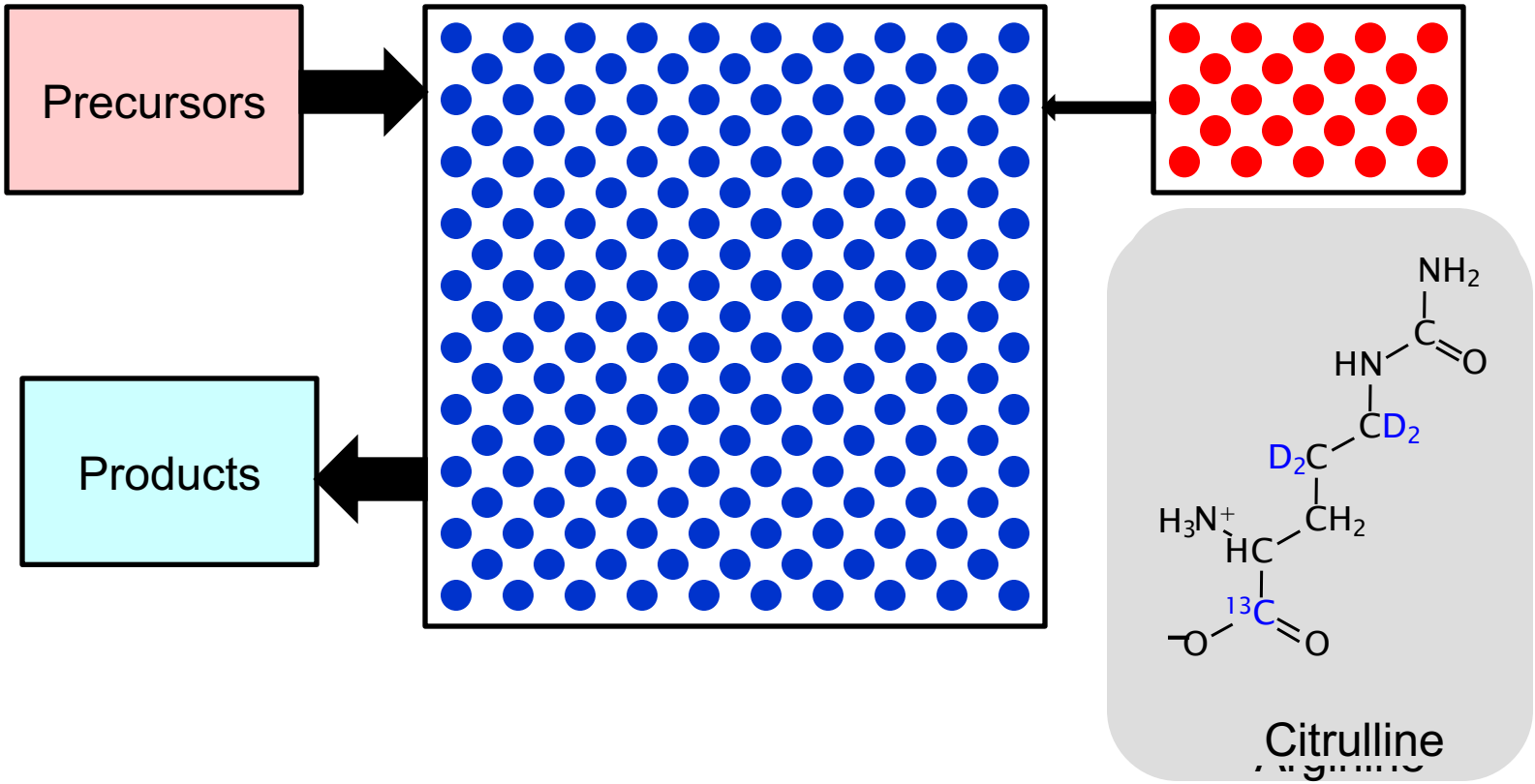
Sample Analyses

- 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

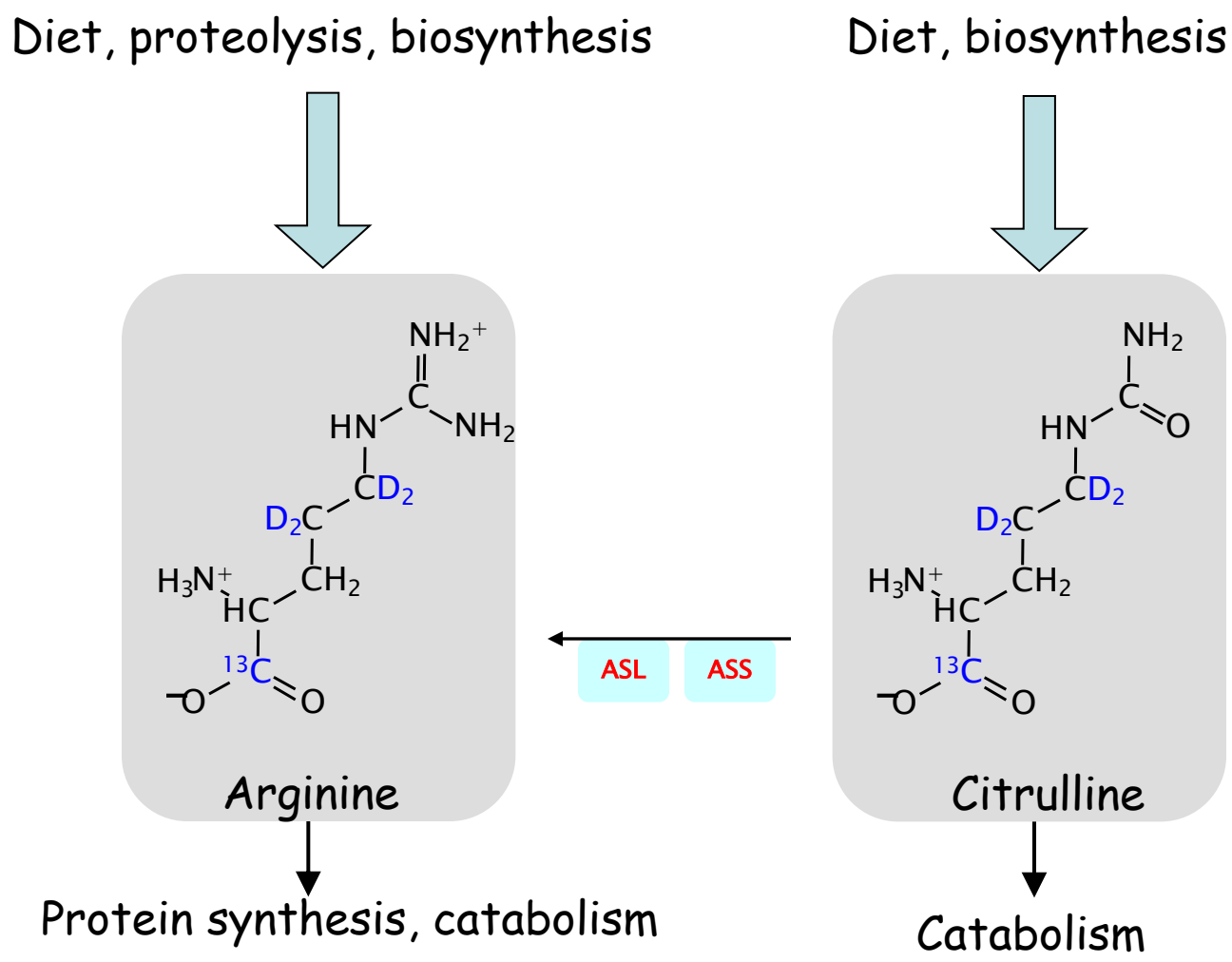
- Using cadmium and acetic acid, nitrate was reduced to nitrite which was subsequently converted to its pentafluorobenzyl derivative and extracted with toluene

