

## Dityrosine: A Non-invasive Biomarker to Monitor Aging?



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Prepared for: [The Kronos™ Science Laboratory](#)

## Studies in The Biochemistry of Aging Laboratory

- 1. Mitochondrial Oxidative Stress and Aging
- 2. Mitochondrial-induced Apoptosis and Aging
- 3. Toxicity of Doxorubicin on the Heart

## Human Studies on Aging, Nutrition, Exercise/Inflammation

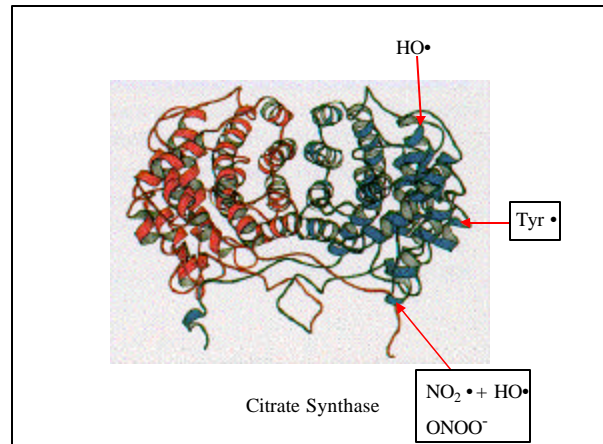
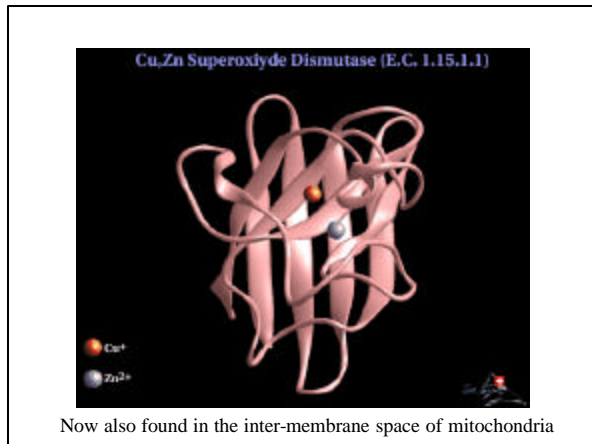
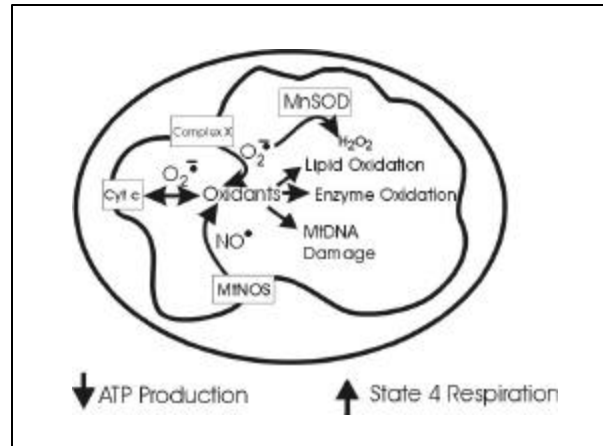
- Inflammation in healthy humans
- Nutritional interventions
- Finding non-invasive markers of oxidative stress to monitor aging and disease states in humans

