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GENE EXPRESSION IN BLOOD, LUNG, AND HEART FOLLOWOING EXPOSURE OF RATS TO HYPOBARIA

Gary Fiskum

University of Maryland School of Medicine

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Final Report

AIR FORCE RESEARCH LABORATORY
711TH HUMAN PERFORMANCE WING,
AIRMAN SYSTEMS DIRECTORATE,
WRIGHT-PATTERSON AIR FORCE BASE, OH 45433
AIR FORCE MATERIEL COMMAND
UNITED STATES AIR FORCE

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ALICIA BURKE, Program Manager
Product Development Branch
Airman Biosciences Division

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THEREA A. BEDFORD, Maj, USAF, NC, FNP-BC
Nurse Scientist, En Route Care
Product Development Branch
Airman Biosciences Division

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14. ABSTRACT
Results obtained from rodent traumatic brain injury models indicate that exposure to air-evacuation-relevant hypobaria within a few days following injury worsens neurologic outcomes and exacerbates inflammatory damage to the brain, lungs, and heart. The causes for these changes are not well understood but could involve changes in gene expression in response to moderate hypobaria and hyperoxia, which often occur during air evacuation. This study tested the hypothesis that exposure of normal healthy rats to hypobaria (4000 or 8000 ft altitude) for as short as 5 hr and as long as 10 hr results in tissue-selective changes in gene expression that could provide insight into the mechanisms responsible for these effects. Microarray analysis of RNA extracted from normal brain, lungs, and heart and blood detected greater than 500 messenger RNAs that were either up- or down-regulated in response to hypobaria or hyperoxia. Several gene expression pathways were identified, including effects on iron homeostasis. To our knowledge, these are the first analyses of tissue selective transcriptomics in response to moderate levels of hypobaria relevant to commercial and military flights. Future studies will compare these results to those obtained following traumatic injury and subsequent exposure to hypobaria.

15. SUBJECT TERMS
Hypobaria; hyperoxia, transcriptome; quantitative polymerase chain reaction; oxidative stress; iron homeostasis

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Contents

1.0 SUMMARY

Background. The views expressed are those of the authors and do not reflect the official guidance or position of the United States Government, the Department of Defense or of the United States Air Force. Exposure of humans and lab animals to high altitude, e.g., 10,000 feet (ft), results in changes in gene expression in tissues and blood cells that may mediate adaptation to high altitude or potentially contribute to altitude sickness. This study tested the hypothesis that exposure of rats to flight-relevant hypobaria (8000 to 4000 ft) causes acute changes in the gene transcripts present in blood, lungs, heart, and brain (hippocampus) and that these transcriptomes are affected by different oxygen (O_2) levels present during exposure to hypobaria. **Methods.** Adult male rats were placed in a flight chamber and exposed, or not, to hypobaria (8000 or 4000 ft) under room air or 100 percent (%) O_2 for 5 or 10 hours (hr). Blood and organs were immediately processed for microarray measurements of 40,000 messenger ribonucleic acids (mRNAs). Expression of 10 selected genes was measured using quantitative polymerase chain reaction (qPCR) to validate microarray results. **Results.** Microarray measurements of gene expression in the blood, lung, heart, and brain detected several hundred differentially-expressed (DE) transcripts across 9 experimental group comparisons to controls maintained at 250 ft altitude under room air. The changes were validated using qPCR analysis of 10 targeted transcripts. Seventy percent of the transcripts tested both agreed with the direction (increase or decrease) of the expression. Although there was no specific trend in the pathway enrichment caused by hypobaria or hyperoxia over "flight time", iron homeostasis and heme biosynthesis signaling were enriched by hyperoxia while anti-oxidant gene expression was enriched by exposure to hypobaria. **Conclusions.** Exposure of rats to 4000 ft hypobaria for 5 hr results in changes in gene expression in blood and vital organs, while exposure to 8000 ft hypobaria elicits a greater response. Gene expression pathway analysis indicates that antioxidant gene expression and iron/redox homeostasis are affected by both hypobaria and hyperoxia. Measurements of changes in gene expression in other organs and in blood are needed to better understand the acute effects of flight-relevant altitude on the health of flight crew and patients undergoing aeromedical transport. Generation of the transcriptomes associated with relatively mild hypobaria and hypoxia or hyperoxia, will allow for future comparisons between the transcriptomes of normal rats to those following trauma, e.g., traumatic brain injury. These analyses will help us determine which gene expression pathways could be modulated to optimize the therapeutic efficacy of the body's endogenous protection and repair pathways.