Stable isotope infusion technique

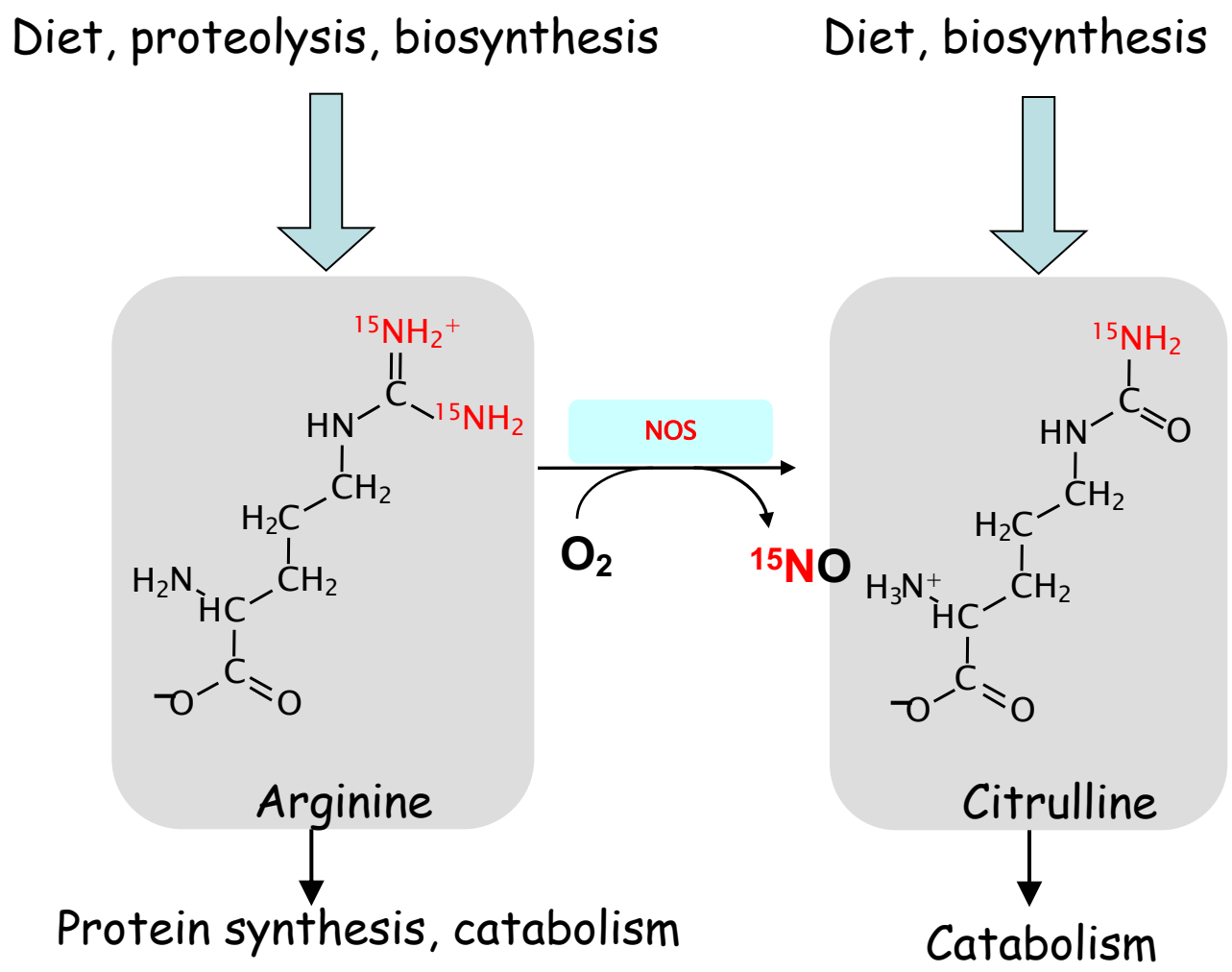


- At plateau of isotopic enrichments, the flux of a metabolite = (rate of infusion)/(isotopic enrichment)

Lanpher et al., Nat Rev Genet 2006



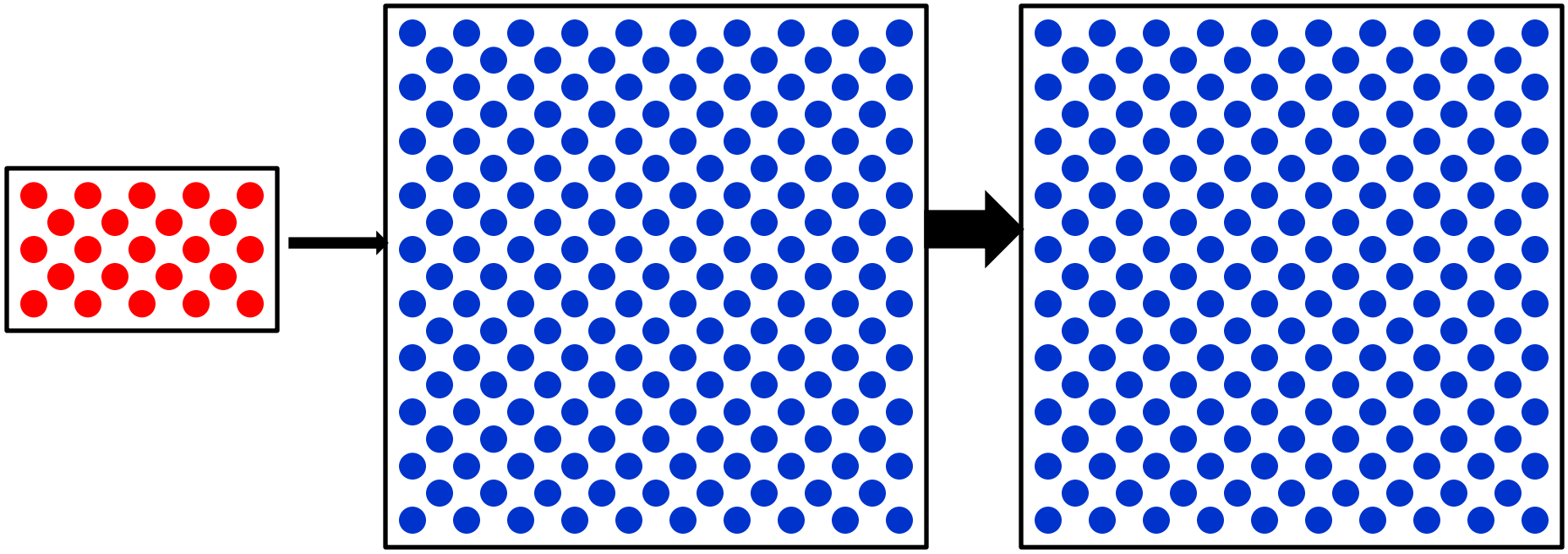
De novo arginine synthesis =
Citrulline-to-arginine flux (QCit→Qarg)



