SUPPLEMENTARY MATERIAL

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Supplementary Materials and Methods - Extended

Methods

Clinical Study: Twelve healthy donor volunteers were enrolled at the Hoxworth Blood Center under FDA Investigational New Drug application and Institutional Review Board approved protocol of the University of Cincinnati number 2017-6190 and in conformity with the Declaration of Helsinky. Each subject donated two units within a minimum of 8 weeks following FDA and AABB regulations. Both units were processed to obtain log4 leukocyte-filtered RBC products (Haemonetics, Braintree, MA), which were randomized to be stored either under standard conditions in CP2D/AS-3, or under hypoxic storage at controlled SO_2 < 20%. Deoxygenation of the units was performed through proprietary Hemanext Inc. technology and workflows, as extensively described.[1–3] At completion of the two-arm cross-sectional study, 24 units were thus stored under standard (n=12) or hypoxic conditions (n=12) for up to 42 days, with each donor being their own control. Each unit was sterilely docked and sampled at storage day 0, 21 and 42 for metabolomics analyses, SO_2, pO_2, pCO_2, hemolysis, pH, ATP, 2,3-DPG and lactate measurements, as previously described[4]. Metabolomics and lipidomics analyses were performed on RBC and supernatants from these units, separated via centrifugation for 10 min at 4ºC at 1,500 g.

Determination of post-transfusion recovery via ^{51}Cr labeling: Autologous in vivo post-transfusion recovery studies were performed at Hoxworth Blood Center, as previously described.[5] On days 42, each unit was inspected for any signs of unusual hemolysis or discoloration indicative of bacterial growth. The unit was well mixed by hand (1 min), and approximately 15 mL of the RBCs removed and labeled with approximately 15µCi of chromium-51 (^{51}Cr) with standard techniques.[5] The labeling agent, ^{51}Cr sodium chromate, was mixed aseptically with the RBCs at roomtemperature for 30 minutes. One double-volume saline wash was conducted. An aliquot of the final volume was reserved for assay as a standard, and the remaining labeled cells (approx. 10 mL) were injected into a free-flowing peripheral vein. Samples (5 mL each) were taken from a contralateral vein at time intervals within the first 30 minutes after infusion as well as at 24 hours through a butterfly needle. The samples were counted in a gamma counter to determine ^{51}Cr activity. The activity of the samples from the first 30 minutes was back-extrapolated to determine a T0 activity. The amount of RBCs and the amount of radioactivity infused were determined basedon the methods of Moroff and coworkers[6] and the International Committee for Standardization in Hematology.[7] All ^{51}Cr specimens used for survival analysis were counted at the same time and corrected for decay. Areas under the curve (AUC) were calculated by integrating the

slope of ^{51}Cr as a function of time over the first 24h. Post-transfusion recoery at 24h and AUC were used for correlation to metabolomics and lipidomics measurements.

Sample processing and metabolite extraction: A volume of 50 µL of frozen RBC and supernatant aliquots was extracted 1:10 or 1:25, respectively in ice cold extraction solution (methanol:acetonitrile:water 5:3:2 *v/v*)[8] or pure methanol[9]. Samples were vortexed and insoluble material pelleted, as described,[10,11] prior to further analysis via ultra-high pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS).

Ultra-High-Pressure Liquid Chromatography-Mass Spectrometry (MS) metabolomics and lipidomics: The analytical platform employs a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Sample processing, metabolite extraction and specific methods for metabolomics and lipidomics are presented in the Supplementary Information document.