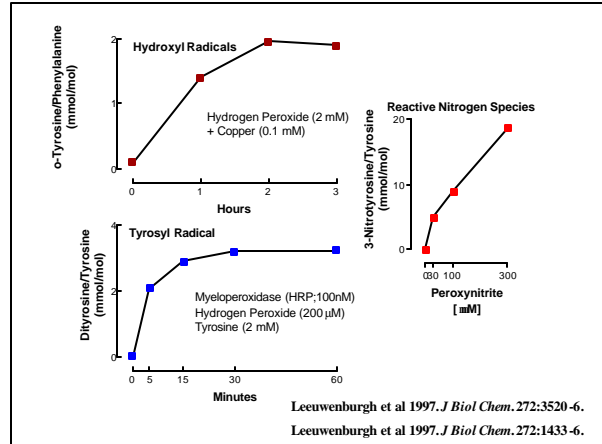
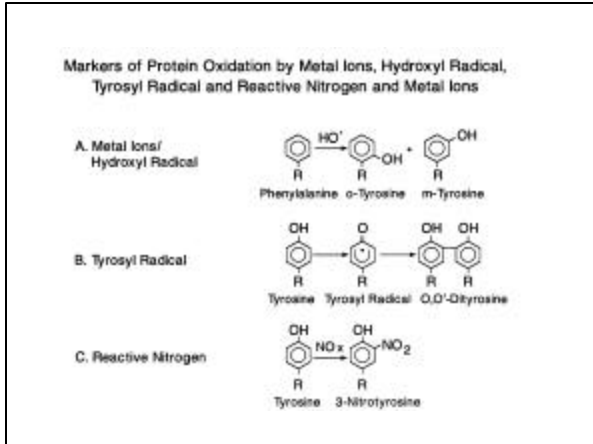


<b>Clinical Research</b>	<b>Analytical Chemistry</b>	<b>Molecular Biology-Biochemistry</b>
Neeharika, Choudry, M.D.	Barry Drew, Ph.D.	Rajani Shelke, Ph.D.
Tracey Philips*	Mina Hiona*	Sharon Phaneuf*
* Ph.D. students		Amie Dirks*

## Outline

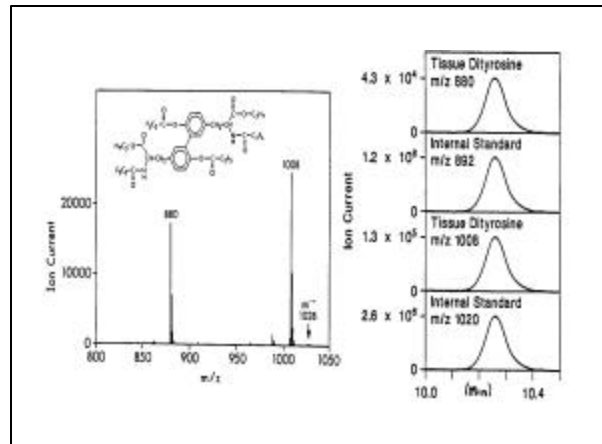
- **Animal Studies**  
Aging, Caloric Restriction and Dityrosine  
Antioxidant Therapy and Dityrosine
- **Human Studies**  
Kwashiorkor and Dityrosine  
Pilot data on Aging Humans and Urinary  
levels of Dityrosine





**Quantification of Oxidized Amino acids by Gas Chromatography-Mass Spectrometry**

- Acid Stable  $^{13}C$  Labeled Internal Standards
- HCl-Propanol / Heptafluorobutyric Anhydride.
- Negative Chemical Ionization (Methane)
- DB-1 capillary column 12 meter
- Selected Ion Monitoring
- Quantification: Ratio of Authentic and Corresponding Labeled Standard



## *In vitro*

- Gas Chromatography Mass Spectrometry is used to detect oxidative stress “fingerprints”
- Hydroxyl radicals, tyrosyl radicals, and reactive nitrogen increase levels of *o*-tyrosine, *o,o'*-dityrosine, and 3-nitrotyrosine *in vitro*

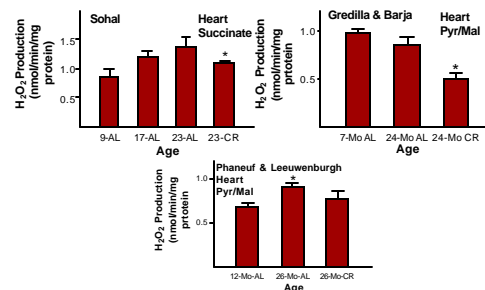
Aging, oxidant production, antioxidant defenses and detection of oxidative damage



## What is the oxidants production in a day?

- Using 3.5ml O<sub>2</sub>/kg/min gives at Rest
- O<sub>2</sub> consumption of 352.8L/day (70kg; male)
- 14.7 moles oxygen a day
- If 1% of oxygen becomes a superoxide radical
- Hypothetically 0.147 moles of superoxide is produced in a day and half of this would form hydrogen peroxide.
- Has this ever been measured in mitochondria?