Nitric oxide synthesis =

Arginine-to-citrulline flux (QArg→Qcit)

NO fractional synthesis rate (FSR)



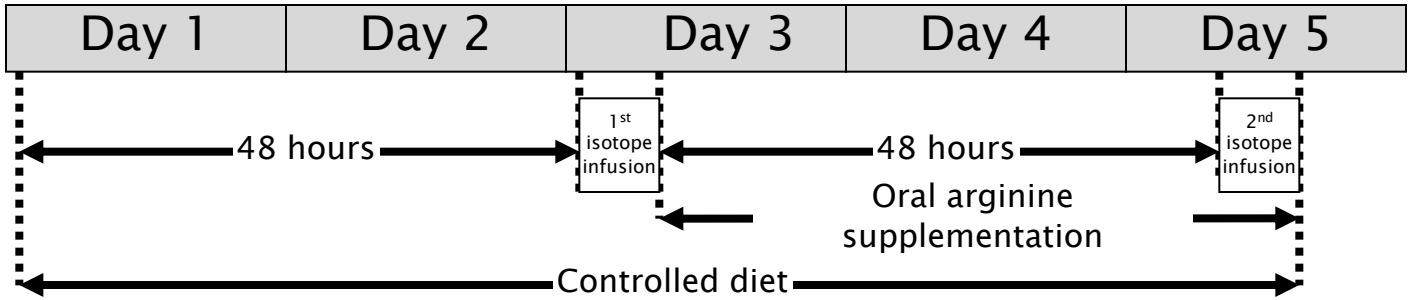
$$\text{FSR of nitric oxide (\%/hr)} = \frac{\text{IENOxt6 - IENOt3}}{\text{IEArgPl}} \times \frac{100}{\text{t6-t3}}$$

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

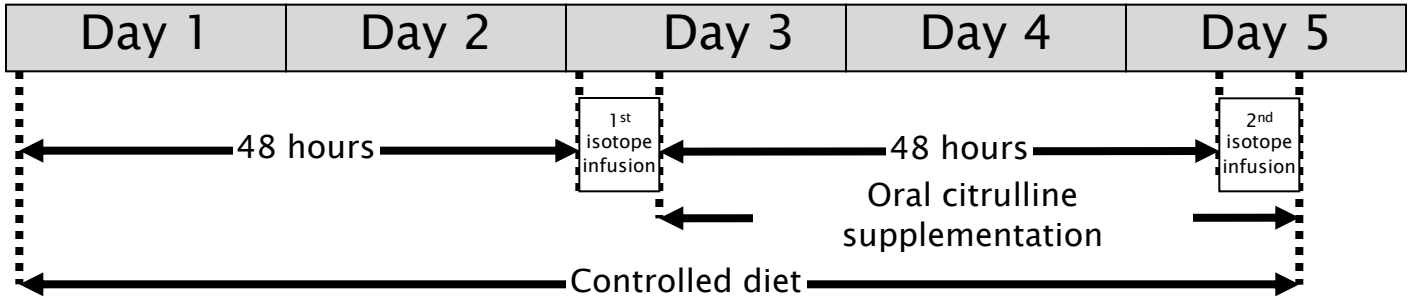
Lanpher et al., Nat Rev Genet 2006

Design

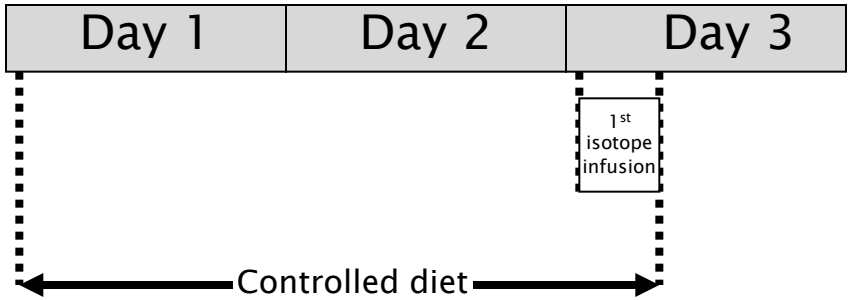
Subjects with MELAS first admission



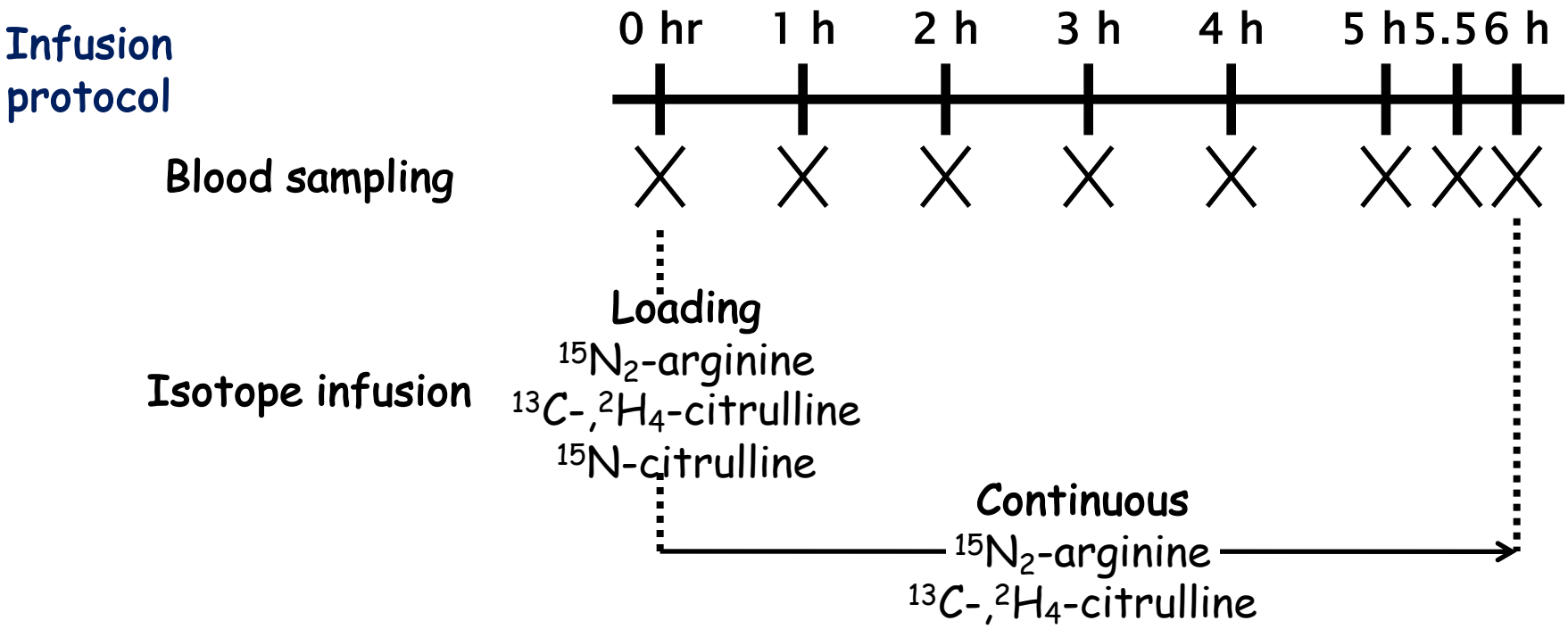
Subjects with MELAS second admission



Control subjects



Design


Infusion protocol
0 hr
1 h
2 h
3 h
4 h
5 h
5.5
6 h
Blood sampling
Isotope infusion
Loading
$^{15}N_2$-arginine
^{13}C-,$^{2}H_4$-citrulline
^{15}N-citrulline
Continuous
$^{15}N_2$-arginine
^{13}C-,$^{2}H_4$-citrulline

