A randomized trial of glutamine and antioxidant supplementation in critically ill patients

This study is registered at Clinicaltrials.gov. Identification number NCT00133978
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  Aurora, CO 80045

  For specific questions related to processing samples please contact:

  Kelly Queensland  
  Tel: 303-724-2945  
  Email: Kelly.Queensland@ucdenver.edu
# Glossary

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>Acid Citrate Dextrose</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute (rotating speed of centrifuge)</td>
</tr>
</tbody>
</table>

RCF = \(0.00001118 \times \text{rotor radius} \times \text{RPM}^2\)
Lab Study Overview

In a sub-study of 200 consecutive patients enrolled at 4-5 centers in the United States and Europe, we will draw blood at baseline (pre-treatment), day 4, and day 7 following enrolment into the REDOXS© Study. The purpose is to measure plasma levels of study nutrients (for e.g. glutamine, selenium) and biochemical markers, which may give insight as to the putative mechanisms of benefit of these nutrients (for e.g. glutathione, lipid peroxidation [TBARS], Interleukin-6, and mitochondrial function [mitochondrial DNA/nuclear DNA ratio]). In addition we will evaluate in blood derived buffy coat cells other specific protective proteins that may mediate the beneficial effects of glutamine including heat shock proteins. We will perform analysis of RNA gene expression to evaluate gene expression changes in critically ill patients receiving the study nutrients. Finally, we perform Single Nucleotide Polymorphism analysis (SNP analysis) on specific inflammatory and heat shock protein SNPs. This will examine if there are specific genotypes that are pre-disposed to benefit from the study nutrients in states of critical illness. The results of the dose-escalating study suggested that increasing doses of the study nutrients would be associated with favourable changes to these parameters. In the context of this randomized trial, we plan to confirm these preliminary observations. Given the central role of mitochondrial dysfunction in the pathogenesis of multiorgan failure and the possible improvement in mitochondrial function observed in our dosing study, the primary outcome for this sub-study will be a marker of mitochondrial function. Based on previous in vivo laboratory work, the key secondary outcome markers will be cellular heat shock protein expression and inflammatory cytokine expression.

Blood Draw Visits

Each REDOXS© patient enrolled in the lab study should have blood draws done on the following study days:

1. Day 1 (baseline, pre-treatment)
2. Day 4
3. Day 7

Study Day 1 = ICU admission date. If the patient is not randomized to the REDOXS© study until study day 2, please obtain the baseline sample prior to initiation of study treatment.

If a blood draw visit day falls on a weekend, and it is not possible to obtain and process the samples on the weekend, the sample should be drawn on the closest working day. For example, blood draws due on Saturdays can be drawn on Fridays. Blood draws due on Sundays can be drawn on Mondays.
## Tube Kit Contents

Each patient will have 1 white box containing 3 tube kit bags. Each tube kit is labelled according to the blood draw visit day (1, 4, or 7).

The tube kit contents are as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Contents</th>
</tr>
</thead>
</table>
| **Day 1**<br>(Baseline, pre-treatment) | 1 x Red/green **speckled** top tube  
1 x Blue/black **speckled** top citrate tube  
1 x Royal blue top trace element tube  
3 x Purple top tubes (only 2 need to be filled and processed)  
1 x PAXGENE RNA tube  
1 x PAXGENE DNA tube  
38 Cryovials:  
one 10 x green  
one 10 x orange  
one 12 x purple  
one 6 x blue  
Cryovial labels |
| **Day 4** | 1 x Red/green **speckled** top tube  
1 x Blue/black **speckled** top citrate tube  
1 x Royal blue top trace element tube  
2 x Purple top tubes (only 2 need to be filled and processed)  
1 x PAXGENE RNA tube  
1 x PAXGENE DNA tube  
37 Cryovials:  
one 10 x green  
one 10 x orange  
one 11 x purple  
one 6 x blue  
Cryovial labels |
| **Day 7** | 1 x Red/green **speckled** top tube  
1 x Blue/black **speckled** top citrate tube  
1 x Royal blue top trace element tube  
2 x Purple top tubes (only 2 need to be filled and processed)  
1 x PAXGENE RNA tube  
1 x PAXGENE DNA tube  
37 Cryovials:  
one 10 x green  
one 10 x orange  
one 11 x purple  
one 6 x blue  
Cryovial labels |

If you identify that tubes within your kits are expired, please contact Elizabeth Luzier to obtain replacement tubes.

Elizabeth Luzier  
Tel: (303) 724-3597  
Email: Elizabeth.Luzier@ucdenver.edu
**Cryovial Labels**

Each cryovial label includes the patient ID and a reference to the study visit day.