## There is a chronic exposure to oxidants during a life-span

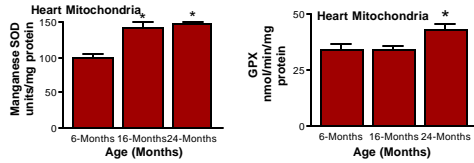


Sohal et al. Mech Ageing Dev 74 (1-2):121-33, 1994.

Gredilla R, Sanz A, Lopez-Torres M, and Barja G. FASEB J 15(9):1589-91, 2001.

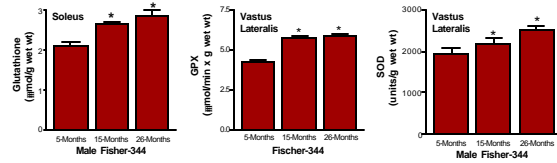
Phaneuf & Leeuwenburgh (Unpublished Data)

**Cardiac mitochondrial SOD and GPX are up-regulated with age**



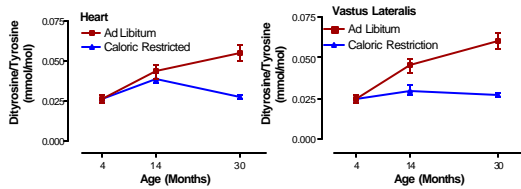
**Phaneuf, S, and Leeuwenburgh, C** Cytochrome c release from the mitochondria in the aging heart: A possible mechanism for apoptosis with age? *Am J Physiol*. 2001, (Accepted).

**Antioxidant defenses are not compromised in aged skeletal muscle**



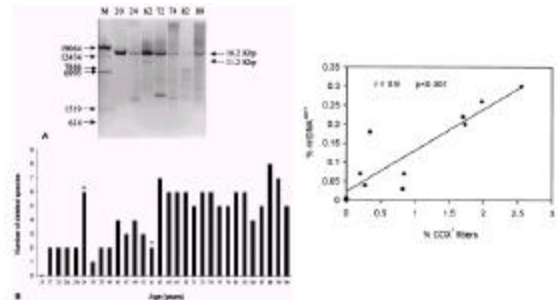
**Leeuwenburgh, C. et al.** Aging and exercise training in skeletal muscle: responses of glutathione and antioxidant enzyme systems. *Am J Physiol*. 267:R439-45, 1994.

**Oxidative Damage in Heart and Skeletal Muscle is Increased with Aging and is Reduced by Caloric Restriction**



**Leeuwenburgh, C. et al.** Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch Biochem Biophys*. 346:74-80. 1997.

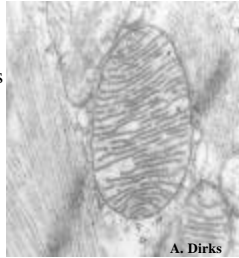
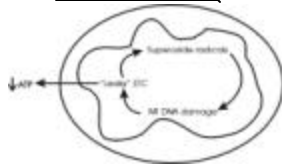
**Mitochondrial Deletions**



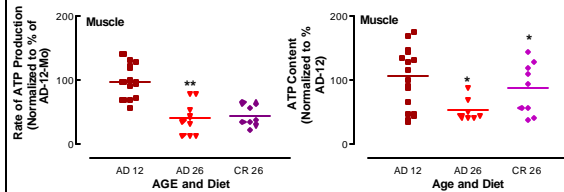
Pesce V, Cormio A, Fracasso F, Vecchiet J, Felzani G, Lezza AM, Cantatore P, Gadaleta MN. *Free Radic Biol Med*, 1:30(11):1223-33, 2001.

## Mitochondrial Dysfunction

- ↑ Oxidants
- ↑ Calcium levels
- ↑ mtDNA damage/deletions
- ↑ Protein Oxidation



Does this lead to a reduction in ATP?