Results

	Age (year)	Weight (kg)	Height (cm)	BMI
Control (n=10)	**29.7** ±9.6 (20 – 46)	**68.0** ±11.9 (57.4 – 82.9)	**172.8** ±9.0 (160.7 – 187.9)	**22.6** ±1.9 (20.7 – 25.4)
MELAS (n=10)	**21.8** ±11.8 (18 – 57)	**56.3** ±10.1 (44.5 – 72.1)	**162.0** ±8.6 (149.7 – 170.9)	**21.4** ±3 (18.0 – 27.4)
P value	NS	<0.05	<0.05	NS

Subjects with MELAS vs controls

	Control subjects	Subjects with MELAS	P value
Plasma concentrations			
Arginine (μmol · L^{-1})	77.8 ±4.4	57.2 ±3.2	<0.005
Citrulline (μmol · L^{-1})	28.1 ±1.1	22.6 ±1.7	<0.005
NOx (μmol · L^{-1})	17.3 ±1.4	15.3 ±1.5	0.2
ADMA (μmol · L^{-1})	0.38 ±0.02	0.54 ±0.04	<0.05
Arginine and citrulline flux and clearance			
Arginine flux (µmol · 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 (µmol · 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 (µmol · 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 (µmol · kg^{-1} · h^{-1})	0.100 ±0.013	0.067 ±0.008	<0.05
ASR of NOx (µmol · L $plasma^{-1}$ · h^{-1})	0.49 ±0.03	0.35 ±0.05	<0.01

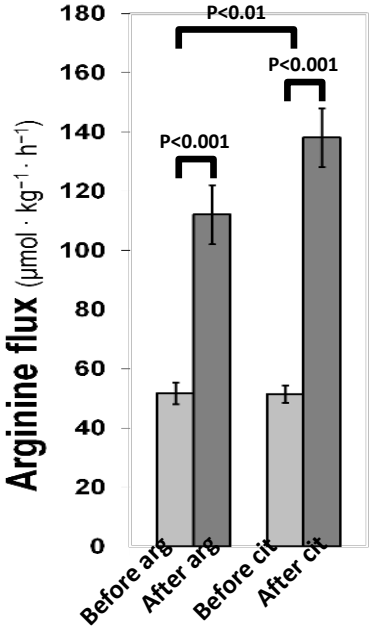
Discussion

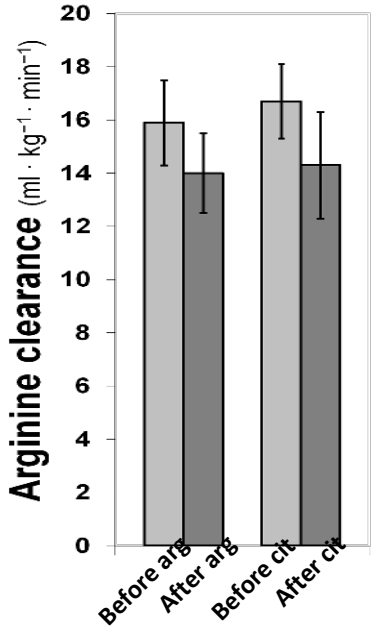
- Lower NO production in subjects with MELAS syndrome may be due to :
  - Increased ADMA concentration (NOS inhibitor)
  - Increased arginine clearance results in lower plasma arginine
  - ↓ citrulline flux (*de novo* citrulline synthesis) → ↓citrulline availability → ↓ *de novo* arginine synthesis →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

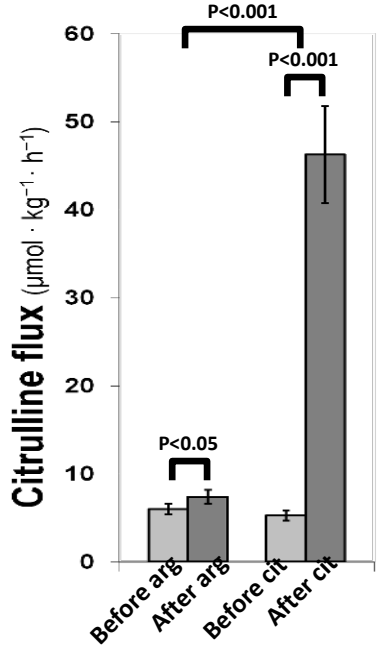
Effect of arginine and citrulline supplementation

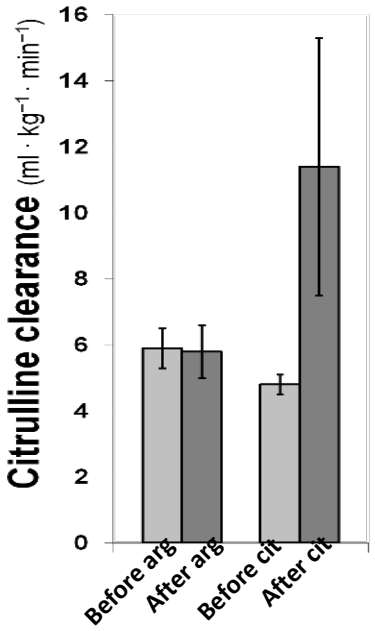
	L-arginine supplementation			L-citrulline supplementation		
	Before	**After**	**P value**	**Before**	**After**	**P value**
Arginine	**61.1** ±3.3	**143.8** ±8.1	**<0.001**	**57.5** ±2.5	**184.5** ±20.8	**<0.001**
Citrulline	**20.3** ±1.3	**25.4** ±2.4	**<0.05**	**21.9** ±2.0	**106.5** ±27.8	**<0.01**
NOx	**16.8** ±1.7	**18.8** ±1.9	0.07	**17.2** ±2.1	**19.9** ±2.1	**<0.05**
ADMA	**0.60** ±0.04	**0.58** ±0.03	0.63	**0.57** ±0.03	**0.61** ±0.03	0.12

Arginine and to a greater extent citrulline supplementation increased fluxes of arginine and citrulline