2.0 INTRODUCTION

Rapid aeromedical evacuation (AE) of critically injured US warfighters from combat zones to high level trauma centers enabled a doctrinal revolution in the military medical system (1). Through the US Air Force Medical Service Critical Care Air Transport Team, AE became the standard operating procedure for the long-distance military transport of patients (2). Even so, the potentially negative physiologic and cognitive impacts of the flights are incompletely understood, and there is both anecdotal and experimental evidence that the transport itself could contribute to secondary injury (3, 4). Expanding knowledge on the comprehensive physiologic effects of flight on injured patients has been identified as an Air Force specific research gap. These efforts will potentially improve patient safety and outcomes by facilitating more accurate risk assessment related to the timing of evacuation and development of mitigation strategies to counteract any identified negative effects.

Like most planes, the cabins of aircraft used for AE are pressurized to an altitude of about 8000 ft (2438 meters), which is generally well-tolerated by healthy individuals. However, there is a growing body of evidence in a plurality of animal models that exposure to AE-relevant hypobaria after several forms of trauma results in physiological and cognitive deficits (5-10). These effects are particularly evident in animal models of traumatic brain injury (TBI) (4, 6). The mechanisms responsible for the adverse effects of AE-relevant hypobaria following trauma are not well-understood, as most studies into the physiological effects of hypobaria are based on the extreme altitudes (>5000 meters) experienced by mountain climbers to study the molecular basis for acute mountain sickness (11, 12).

The most commonly cited explanation for adverse effects of hypobaria following trauma is systemic hypoxia and associated impairment of O_2 delivery to vital organs (13, 14). Thus, Critical Care Air Transport (CCAT) members and other critical care practitioners often provide supplemental O_2 to even mild TBI patients and may at times provide up to 100% to avoid hypoxia. However, supplemental O_2 in excess of that necessary to avoid hypoxia can be toxic. Hyperoxia is associated with increased mortality in patients with stroke, TBI, and resuscitation from cardiac arrest (15, 16). Moreover, one large observational study found that early hyperoxia in trauma patients is associated with reduced adjusted in-hospital mortality (17).

Previous research performed in our lab and others indicates that exposure of animals to AE-relevant hypobaria results in worse neurological and histopathological outcomes compared to normobaria (5-9). One possible response to mild, AE-relevant hypobaria is changes in gene expression, based on the myriad of pathologies and genetic pathways that are affected by more severe hypobaria (4, 9, 18). The goal of this study was to identify alterations in genomic pathways that occur in the lungs, heart, blood, and hippocampus immediately following exposure of rats to mild and moderate hypobaria, i.e., 4000 and 8000 ft, for durations of 5 and 10 hr, under normoxic and hyperoxic conditions.

3.0 METHODS

3.1 Exposure of rats to hypobaria and/or hyperoxia

The protocol was reviewed and approved by the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance (FWR-2018-0001A) and by the University of Maryland, Baltimore Institutional Animal Care and Use Committee (1217007). These studies were conducted in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and were performed in compliance with DODI 3216.1.

Adult male Sprague Dawley rats weighing 300-350 gram (g) were placed in a hypobaria chamber and exposed to normobaria (= 250 ft altitude), or two levels of hypobaria, equivalent to 4000 ft or 8000 ft altitude, for 5 or 10 hr. Pressure was decreased incrementally over the initial 30 minute (min) to simulate ascent and increased over the final 30 min to simulate descent. The chamber was also perfused with either air (21% O_2; normoxia) or 100% O_2 (hyperoxia) throughout the simulated "flight." Immediately after the exposure to hypobaria, rats were euthanized in accordance with the UMSOM and the AFRL IACUC-approved protocols. Rats were first anesthetized with 4-5% isoflurane. Once they were deeply anesthetized, a thoracotomy was performed, resulting in immediate death, followed by removal of blood and tissue samples. Lung, heart, and hippocampus tissue was collected and snap frozen on dry ice before storage at -80 degrees centigrade (°C). Blood was collected in PAXgene tubes (Qiagen) and stored at -80°C for ribonucleic acid (RNA) stabilization.