Drew, B., A. Dirks, & C. Leeuwenburgh (unpublished data)

## Conclusions

- Aging Results in an increase in oxidant production (heart, brain, liver)
- Antioxidant enzymes (SOD, GPX) and GSH increase in response to the chronic oxidative stress
- In addition, MtDNA deletions increase in several tissues – Was this may mean for mitochondrial function is yet unclear
- There is a decline in maximum rate of ATP production
- Despite the adaptive response against oxidative stress, there is oxidative damage to lipid, DNA, and proteins.

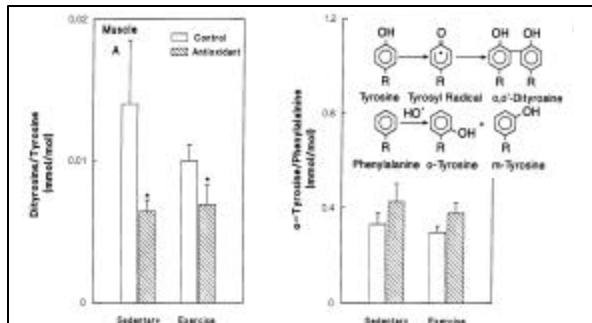
Can we attenuate oxidative damage with (additional) antioxidants?

## Animals and Diet

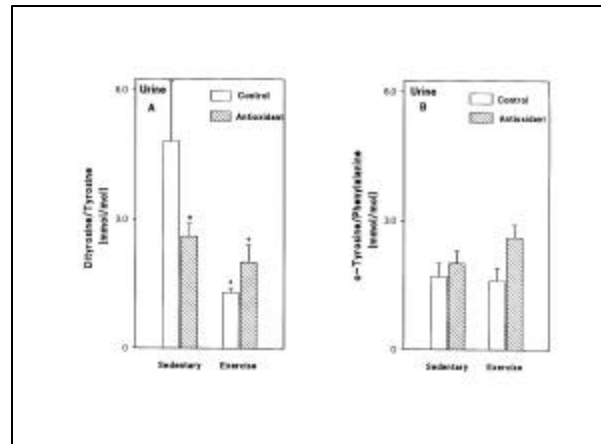
- |   |   |
|---|---|
| <p><u>Animals:</u></p> <ul style="list-style-type: none"> <li>• Female Colony-bred Long Evans/Wistar</li> <li>• Age 24 Months</li> <li>• At 5 months of age animals began the antioxidant diet and life-long voluntary wheel running</li> </ul> | <p><u>Antioxidant Diet:</u><br/><u>Harlan-Tekland</u></p> <ul style="list-style-type: none"> <li>• Ascorbic acid</li> <li>• <math>\alpha</math>-Tocopherol</li> <li>• BHT</li> <li>• <math>\beta</math>-Carotene</li> </ul> |
|---|---|

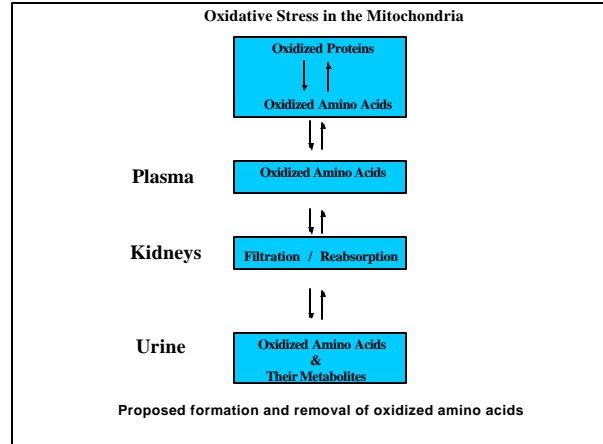
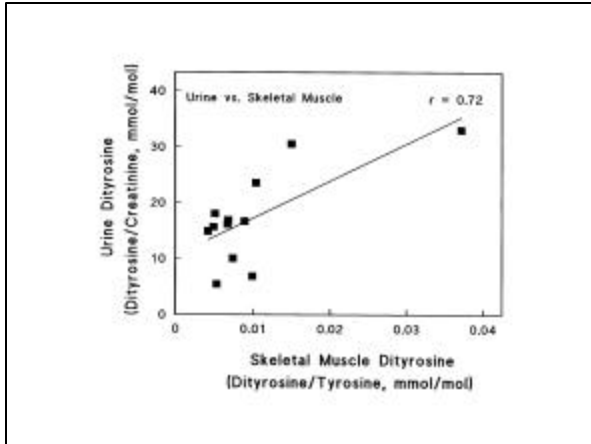
## Hypothesis