Example: For patient # 12

![Blood draw number diagram]

**Blood Draw Instructions**

Using a central line or standard venipuncture techniques, draw blood in the following order:

1. 1 x Red/green speckled top tube
2. 1 x Blue/black speckled tube
3. 1 x Royal blue top trace element tube
4. 2 x Purple top tubes (Draw at least 4 mL of blood into each of the tubes)
5. 2 x PAXGENE tubes (Hold vertically below patient’s arm during collection)

Invert all tubes 8-10 times.
Blood Processing Instructions

While blood is spinning in centrifuge (refer to tube-specific instructions below), place provided labels on cryovials:

<table>
<thead>
<tr>
<th>Cryovials</th>
<th>Labels</th>
</tr>
</thead>
</table>
| 10 orange cryovials | 8 x ACD plasma labels  
                           2 x ACD PBMC labels |
| 10 green cryovials  | 8 x heparin plasma labels  
                           2 x heparin PBMC labels |
| 6 blue cryovials   | 6 x trace plasma labels               |
| 12 purple cryovials| 11 x EDTA plasma labels  
                           1 x Liege label (only for draw #1) |

ACD = Acid Citrate Dextrose  
PBMC = Peripheral Blood Mononuclear cells  
EDTA = ethylenediaminetetraacetic acid

**Speckled Red/Green top & Blue/Black tube (CPT tubes)**

1 x Red/green speckled top tube  
1 x Blue/black speckled tube

- Tubes should be processed as soon as possible following collection  
  - Tubes can be kept at room temperature for a maximum of 2 hours  
- Centrifuge at room temperature for 30 minutes at 1800 RCF  
  - RCF = \(0.00001118 \times \text{rotor radius} \times \text{RPM}^2\)  
- Mononuclear cells & platelets are in whitish layer just under the plasma layer (see Figure 1).  
  - Remove the plasma from each tube without disturbing the cell layer and aliquot into the appropriate colored cryovials. Discard any extra plasma.  
    - 0.5mL (500μL) of plasma from the Blue/Black tube into each of the 8 labeled (ACD) orange cryovials.  
    - 0.5mL (500μL) of plasma from the Red/Green tube into each of the 8 labeled green cryovials.
Speckled Blue/Black Top Tube ONLY

Using the already processed Blue/black speckled tube:

- Gently resuspend the cells above the gel and transfer to 2 labelled (ACD PBMC) orange cryovials.
- Estimate the volume (should be ~ 0.8-1.5mL) and gently add the preserving solution (DMSO) to a final concentration of 10% (see table).
- Cap and mix gently by inverting tube 5 times.
- Freeze at -20°C for approximately one day, then at -80°C prior to shipment to the Central lab.

Table 1: DMSO Table

<table>
<thead>
<tr>
<th>Volume of cell/plasma mixture (μL)</th>
<th>Volume of DMSO to add (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>88</td>
</tr>
<tr>
<td>900</td>
<td>99</td>
</tr>
<tr>
<td>1000</td>
<td>111</td>
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<tr>
<td>1100</td>
<td>122</td>
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<tr>
<td>1200</td>
<td>133</td>
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<tr>
<td>1300</td>
<td>144</td>
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<tr>
<td>1400</td>
<td>155</td>
</tr>
<tr>
<td>1500</td>
<td>166</td>
</tr>
<tr>
<td>1600</td>
<td>177</td>
</tr>
<tr>
<td>1700</td>
<td>188</td>
</tr>
</tbody>
</table>
Speckled Red/Green Top Tube ONLY

Using the already processed Red/Green speckled tube:

- Pull out white cell layer & add to a new 15 mL centrifuge tube
  - This is the second layer in the vacutainer, it will be turbid and sitting above the plug
  - Do not be concerned if contaminated with a small amount of plasma above it
- Add PBS (Phosphate-buffered Saline) to bring volume of collected cells in the 15mL conical centrifuge tube to a total of 15mL
- Cap tube, mix by inverting tube 5 times
- Centrifuge for 15 minutes at 600 RCF
  - RCF = 0.00001118 x rotor radius x RPM²
  - ONLY if a pellet does not form, increase the RCF to 1300
- Aspirate as much supernatant as possible without disturbing cell pellet
- Resuspend cell pellet by gently vortexing or tapping tube with finger
- Add PBS to bring volume to 10mL
- Cap tube, mix cells by inverting 5 times.