3.2 RNA isolation and quantification

Samples of frozen tissue were cut and placed into -80°C RNAlater™ ICE (Thermo Fisher Scientific, Waltham, MA) and stored at -20°C overnight. The following day, tissue was homogenized on ice and RNA was isolated with TRIzol® (Thermo Fisher) and PureLink™ mRNA mini kit (Thermo Fisher) according to manufacturer's instructions. Blood mRNA was isolated according to manufacturer's PAXgene Blood RNA protocol. Deoxyribonuclease (DNase) was added to columns to prevent deoxyribonucleic acid (DNA) contamination. Isolated RNA was then quantified using a NanoDrop ND-1000 (Thermo Fisher). Quality and integrity of some RNA samples was assessed by an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) at the University of Maryland Baltimore (UMB) Center for Innovative Biomedical Resources (CIBR). RNA was required to achieve a RIN (RNA integrity number) of 7 or higher before microarray analysis.

3.3 Microarray profiling, data, and pathway analysis

Microarray profiling of lung, heart, and blood mRNA was performed using Clariom S Rat Assays (Thermo Fisher), while profiling of hippocampus RNA was performed using Rat Gene 2.0 ST Array chips (Thermo Fisher), according to manufacturer's instructions. Briefly, 150 nanograms (ng) RNA was transcribed to double-stranded complementary DNA (cDNA), followed by synthesis and purification of complementary RNA (cRNA). 2nd-cycle single-stranded complementary DNA (ss-cDNA) was then synthesized and then purified after RNA hydrolysis

using ribonuclease H (RNase H). ss-cDNA was fragmented and labeled before hybridization onto the microarray chips, which were washed and processed using an Affymetrix GeneChip system 3000 7G (Thermo Fisher).

Affymetrix image data (.CEL files) was further analyzed by the UMB Institute for Genome Sciences (IGS) using the R statistical analysis tool. The R 'oligo' package (19) was used to access the intensity values which were normalized using the 'Robust Multichip Average (RMA)' normalization method provided by the R 'limma' package (20). The samples were assessed and corrected for any batch effects before downstream analyses. Principal component analysis was utilized to identify and exclude any outlier samples. A general linear model was utilized to fit the expression values for each gene. The R 'limma' package uses a moderated t-statistic test based on a Bayesian model to assess for differential expression. Differential expression of genes was computed between each hypobaric condition compared to the baseline (Altitude: 250 ft; Duration: 5 hr; Oxygen: 21%). Significant DE genes were detected using a p-value cut-off of 0.1 and a 1.5x fold-change cut-off. The selected DE genes were then utilized to assess pathway enrichment using the Ingenuity Pathway Analysis (IPA) tool (QIAGEN Inc., Santa Clara, CA; https://www.qiagenbioinformatics.com /products/ingenuity-pathway-analysis) (21). The significant pathways enriched were selected using a 5% p-value cut-off. All figures were generated using the R statistical analysis tool.

3.4 Reverse transcription and microarray validation with qPCR

Reverse transcription of 1 microgram (μg) RNA was carried out with High Capacity cDNA Reverse Transcription kit (Thermo Fisher) according to manufacturer's instructions. qPCR was performed using primers designed through the National Center for Biotechnology Information (NCBI) primer design tool and purchased from IDT (Integrated DNA Technologies, Coralville, IA). Primers and annealing temperatures (T_a) were optimized to ensure efficiency was between 90% and 110% (Table 1). qPCR was performed with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher) on a QuantStudio 3 qPCR machine (Thermo Fisher) using the following protocol: initialized with 50 °C for 2 min then 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 seconds (s), 15 s at the primers' respective annealing temperatures (Table 1), and 72 °C for 1 min.

Table 1: Primer pairs designed with rat mRNA sequences from the NCBI database and their respective annealing temperatures (T_a).