Metabolomics: UHPLC-MS metabolomics analyses were performed using a Vanquish UHPLC system coupled online to a high-resolution Q Exactive mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were resolved over a Kinetex C18 column (2.1x150 mm, 1.7 µm; Phenomenex, Torrance, CA, USA) at 45°C. A volume of 10 ul of sample extracts was injected into the UHPLC-MS. Each sample was injected and run four times with two different chromatographic and MS conditions as follows: 1) using a 5 minute gradient at 450 µL/minute from 5-95% B (A: water/0.1% formic acid; B:acetonitrile/0.1% formic acid) and the MS was operated in positive mode and 2) using a 5 minute gradient at 450 µL/minute from 5-95% B (A: 5% acetonitrile, 95%water/1 mM ammonium acetate; B:95%acetonitrile/5% water, 1 mM ammonium acetate) and the MS was operated in negative ion mode. The UHPLC system was coupled online with a Q Exactive (Thermo, San Jose, CA, USA) scanning in Full MS mode at 70,000 resolution in the 60-900 m/z range, 4 kV spray voltage, 15 sheath gas and 5 auxiliary gas, operated in negative or positive ion mode (separate runs). Stable isotope-labeled internal standards were used as an internal quality control for quantitation, as described.[8]

Lipidomics: Samples were resolved over an ACQUITY HSS T3 column (2.1 x 150 mm, 1.8 µm particle size (Waters, MA, USA) using an aqueous phase (A) of 25% acetonitrile and 5 mM ammonium acetate and a mobile phase (B) of 50% isopropanol, 45% acetonitrile and 5 mM ammonium acetate. Samples were eluted from the column using either the solvent gradient: 0-1 min 25% B and 0.3 mL/min; 1-2 min 25-50% B and 0.3 mL/min, 2-8 min 50-90% B and 0.3 mL/min, 8-10 min 90-99% B and 0.3 mL/min, 10-14 min hold at 99% B and 0.3 mL/min, 14-14.1 min 99-25%

B 1and 0.3 mL/min, 14.1-16.9 min hold at 25% B and 0.4 mL/min, 16.9-17 min hold at 25% B and resume flow of 0.3 mL/min. isocratic elution of 5% B flowed at 250 µl/min and 25ºC or a gradient from 0- 5% B over 0.5 min; 5-95% B over 0.6 min, hold at 95% B for 1.65 min; 95-5% B over 0.25 min; hold at 5% B for 2 min, flowed at 450 µl/min and 35ºC[9]. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated independently in positive or negative ion mode, scanning in Full MS mode (2 µscans) from 150 to 1500 m/z at 70,000 resolution, with 4 kV spray voltage, 45 shealth gas, 15 auxiliary gas. When required, dd-MS2 was performed at 17,500 resolution, AGC target = 1e5, maximum IT = 50 ms, and stepped NCE of 25, 35 for positive mode, and 20, 24, and 28 for negative mode.

Quality control and data processing: Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was then converted from .raw to .mzXML file format using Mass Matrix (Cleveland, OH, USA). Samples were analyzed in randomized order with a technical mixture injected incrementally to qualify instrument performance. This technical mixture was also injected three times per polarity mode and analyzed with the parameters above, except CID fragmentation was included for unknown compound identification.

Metabolite assignment and relative quantitation: Metabolite assignments were performed using MAVEN (Princeton, NJ, USA),[12] as determined from high-resolution accurate intact mass spectra and isotopic pattern deconvolution, against the KEGG pathway, HMDB, ChEBI, and ChEMBL databases. Assignments were further confirmed against an in house library of unlabeled, ^{13}C, ^{15}N isotopes or deuteratium-labeled standards (IROATech; Cambridge Isotopes; SPLASH® LIPIDOMIX® Mass Spec Standard, Avanti Lipids).[8,9] Graphs and statistical analyses (either t-test or repeated measures ANOVA), as well as network analyses and circos plots of correlations were prepared with GraphPad Prism 8.0 (GraphPad Software, Inc, La Jolla, CA), GENE E (Broad Institute, Cambridge, MA, USA), the Metscape 2.0 plugin for Cytoscape,[13] MetaboAnalyst 4.0[14] and the OmicsNet plugin.[15]