Directions if you have the ability to spin small cryovials:

- Centrifuge the above tube for 10-15min @ 600RCF
- Aspirate the supernatant without disturbing the pellet.
- Resuspend cell pellet in 1 ml of PBS
- Transfer 500ul of liquid to 2 labelled (heparin PBMC) green cryovials
- Centrifuge small tubes for at least 15 min @ 600 RCF
  - Be sure the cell pellet has formed before stopping the spinning step
- Aspirate supernatant without disturbing cell pellet
- Freeze cell pellets at -80ºC prior to shipment to central lab.

Directions if you can only spin larger tubes

- Leave the liquid in the 15mL conical and spin the entire volume together to form one large pellet.
  - Label and freeze the pellet in the 15mL conical (pellet transfer may cause loss of specimen)
- Aspirate as much supernatant as possible without disturbing cell pellet
- Freeze cell pellets at -80ºC prior to shipment to central lab

Royal Blue top (trace element – small tube)

1 x Royal blue top tubes

- Centrifuge royal blue top tube for 10 min @ 1300 RCF (spin together with non-DNA purple top tubes)
  - These can be spun cold if your centrifuge is already refrigerated.
- Place 3 to 6 0.5mL aliquots of plasma into each of the 6 labelled (trace plasma) blue cryovials
- Store plasma at -80ºC, discard pellet
- Send plasma to central lab
**Purple Tops for Plasma**

2 x purple top tubes

- Centrifuge purple top tubes for 10 min @ 1300 RCF at room temperature (spin together with royal blue top)
  - *These can be spun cold if your centrifuge is already refrigerated.*
- Remove plasma
- Aliquot 0.5mL plasma into each of the **11 labelled (EDTA plasma) purple cryovials**
- Remove (preferably with a wide bore tip) and combine buffy coats from both tubes into single cryovial labeled **Lighe WBC**
- Process pellet from one of the purple top tubes

**Pellet Processing:**

- Add normal saline 0.9% (until half a centimetre below full), mix well
- Centrifuge 10 min @ 1300 RCF
- Discard top layer
- Repeat wash x 2
- Remove 500μL red blood cells into 15mL centrifuge tube
- Add 2mL ice-cold water
- Mix well, refrigerate for 15 mins
- Centrifuge 10 min @ 1300 RCF
- **Store top layer in self-provided 2 mL cryovial labelled with patient number and RBC @ -80ºC prior to shipping to central lab**

**PAXGENE RNA Tube**

1 x PAXGENE RNA Tube

- Freeze PAXGENE RNA tube upright in wire rack at -20ºC for 24 hours (do **NOT** freeze in Styrofoam, tube will crack)
- Transfer tubes to -70ºC or -80ºC freezer
- Send to the central lab

**PAXGENE DNA Tube**

1 x PAXGENE DNA Tube

- Freeze PAXGENE DNA tube upright in wire rack at -20ºC for 24 hours (do **NOT** freeze in Styrofoam, tube will crack)
- Transfer tubes to -70ºC or -80ºC freezer
- Send to the central lab
Data Entry

A minimal amount of data entry is required in the EDCS. It is as follows:

Select the Lab Sub-Study link in the Screening/Baseline forms section of the EDC System.

When you select the link you will see the following question:

If you select “NO” all you have to do is save the form. If you select “YES” then you will need to complete the following screen:

Complete the form. The SeraCare Patient ID# is the identification number found on the labels in the tube kits. See Labelling section for more details.

SAVE the entered data.
Packaging & Shipping

Containers must be securely packed in their shipping box. Please check by gently shaking the box after packaging. If the containers are loose, repack the box by filling the empty spaces with paper.

Mail processed study specimens on dry ice to the Wischmeyer TPN lab at:

Wischmeyer Translational PharmacoNutrition Laboratory  
UCD Dept. of Anesthesiology  
12700 E. 19th Ave.  
RC2 Room P15 7490D  
Aurora, CO 80045

The FedEx account number to be used to ship samples to the Wischmeyer lab is 464718125.

Please be sure to inquire about your local service schedules and the latest time of day that you can call to arrange for a pick-up. Timely pick-ups will ensure samples are shipped within stability for testing.

Important Considerations

Local courier service (pickup and delivery) may be limited prior to, during and following observed holidays. It is imperative that you check local service schedules in advance of the holiday.

- Specimens should be shipped from Mondays through Wednesdays only.
- Frozen samples should NOT be shipped on the day before an observed holiday.