Gene name (gene symbol)	NCBI accession number	T_a (°C)	Primer sequences
Hypoxia inducible factor-3α (HIF-3α)	NM_022528	53	5'-AACGGAGCAACATACTCGCA-3' 3'-TCCCATAGGGTCCGTTGTCA-5'
Oxidative stress induced growth inhibitor 1 (Osgin1)	NM_138504	52	5'-TGCGGAAGAAATGCAGAGGC-3' 3'-TAGCGGGTGATGATGTCCCT-5'
Superoxide dismutase 3 (SOD3)	NM_012880	52	5'-GCTGGGTCTGTCCTGTACTT-3' 3'-GAACAGTCCACACCTTGGAGTC-5'
Neurotrophic tyrosine kinase, receptor, type 2 (NTRK2)	NM_001163168	55	5'-AAGATCCCCCTGGATGGGTAG-3' 3'-GGGGCACTCGACTTGAGGA-5'
Zinc finger and BTB domain containing 16 (ZBTB16)	NM_001013181	53	5'-CGATCCAGGCACACCCCTAA-3' 3'-GGGTGGAACGTCTCGTCTC-5'
Tocopherol (alpha) transfer protein (TTPA)	NM_013048	52	5'-CCTGGCCTGGCGCTTAATG-3' 3'-ACCTGAAGACTTCCGACCGA-5'
Ferrochelatase (FECH)	NM_001108434	55	5'-CCAATCGGGTCCAGCAGTGGT-3' 3'-TCCTCCTTCGGGTTTTGCCC-5'
Natriuretic peptide receptor 3 (NPR3)	NM_012868	52	5'-TGCAAATCATGTGGCCTAGAAG-3' 3'-ACCAAACGATTACCGGAAGATGA-5'
Solute carrier family 4 (anion exchanger) (SLC4A1)	NM_012651	52	5'-AAGCTCTGCTCAACCTGGTG-3' 3'-CGTAATCGTCTGCCCCCTTT-5'
5-aminolevulinate synthase 2 (ALAS2)	NM_013197	52	5'-GTGGATGAAGTCCATGCCGTA-3' 3'-CCTTGAGAACCGTTCCGGAA-5'
Actin, β (ACTB)	NM_031144	52	5'-GGGACGATATGGAGAAGATTTG-3' 3'-GACATACGGAGACCAGCAT-5'

4.0 RESULTS

4.1 Microarray Results

Lung, heart, and blood mRNA samples were run on Clariom S Assays that quantify the expression of almost 23,000 transcripts, while the Rat Gene 2.0 ST Arrays quantified over 50,000 hippocampus transcripts. Figure 1 shows the clustering of lung mRNA samples using Principal Component Analysis (PCA) of the gene expression. 5 outlier samples were identified and excluded from the lung data set (Figure 1A; within the red square): 2 from the group exposed to 21% oxygen at 4000 ft for 10 hr, 2 from the group exposed to 21% O_2 at 8000 ft for

10 hr, and one from the group exposed to 100% O_2 at 8000 ft for 5 hr. Figure 1B shows the distribution of the remaining samples after the outliers were removed. The absence of a noticeable trend indicates that there was no obvious driver of sample distribution beyond biological variation. The same analysis was performed for all tissues with final n-values listed in Table 2.

Microarray transcript values were compiled and compared to the normobaria, normoxia baseline group (21% oxygen, 0 ft, 5 hr) to identify DE transcripts. In order to focus on transcripts that showed the greatest change and therefore most likely reflect physiological response to the treatments, initial thresholds were set at > 1.5x change (positive or negative) with p-values < 0.1 compared to the baseline group. In the lung, for example, this resulted in 638 unique DE transcripts across 9 comparisons. The total number of transcripts that were significantly up- or down-regulated in each tissue based on treatment group is listed in Table 2.