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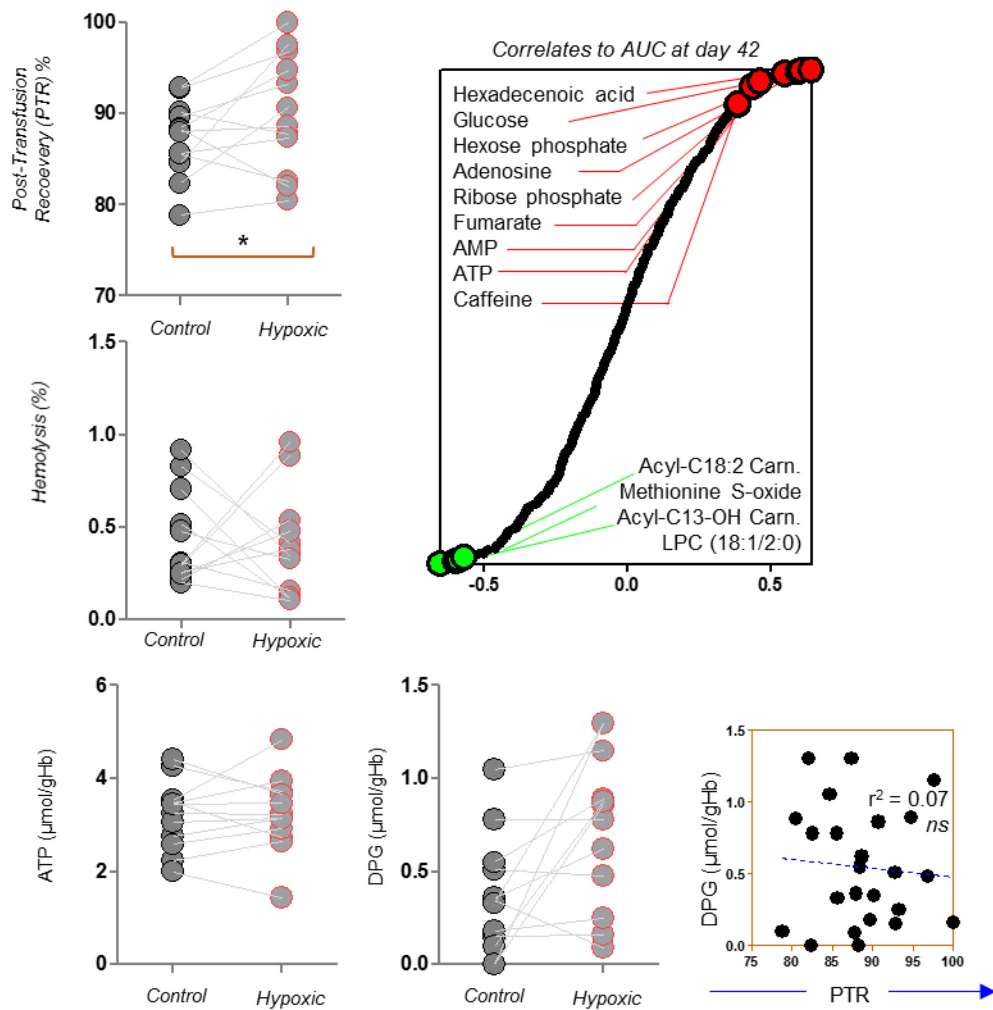
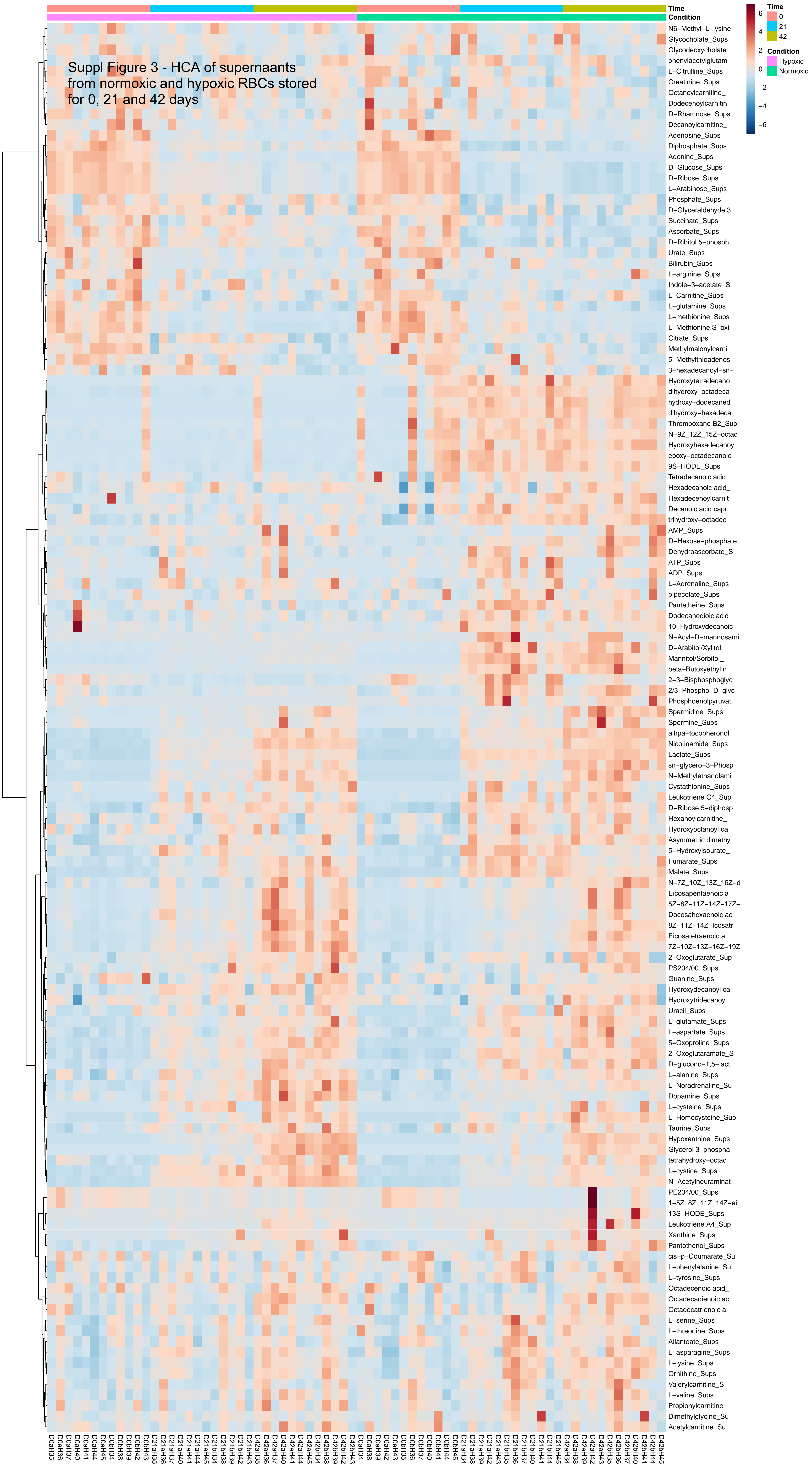