- Antioxidant therapy will reduce both tissue and urine level of oxidized amino acids providing markers to monitor protein oxidation non-invasively *in vivo*



Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW. Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress *in vivo*. *American Journal of Physiology* 276:R128-R135, 1999.





## Conclusions

- Oxidized amino acids may be recognized by proteolytic enzymes degraded, released, and excreted into the urine
- Quantification of the levels of oxidized amino acids in urine may thus serve as an integrated, noninvasive measure of oxidative stress *in vivo*

## Clinical Studies: Increased Oxidative Stress in Kwashiorkor

Manary, M.J., C. Leeuwenburgh,  
and J.W. Heinecke. Increased  
oxidative stress in kwashiorkor.

*J Pediatr.* 137:421-4, 2000.

## Kwashiorkor

- Characteristics:

- Edema
- ↓ Vitamin E
- ↓ Glutathione
- ↓  $\beta$ -carotene



## Question?

- Is oxidative stress involved in Kwashiorkor
- Non-invasive markers
  - *o-o'*-Dityrosine as well as *ortho*-Tyrosine in the urine of children with Kwashiorkor

## Methods

- Children approximately three years old were classified as either
  - Well nourished (n = 5)
  - Cerebral malaria (n = 6)
  - Kwashiorkor (n= 8)
  - Kwashiorkor with infection (n=17)

## Methods

- Urine was collected using a sterile catheter
- Samples were mixed with antioxidant buffer containing 0.1% phenol and diethylenetriaminepentaacetic acid (DTPA)
- Immediately frozen in dry ice and stored at  $-80^{\circ}\text{C}$  until analysis

## Methods

- Oxidized amino acids were measured in the urine using isotope dilution negative-ion electron capture gas chromatography/mass spectrometry with selected ion monitoring
- Amino acids were normalized to amino acid precursors and to creatinine in order to correct for differences in glomerular filtration rates

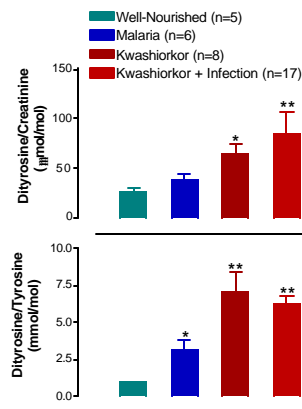
## Isolation of Oxidized Amino Acids from Tissue and Urine

### Tissue

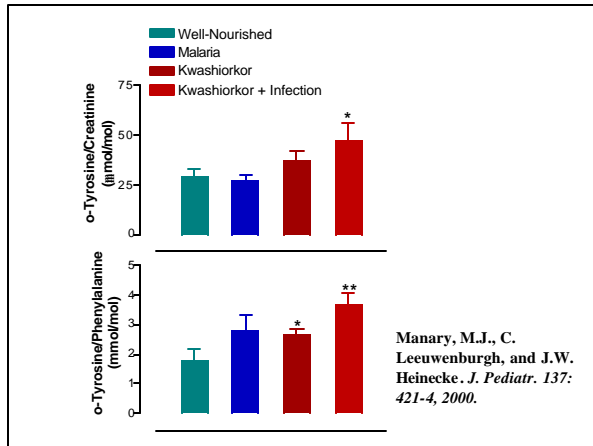
- 5 mg protein
- Dialysis
- HCl Hydrolysis
- Amino Acid Isolation (Supelco Column)
- Derivatization
- GC-MS analysis

### Urine

- 0.5-1 ml urine ( $^{13}\text{C}$  standard)
- TCA precipitation
- Amino Acid Isolation (Supelco Column C18)
- Derivatization
- GC-MS analysis



Manary, M.J., C. Leeuwenburgh, and J.W. Heinecke. *J. Pediatr.* 137: 421-4, 2000.