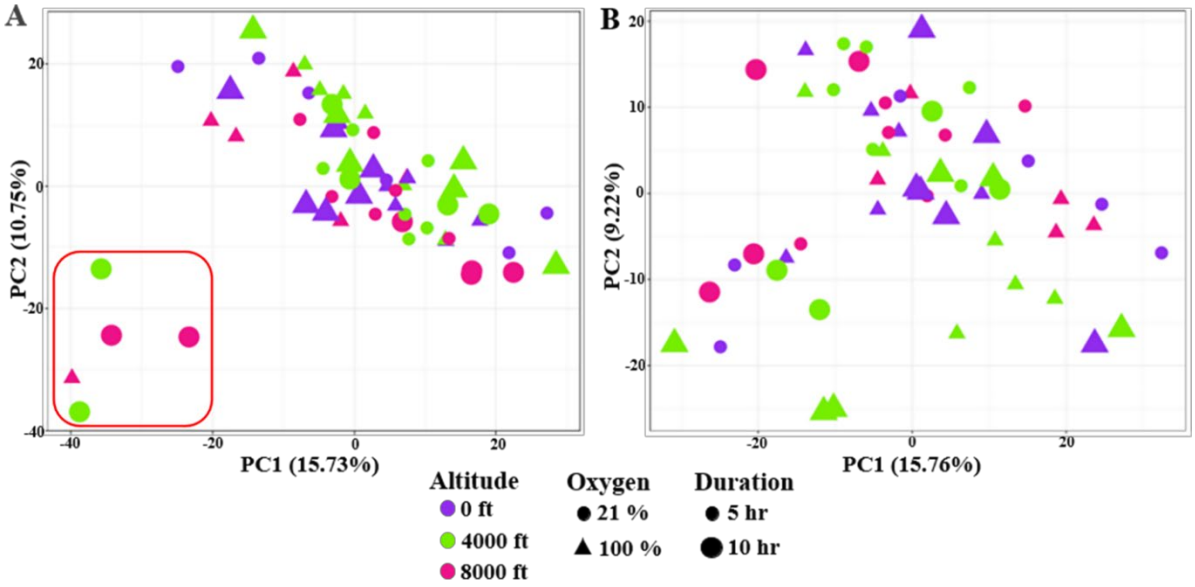


Figure 1: PCA plots showing clustering of lung RNA samples from all treatment groups. Samples are characterized into simulated altitude (0 ft, 4000 ft, or 8000 ft), oxygen (21% or 100%), and duration of exposure (5 hr or 10 hr).
A) The first and second components of all 60 samples. Samples within the red square were identified as outliers and were not considered for differential expression analysis or qPCR validation. B) First and second components of samples after removing outliers.

Table 2: The number of different transcripts that exhibited at least 1.5 x change ($p < 0.1$) compared to baseline (0 ft, 21% oxygen, 5 hr) in response to the listed treatments in **rat lung, heart, blood, and hippocampus** tissue from microarray analysis. The number of transcripts that were upregulated (↑) or downregulated (↓) is listed. n = 4-6 for all groups in all tissues.

Altitude (ft)	Oxygen (%)	Duration (hr)	Lung	Heart	Blood	Hippocampus
0	100	5	↑16, ↓23	↑7, ↓0	↑33, ↓65	↑177, ↓99
0	100	10	33, 86	6, 9	7, 35	50, 119
4000	21	5	15, 32	7, 53	9, 243	116, 150
4000	21	10	53, 39	126, 89	22, 92	146, 42
4000	100	5	8, 23	21, 37	31, 40	128, 158
4000	100	10	29, 40	47, 21	14, 39	50, 43
8000	21	5	56, 27	17, 8	76, 284	58, 65
8000	21	10	214, 141	14, 8	63, 536	60, 66
8000	100	5	14, 68	24, 55	30, 93	60, 28

Some of the highest and lowest changes in gene expression is highlighted in red, covering both increased expression (↑) and decreased expression (↓). The greatest changes in expression were observed for samples obtained from blood under room air at 8000 ft for 10 hr (↑63 and ↓536). A similar large change in gene expression was observed with lung under the same conditions (↑214 and ↓141). The elevated number of specific transcripts tends to be the highest following exposure to 8000 ft hypobaria under 21% O_2, likely due to mild hypoxia. The elevated transcripts seen following exposure to hypobaria for 10 hr is probably due to the time taken for stress to induce a more complete level of gene transcription. With few exceptions, the number of different transcripts in the heart was lower than that of other tissues (see e.g., ↑7 and 0) for zero altitude under 100% O_2 for 5 hr. Surprisingly, the number of transcripts present following exposure of rats to 100% O_2 was not particularly different than that seen under normoxic conditions (↑8 and ↓23) in lungs and heart (↑7 and ↓35). Finally, the changes in gene expression evoked by only 4000 ft altitude indicates that even very mild hypobaric hypoxia elicits the expression of genes that could have physiological effects that are important for flight crew performance, normal passengers, and air-evacuated patients. See (↑126 and ↓89) for heart following exposure to 4000 ft under 21% O_2 for 10 hr.

4.2 qPCR Validation

In order to validate the microarray results, certain DE gene transcripts were selected for qPCR analysis. Validation focused on the lung mRNA results. Candidates selected for validation were DE genes that either are known to be responsive to oxidative stress, such as

hypoxia inducible factor-3α (HIF-3α), oxidative stress induced growth inhibitor 1 (OSGIN1), and superoxide dismutase 3 (SOD3). or were identified as DE genes in 2 or more treatment groups. Gene expression was normalized to β-Actin (ACTB) and compared to baseline samples to determine fold-change. In total, 69% of the transcripts tested both agreed with the direction (increase or decrease) of the expression and exceeded the fold-change of the microarray analysis compared to baseline. 28% of transcripts matched with the direction of differential expression but the magnitude of the fold-change did not meet that of the microarray analysis. Only 1 transcript in 1 group completely contradicted the microarray results (Table 3). Considering the increased sensitivity of qPCR as compared to microarray, we concluded that these results are adequate to validate the microarray analysis.