Supplementary Figure 1 – Line plots of end of storage PTR and hemolysis of paired control (normoxic) and hypoxic RBC units. On the right hand side, metabolic correlates to AUC of PTR at day 42 for combined normoxic and hypoxic units.

Suppl Figure 2 - HCA of
normoxic and hypoxic
RBCs at storage day 0, 21 and 42
Time
Condition
Time
0
21
42
Condition
Hypoxic
Normoxic
6
4
2
0
−2
−4
−6
Sphingosine 1−phos
Sphinganine 1−phos
4−Pyridoxate
2−Deoxy−alpha−D−gl
Glutathione disulf
CMP−N−acetylneuram
L−asparagine
L−aspartate
Dimethylglycine
sn−glycero−3−Phosp
D−Ribitol 5−phosph
L−glutamate
Creatinine
L−Citrulline
Glycerol 3−phospha
N−formyl kynurenin
GDP
NADPH
Phosphoethanolamin
5−10−Methenyltetra
Urate
Cholesterol sulfat
GMP
D−Glyceraldehyde 3
threo−3−Hydroxy−L−
L−methionine
L−Methionine S−oxi
IDP
Citrate
Oxaloacetate
Carnosine
Propionylcarnitine
Acetylcarnitine
UDP−D−glucuronate
Phosphocreatine
Spermine
alpha−D−Glucosamin
UDP−N−acetyl−D−glu
2/3−Phospho−D−glyc
Phosphoenolpyruvat
Biliverdin
2−3−Bisphosphoglyc
NADP
D−Fructose 1−6−bis
L−glutamine
Glutathione
Cys−Gly
GTP
UDP−glucose
Adenine
ATP
ADP
D−Glucose
D−Ribose
1−4−beta−D−Xylan
Deoxyribose tripho
Pyridoxal
Ascorbate
6−Phospho−D−glucon
D−glucono−1,5−lact
Inosine
Pyruvate
D−Hexose−phosphate
Sedoheptulose 7−ph
Pentose phosphates
dihydroxy−octadeca
Prostaglandin F2al
Prostaglandin D3 i
epoxy−octadecanoic
hydroxy−dodecanedi
dihydroxy−hexadeca
Tetradecanoic acid
N−9Z_12Z_15Z−octad
alpha−N−Peptidyl−L
Hexanoic acid capr
D−Arabitol/Xylitol
Octanoic acid capr
Prostaglandin B2
trihydroxy−octadec
10−Hydroxydecanoic
2−Hydroxyglutarate
Hydroxyundecanoyl
Hydroxydecanoyl ca
Hydroxydodecanoyl
Hydroxytetradeceno
Stearidonyl carnit
Tetradecenoylcarni
Hexadecadienoylcar
Dodecanoylcarnitin
N−9Z−hexadecenoyl−
N−Octadecanoyl−eth
N−hexadecanoyl−eth
N−Hexadecanoyl−eth
2−5Z_8Z_11Z_14Z−ei
N−9Z_12Z−octadecad
N−9Z−octadecenoyl−
Glycocholate
Glycodeoxycholate
Hexadecanoic acid
Prostaglandin D2/T
13S−HODE
9S−HODE
N1−Acetylspermidin
Putrescine
Spermidine
Fumarate
Malate
5−6−Dihydrouracil
Decanoic acid capr
Dodecanoic acid
R−S−Lactoylglutath
S−lactoylglutathio
3−hexadecanoyl−sn−
D−Ribose 5−diphosp
Bilirubin
N−Acyl−D−mannosami
N−Acetyl−L−ornithi
L−arginine
Pantetheine
Nicotinamide
D−Alanyl−alanyl−po
N−eicosanoyl−ethan
N−11Z_14Z−eicosadi
gamma−L−Glutamyl−D
Phosphodimethyl−et
S−Adenosyl−L−methi
5−Methylthioadenos
Propenoylcarnitine
L−valine
Methylmalonylcarni
L−tyrosine
cis−p−Coumarate
N−Amidino−L−aspart
L−leucine
L−phenylalanine
PEP−160/00
1−1Z−hexadecenyl−s
IMP
2−3−Dinor−8−iso pr
PE204/00
1−5Z_8Z_11Z_14Z−ei
sn−glycero−3−Phosp
PE181/00
PE160/00
1−hexadecanoyl−sn−
UMP
PS181/00
PS204/00
Octadecenoic acid
Octadecadienoic ac
Octadecatrienoic a
Leukotriene A4
Eicosatetraenoic a
7Z−10Z−13Z−16Z−19Z
Docosahexaenoic ac
Eicosapentaenoic a
5Z−8Z−11Z−14Z−17Z−
tetrahydroxy−octad
Thromboxane B2
L−Homocysteine
8Z−11Z−14Z−Icosatr
alhpa−tocopherol
Mannitol/Sorbitol
N−Acetylneuraminat
Allantoate
Lactate
Hypoxanthine
g−Oxalo−crotonate
L−cysteine
Guanosine
Guanine
glycine
S−Glutathionyl−L−c
PC102/102
2−2E_4E−decadienoy
5−Oxoproline
Shikimate 3−phosph
Kynurenic acid
N−7Z_10Z_13Z_16Z−d