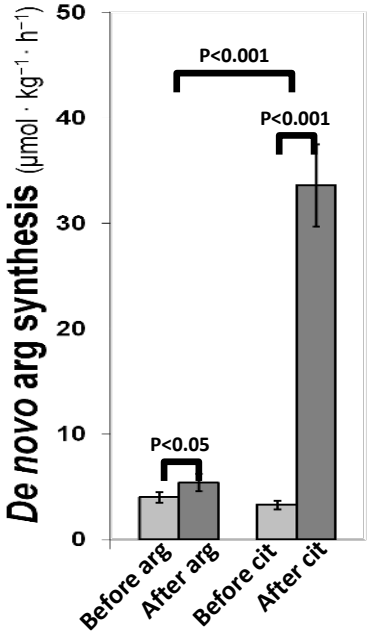


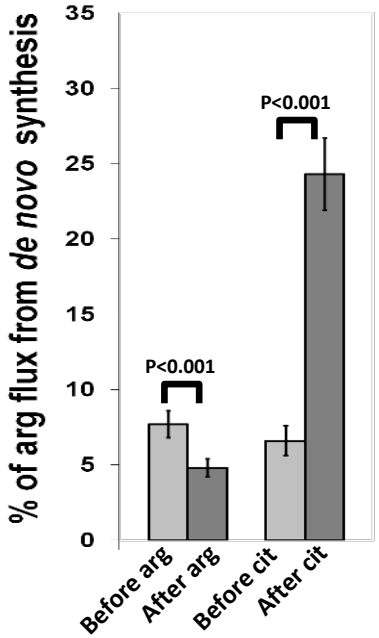


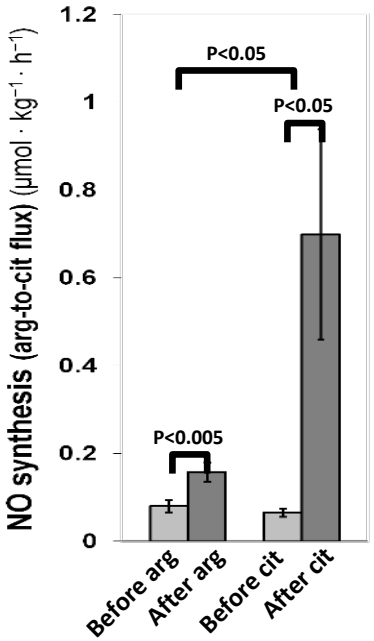


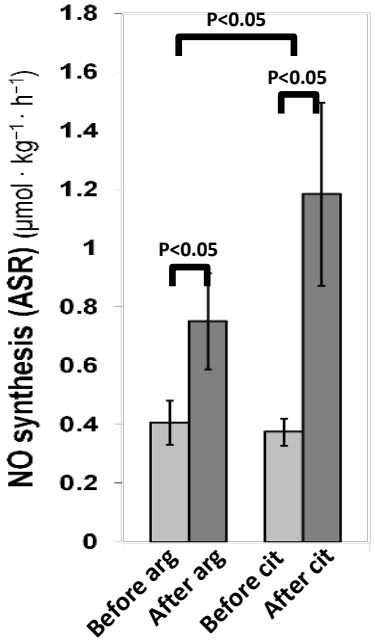


De novo arginine synthesis increased markedly with citrulline supplementation, superior efficacy of citrulline in increasing NO production









De novo arginine synthesis: more important role in NO synthesis than plasma arginine

Comparative Study > Mol Genet Metab. 2012 Apr;105(4):607-14.
doi: 10.1016/j.ymgme.2012.01.016. Epub 2012 Jan 24.

Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation

Ayman W El-Hattab [1], Jean W Hsu, Lisa T Emrick, Lee-Jun C Wong, William J Craigen, Farook Jahoor, 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

	Mean ±SEM	P value	Reduction %
Alanine before arginine (µmol/L)	532 ± 23	<0.05	7%
Alanine after arginine (µmol/L)	496 ± 26		
Alanine before citrulline (µmol/L)	536 ± 19	<0.001	19%
Alanine after citrulline (µmol/L)	434 ± 28		

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 1
Characteristics of the research subjects, presented as mean ± 1SD.

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

NS: not statistically significant.

El-Hattab et al, 2016

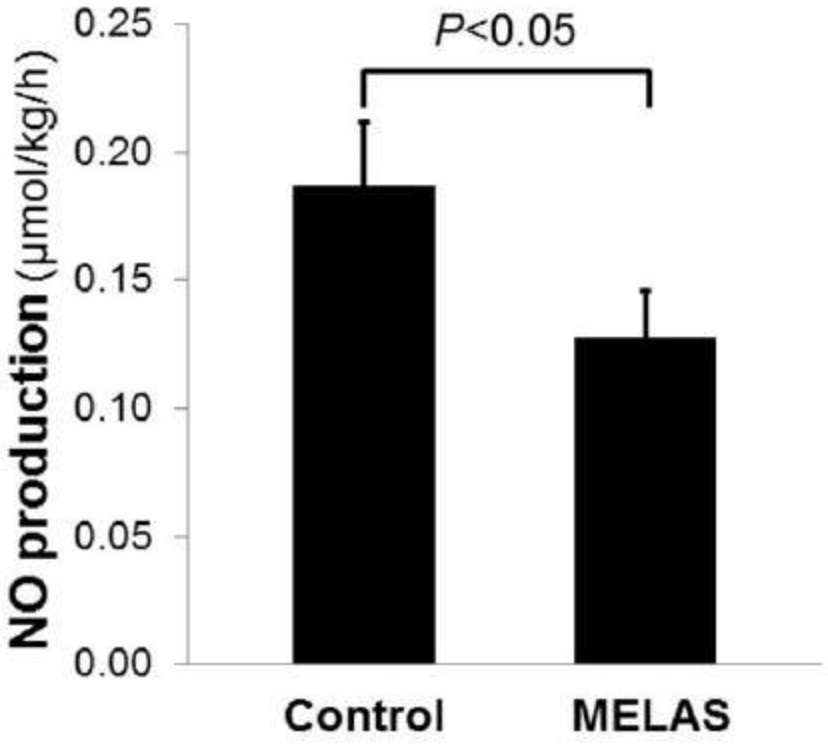
Age differences in flux values

- 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.