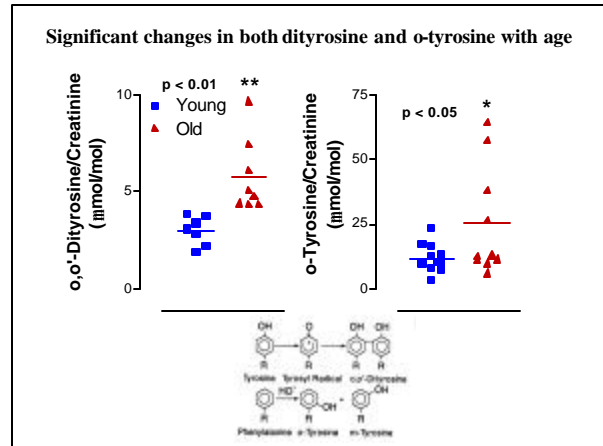
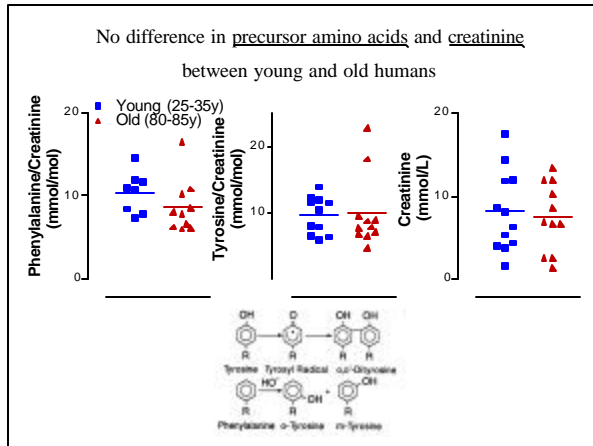
## Conclusions

- Kwashiorkor involves oxidative stress and oxidative damage to proteins
- This disease may be monitored using non-invasive markers in the urine
- Therapies which include antioxidant supplementation may decrease the incidence of Kwashiorkor and the number of malnutrition-related deaths



## Design of Small Pilot Study (Aging Humans)

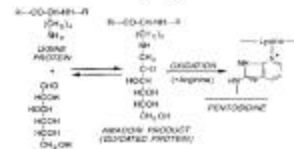
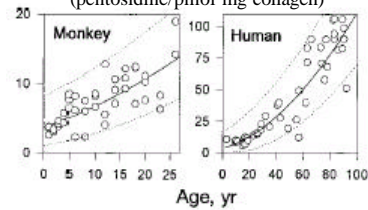
- Urine Collected from:
  - Young males and females (25-35y; n = 5, each)
  - Old females and one male (80-85y; n = 11)
- Healthy subjects with no indication of diabetes and cardiovascular disease



## Clinical Studies

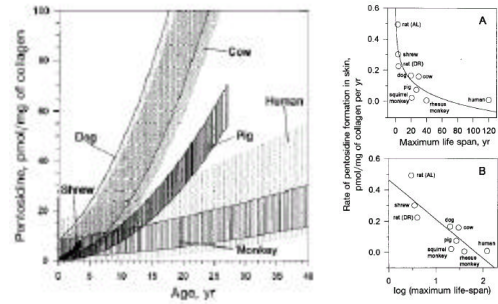
- In humans, urinary levels of both dityrosine and o-tyrosine increase by 100% and 40%, respectively, comparing young and old subjects
- Kwashiorkor (disease characterized by protein and antioxidant deficiency) showed a 200-600% increase in urinary oxidized amino acids compared to well-nourished children
- Therefore, markers for oxidative stress in urine may be useful for non-invasive assessment of aging and several disease states.

Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. Sell et al. *PNAS*, 93, 485-490, 1996  
(pentosidine/pmol mg collagen)



## Future Studies

- Large scale clinical trials are needed to determine range of dityrosine in aging humans before it could function as a marker to monitor aging.



Sell et al. *PNAS*, 93, 485-490, 1996.

The End