Suppl Figure 3 - HCA of supernaants
from normoxic and hypoxic RBCs stored
for 0, 21 and 42 days
Time
Condition
Time
0
21
42
Condition
Hypoxic
Normoxic
6
4
2
0
−2
−4
−6
N6−Methyl−L−lysine
Glycocholate_Sups
Glycodeoxycholate_
phenylacetylglutam
L−Citrulline_Sups
Creatinine_Sups
Octanoylcarnitine_
Dodecenoylcarnitin
D−Rhamnose_Sups
Decanoylcarnitine_
Adenosine_Sups
Diphosphate_Sups
Adenine_Sups
D−Glucose_Sups
D−Ribose_Sups
L−Arabinose_Sups
Phosphate_Sups
D−Glyceraldehyde 3
Succinate_Sups
Ascorbate_Sups
D−Ribitol 5−phosph
Urate_Sups
Bilirubin_Sups
L−arginine_Sups
Indole−3−acetate_S
L−Carnitine_Sups
L−glutamine_Sups
L−methionine_Sups
L−Methionine S−oxi
Citrate_Sups
Methylmalonylcarni
5−Methylthioadenos
3−hexadecanoyl−sn−
Hydroxytetradecano
dihydroxy−octadeca
hydroxy−dodecanedi
dihydroxy−hexadeca
Thromboxane B2_Sup
N−9Z_12Z_15Z−octad
Hydroxyhexadecanoy
epoxy−octadecanoic
9S−HODE_Sups
Tetradecanoic acid
Hexadecanoic acid_
Hexadecenoylcarnit
Decanoic acid capr
trihydroxy−octadec
AMP_Sups
D−Hexose−phosphate
Dehydroascorbate_S
ATP_Sups
ADP_Sups
L−Adrenaline_Sups
pipecolate_Sups
Pantetheine_Sups
Dodecanedioic acid
10−Hydroxydecanoic
N−Acyl−D−mannosami
D−Arabitol/Xylitol
Mannitol/Sorbitol_
beta−Butoxyethyl n
2−3−Bisphosphoglyc
2/3−Phospho−D−glyc
Phosphoenolpyruvat
Spermidine_Sups
Spermine_Sups
alhpa−tocopheronol
Nicotinamide_Sups
Lactate_Sups
sn−glycero−3−Phosp
N−Methylethanolami
Cystathionine_Sups
Leukotriene C4_Sup
D−Ribose 5−diphosp
Hexanoylcarnitine_
Hydroxyoctanoyl ca
Asymmetric dimethy
5−Hydroxyisourate_
Fumarate_Sups
Malate_Sups
N−7Z_10Z_13Z_16Z−d
Eicosapentaenoic a
5Z−8Z−11Z−14Z−17Z−
Docosahexaenoic ac
8Z−11Z−14Z−Icosatr
Eicosatetraenoic a
7Z−10Z−13Z−16Z−19Z
2−Oxoglutarate_Sup
PS204/00_Sups
Guanine_Sups
Hydroxydecanoyl ca
Hydroxytridecanoyl
Uracil_Sups
L−glutamate_Sups
L−aspartate_Sups
5−Oxoproline_Sups
2−Oxoglutaramate_S
D−glucono−1,5−lact
L−alanine_Sups
L−Noradrenaline_Su
Dopamine_Sups
L−cysteine_Sups
L−Homocysteine_Sup
Taurine_Sups
Hypoxanthine_Sups
Glycerol 3−phospha
tetrahydroxy−octad
L−cystine_Sups
N−Acetylneuraminat
PE204/00_Sups
1−5Z_8Z_11Z_14Z−ei
13S−HODE_Sups
Leukotriene A4_Sup
Xanthine_Sups
Pantothenol_Sups
cis−p−Coumarate_Su
L−phenylalanine_Su
L−tyrosine_Sups
Octadecenoic acid_
Octadecadienoic ac
Octadecatrienoic a
L−serine_Sups
L−threonine_Sups
Allantoate_Sups
L−asparagine_Sups
L−lysine_Sups
Ornithine_Sups
Valerylcarnitine_S
L−valine_Sups
Propionylcarnitine
Dimethylglycine_Su
Acetylcarnitine_Su