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

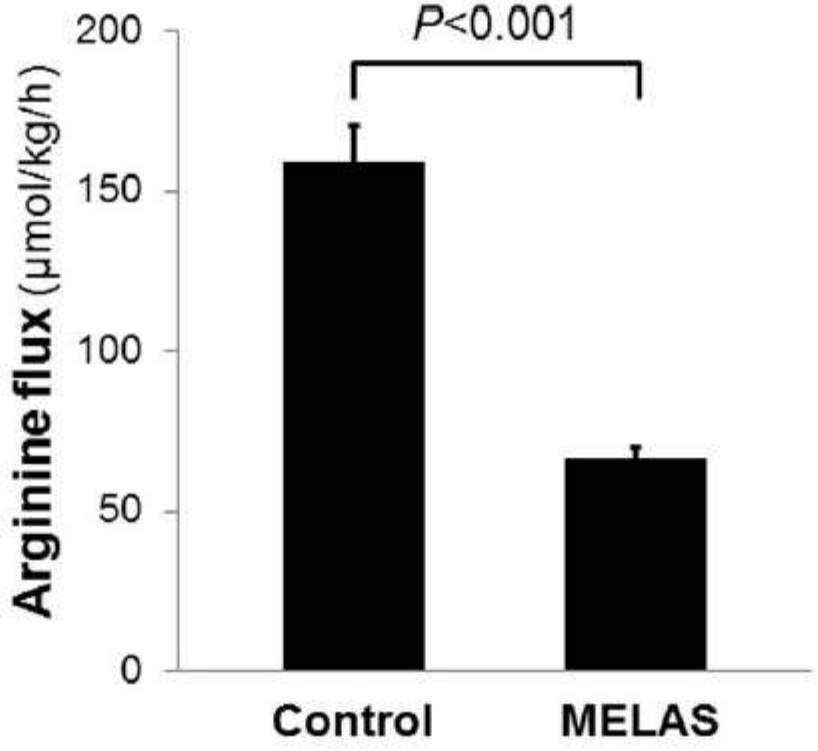
El-Hattab AW, Hsu JW, Emrick LT, Wong LJ, Craigen WJ, Jahoor F, Scaglia F. Restoration of impaired nitric oxide production in MELAS syndrome with citrulline and arginine supplementation. Mol Genet Metab. 2012 Apr;105(4):607-14.

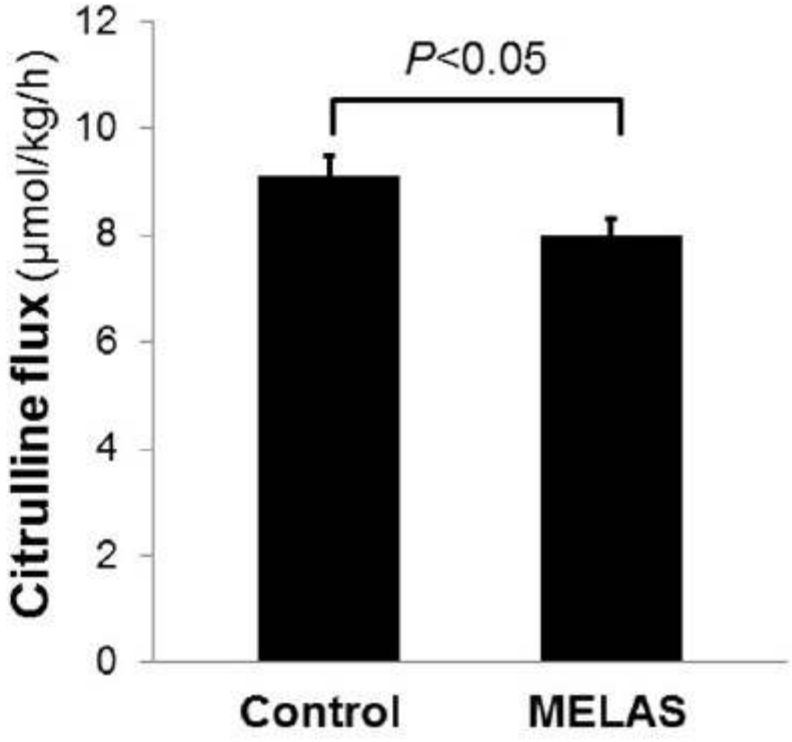
Lower NO production rate in children with MELAS



El-Hattab et al, 2016

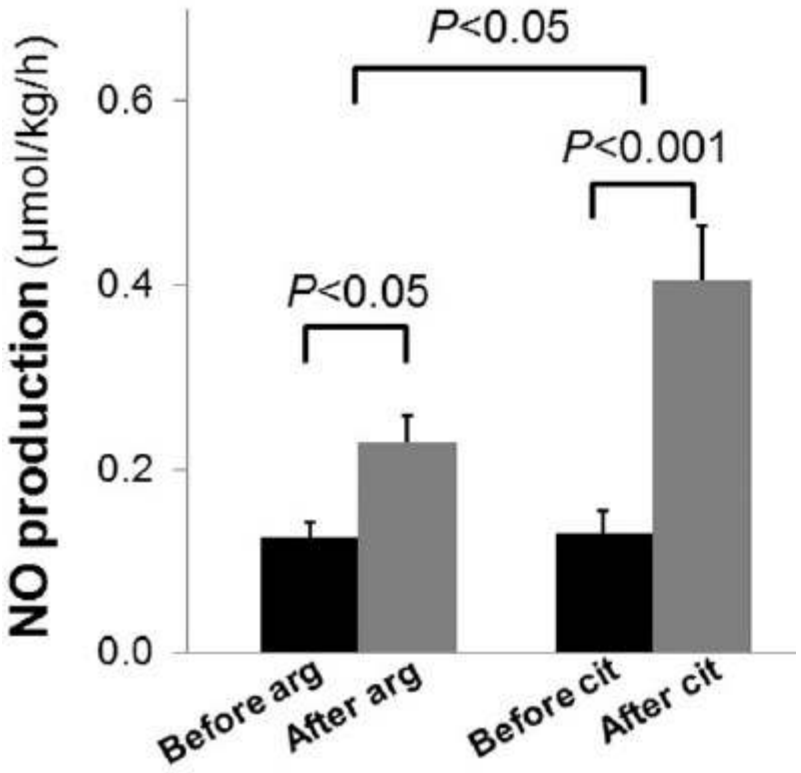
Lower NO production rate in children with MELAS was associated with lower fluxes of arginine and citrulline