Observed Holidays

<table>
<thead>
<tr>
<th>Holiday</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Year's Day</td>
<td>Friday, January 1st</td>
<td>Saturday, January 1st</td>
</tr>
<tr>
<td>Good Friday</td>
<td>Friday, April 2nd</td>
<td>Friday, April 22nd</td>
</tr>
<tr>
<td>Easter Monday</td>
<td>Monday, April 5th</td>
<td>Monday, April 5th</td>
</tr>
<tr>
<td>Labour Day</td>
<td>Monday, September 6th</td>
<td>Monday, September 5th</td>
</tr>
<tr>
<td>Christmas Day</td>
<td>Saturday, December 25th</td>
<td>Sunday, December 25th</td>
</tr>
<tr>
<td>Boxing Day</td>
<td>Sunday, December 26th</td>
<td>Saturday, December 26th</td>
</tr>
</tbody>
</table>

Check with your courier regarding other holidays not listed above.
Appendix

Processing Flow Charts

A – Red/Green Speckled Top Tube & Blue/Black Speckled Top Tube
B – Royal Blue Top
C – Purple Top
D – PAXGENE RNA Tube & PAXGENE DNA Tube
Appendix A - Red/Green Speckled Top Tube & Blue/Black Speckled Top Tube

**Speckled Red/Green Top**

- **Red/Green Top Tube**
  - Centrifuge at room temperature for 30 mins @ 1800 RCF
  - Remove the plasma from each tube (without disturbing the cell layer)
  - Aliquot 0.5mL (500μL) of plasma into the appropriate coloured cryovials
  - **Red/Green**
    - Aliquot 0.5mL (500μL) of plasma into 8 labeled (heparin) green cryovials
  - **Red/Green top tube**
    - Pull out the white cell layer & add to a new 15mL tube
    - Add PBS to bring volume to 15mL. Cap tube & invert 5 times. Centrifuge for 15 mins @ 600 RCF.
    - Aspirate the supernatent. Resuspend the cell pellet by gently vortexing. Add PBS to bring volume to 10 mL. Cap tube & invert 5 times.
    - Spin & freeze pellets at -80°C. Refer to pg. 10 for specific instructions

**Speckled Blue/Black Top**

- **Blue/Black Top Tube**
  - Aliquot 0.5mL (500μL) of plasma into 8 labeled (ACD) orange cryovials
  - **Blue/Black**
    - Aliquot 0.5mL (500μL) of plasma into the appropriate coloured cryovials
  - **Blue/Black top tube**
    - Gently resuspend the cells above the gel
    - Transfer to 2 labeled (ACD PBMC) orange cryovials
    - Estimate the volume remaining in the tube, gently add DMSO (see table) to a final concentration of 10%
    - Cap & invert gently 5 times. Freeze at -20°C for 1 day, then -80°C

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Version: 12-Nov-2010
Appendix B – Royal Blue Top Tube

Royal Blue Top

Centrifuge for 10 mins @ 1300 RCF (spin together with non-DNA purple tops)

Place 3 to 6 0.5mL aliquots of plasma into each of the 6 labeled (trace plasma) blue cryovials

Store plasma at -80°C, discard pellet.
Appendix C - Purple Top Tube

**Purple Top**

Centrifuge for 10 mins @ 1300 at room temperature (spin together with royal blue top)

Remove the plasma from each tube (without disturbing the cell layer) → Aliquot 0.5mL plasma into each of the 11 labeled (EDTA) purple cryovials

**PELLET PROCESSING**

Using the pellet from 1 purple top tube

Add normal saline 0.9% to purple tube until 0.5 cm below full, mix well.

Centrifuge for 10 mins @ 1300 RCF

Discard top layer, repeat wash with normal saline 0.9% x 2.

Remove 500μL red blood cells into 15mL centrifuge tube

Add 2mL ice-cold water, mix well & refrigerate for 15 mins

Centrifuge for 10 mins @ 1300 RCF. Store top layer in self-provided 2mL cryovial labeled with patient ID & RBC. Freeze at -80°C.
**Appendix D – PAXGENE RNA & DNA Tubes**

### PAXGENE RNA Tube

1. **Freeze** PAXGENE RNA tube upright in wire rack at -20ºC for 24 hours (Do NOT freeze in Styrofoam, tube will crack)
2. **Transfer** tubes to a -70ºC or -80ºC freezer
3. **Send to central lab within 8 weeks of drawing samples**

### PAXGENE DNA Tube

1. **Freeze** PAXGENE DNA tube upright in wire rack at -20ºC for 24 hours (Do NOT freeze in Styrofoam, tube will crack)
2. **Transfer** tubes to a -70ºC or -80ºC freezer
3. **Send to central lab within 8 weeks of drawing samples**