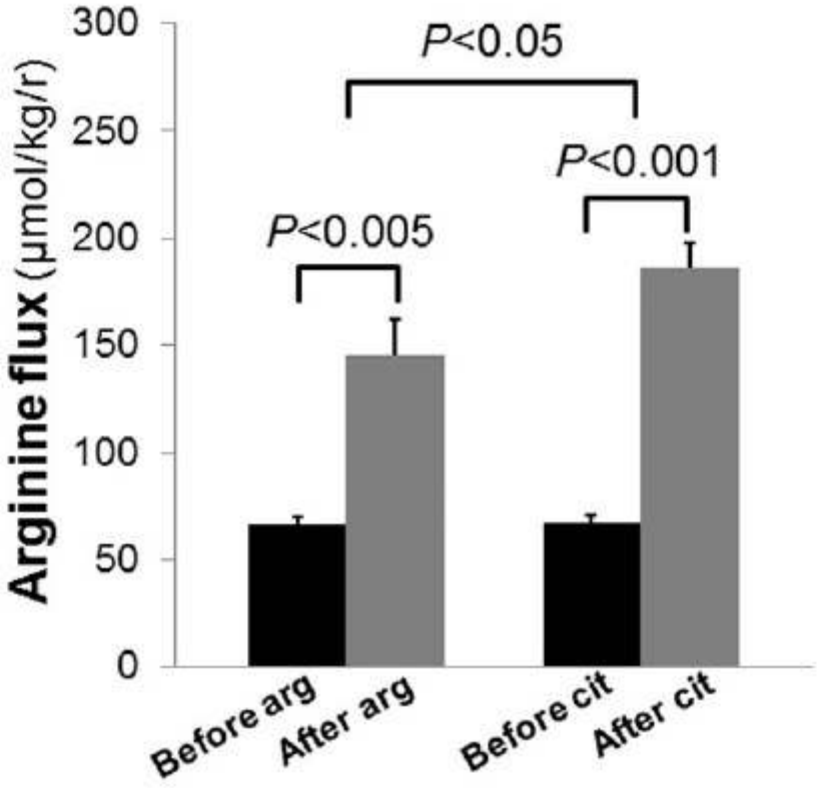
El-Hattab et al, 2016

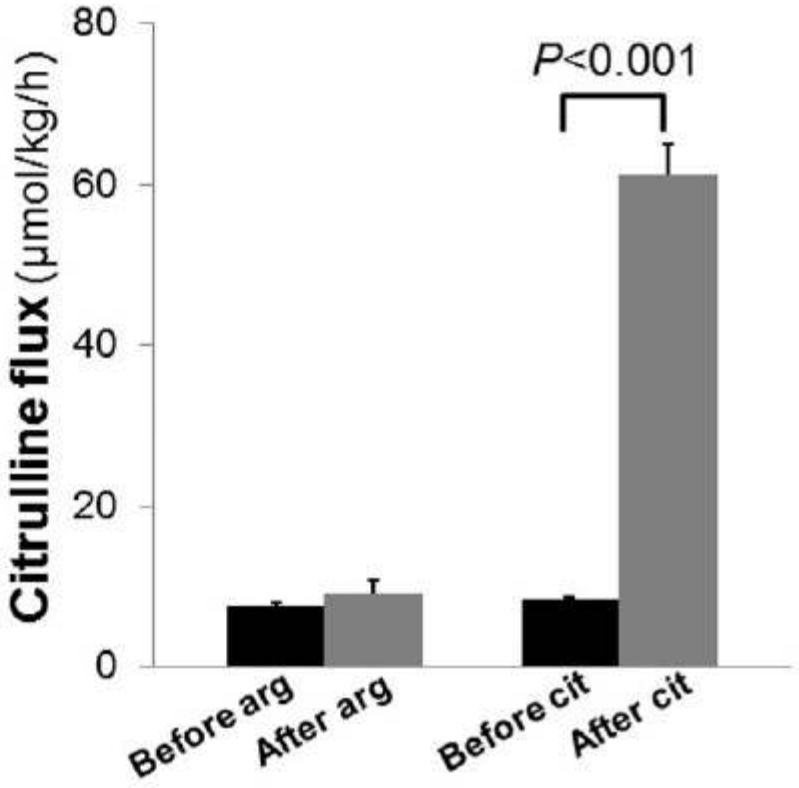
Both citrulline and arginine supplementation resulted in increased NO production rate in pediatric subjects



El-Hattab et al, 2016

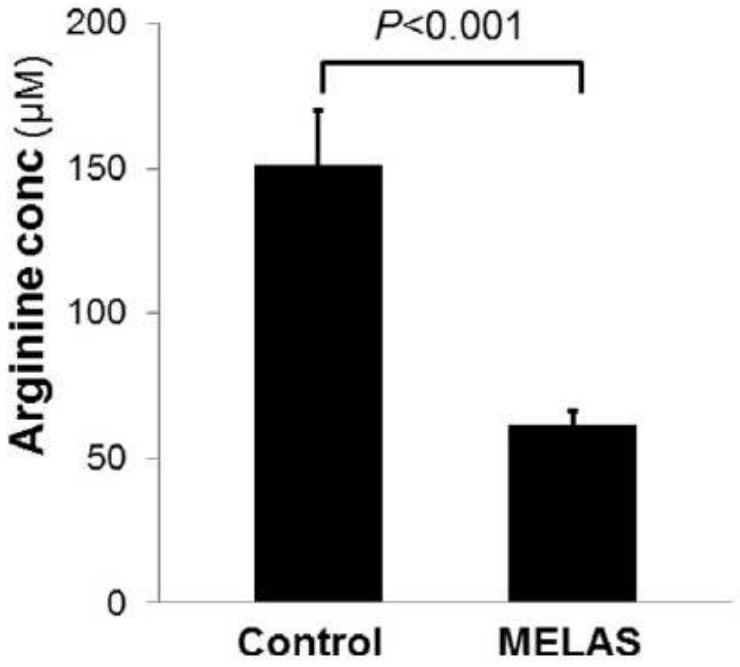
Accompanied with increments in both arginine and citrulline flux with arginine and citrulline supplementation





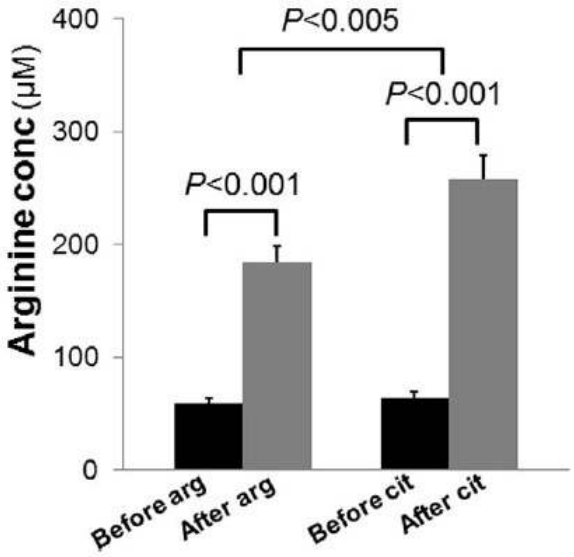
El-Hattab et al, 2016

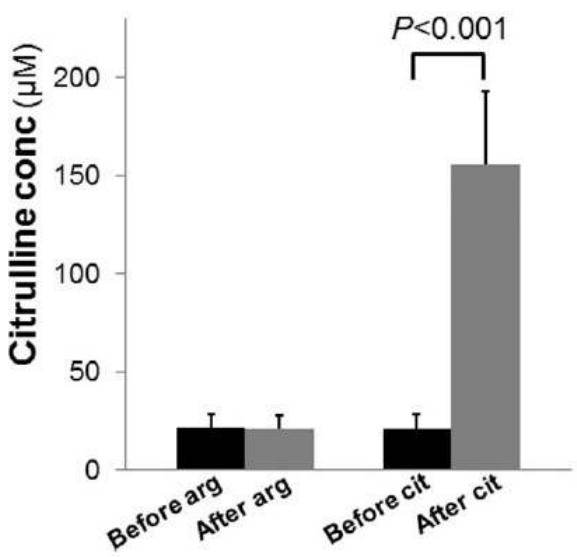
Plasma arginine concentration

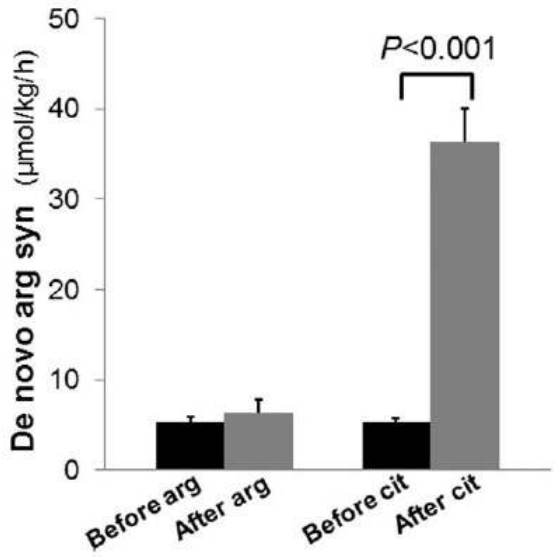


El-Hattab et al, 2016

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

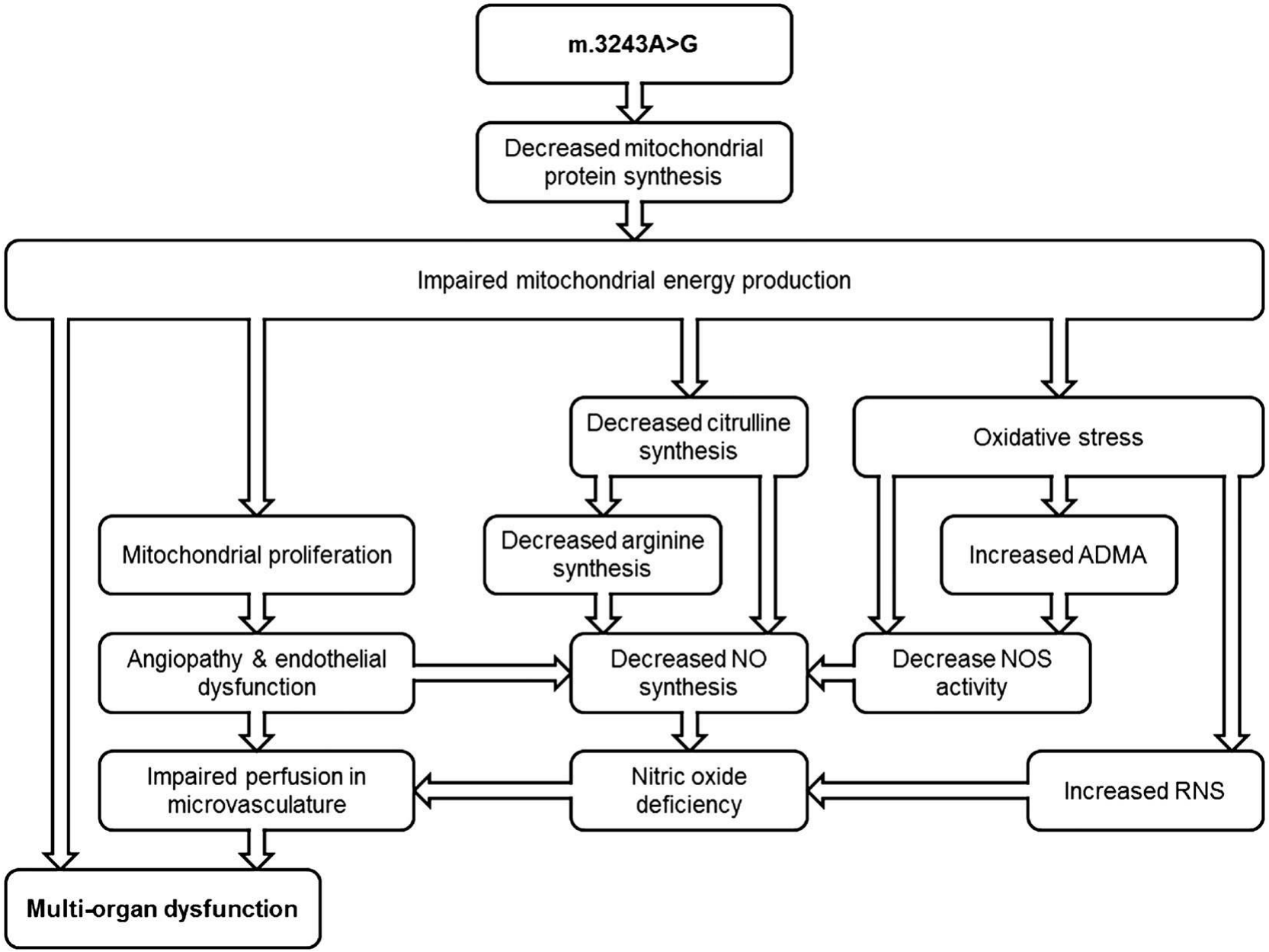
› Mol Genet Metab. 2016 Apr;117(4):407-12. doi: 10.1016/j.ymgme.2016.01.010. Epub 2016 Jan 27.

Impaired nitric oxide production in children with MELAS syndrome and the effect of arginine and citrulline supplementation

Ayman W El-Hattab [1], Lisa T Emrick [2], Jean W Hsu [3], Sirisak Chanprasert [4], Mohammed Almannai [4], William J Craigen [4], Farook Jahoor [3], Fernando Scaglia [5]

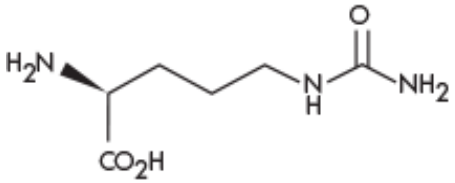
Affiliations + expand

PMID: 26851065 PMCID: PMC4818739 DOI: 10.1016/j.ymgme.2016.01.010



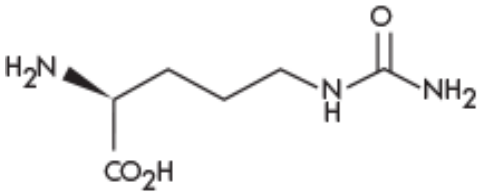
El-Hattab AW, Adesina AM, Jones J, Scaglia F. MELAS syndrome: Clinical manifestations, pathogenesis, and treatment options. Mol Genet Metab. 2015 Sep-Oct;116(1-2):4-12.

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 CO_2

- 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

	Baseline	Week 1	Week 2	Week 3	Week 4 (Final)	Follow-up*
Blood pressure measurement	X	X	X	X	X	X
ASL MRI to measure cerebral blood flow	X				X	
Plasma and urine guanidino compounds	X	X				
Plasma amino acids including plasma alanine [a]	X	X			X	
Plasma lactate	X	X			X	
Blood glucose	X	X	X	X	X	X
Comprehensive metabolic panel	X					
Complete blood count with differential	X					
Renal function tests (blood urea, creatinine, Cystatin C, and eGFR [b])	X					
Physician AE assessment	X	X	X	X	X	X
Seizure diary collection		X	X	X	X	X
Blood sugar diary collection		X	X	X	X	X
Blood pressure diary collection		X	X	X	X	X
Headache calendar collection		X	X	X	X	X
GI diary collection		X	X	X	X	X
Medication pill count (compliance measure)		X	X	X	X	
Neurologic assessment	X	X			X	X
Monitoring Follow Up Call		X	X	X	X	X

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

[a] 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 m^2) = 175 × $(S_{cr})^{-1.154}$ × $(Age)^{-0.203}$ × (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

SIMD/ Hyperion fellowship in IEMs award to Ayman El-Hattab, National Institutes of Health, M01-RR0188, General Clinical Research Center

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Studying Nutrition Today for the Health of Future Generations

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NINDS/NICHD: 5U54NS078059-08

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