Table 3: List of **lung** microarray results validated by qPCR. Microarray results are based on average fold change of the identified transcript in each treatment group compared to baseline (n-values found in Table 1); ACTB expression was used to normalize gene expression data. Arrows indicate an increase (↑) or decrease (↓) in gene expression compared to baseline.

Gene and direction	Biological group	Microarray results (fold change from baseline)	qPCR results (fold change from baseline)
HIF-3α ↑	100% O_2, 0 ft, 10 hr	1.622	2.6
OSGIN 1 ↑	100% O_2, 0 ft, 10 hr	1.534	2.096
SOD3 ↑	21% O_2, 8000 ft, 10 hr	1.669	1.09
NTRK2 ↑ or ↓	21% O_2, 4000 ft, 5 hr	2.604	2.899
	21% O_2, 4000 ft, 10 hr	2.879	3.67
	21% O_2, 8000 ft, 5 hr	11.875	14.572
	21% O_2, 8000 ft, 10 hr	12.122	16.607
	100% O_2, 0 ft, 10 hr	-2.458	-4.31
	100%, 4000 ft, 10 hr	-1.64	-2.985
ZBTB16 ↑	21% O_2, 8000 ft, 5 hr	1.667	-0.9
	21% O_2, 8000 ft, 10 hr	2.32	2.6
	100% O_2, 0 ft, 10 hr	2.218	1.9
	100% O_2, 4000 ft, 10 hr	2.144	1.645
TTPA ↓	21% O_2, 4000 ft, 5 hr	-1.605	-1.908
	21% O_2, 4000 ft, 10 hr	-1.799	-1.27
	21% O_2, 8000 ft, 5 hr	-1.624	-2.445
	21% O_2, 8000 ft 10 hr	-1.527	-2.041
FECH ↓	100% O_2, 4000 ft, 10 hr	-1.607	-2.012
NPR3 ↓	21% O_2, 8000 ft, 5 hr	-2.089	-3.663
	21% O_2, 8000 ft, 10 hr	-2.39	-5
SLC4A1 ↓	21% O_2, 4000 ft, 5 hr	-1.78	-2.577
	21% O_2, 8000 ft, 10 hr	-1.576	-2.353
	100% O_2, 0 ft, 5 hr	-1.597	-1.718
	100% O_2, 0 ft, 10 hr	-1.885	-2.237
	100% O_2, 4000 ft, 5 hr	-2.038	-1.802
	100% O_2, 4000 ft, 10 hr	-2.145	-2.681
ALAS2 ↓	100% O_2, 0 ft, 10 hr	-2.252	-1.543
	100% O_2, 4000 ft, 5 hr	-2.01	-1.429
	100% O_2, 4000 ft, 10 hr	-2.091	-1.667

4.3 Pathway Enrichment Analysis and Transcript Expression

Pathway enrichment analysis was performed across all groups to determine which pathways were the most responsive to the various treatments compared to the baseline group (Figure 2A, Figures 3-5). Although there is no obvious trend in the pathway enrichment caused by hyperoxia or hypobaria over time, pathways such as acute phase response signaling, the coagulation system, and the iron homeostasis signaling pathway were affected in multiple tissues. In the lung, for instance, iron homeostasis signaling and heme biosynthesis signaling are enriched in lungs exposed to hyperoxia while nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress signaling and integrin linked kinase (ILK) signaling are enriched at hypobaric altitudes. Additionally, specific transcripts do display a causal relationship with the treatments. For example, transferrin receptor (TFRC) shows increased expression in all groups that were exposed to 100% O_2 compared to those that received 21% O_2, meanwhile, hemojuvelin bone morphogenic protein co-receptor (HJV) shows slightly increased expression in the groups exposed to 8000 ft hypobaria (Figure 2B). Similarly, several actin genes displayed increased expression in response to 8000 ft hypobaria, while heme oxygenase 1 (HMOX1) showed a slight increase in expression for all treatment groups compared to baseline (Figure 2C), indicating an oxidative stress response in lung tissue. Similar observations can be made for the other tissues as well. Figure 3 lists the top 20 pathways enriched in the heart, Figure 4 lists the pathways in the blood, and Figure 5 lists the pathways in the hippocampus after the different treatments.

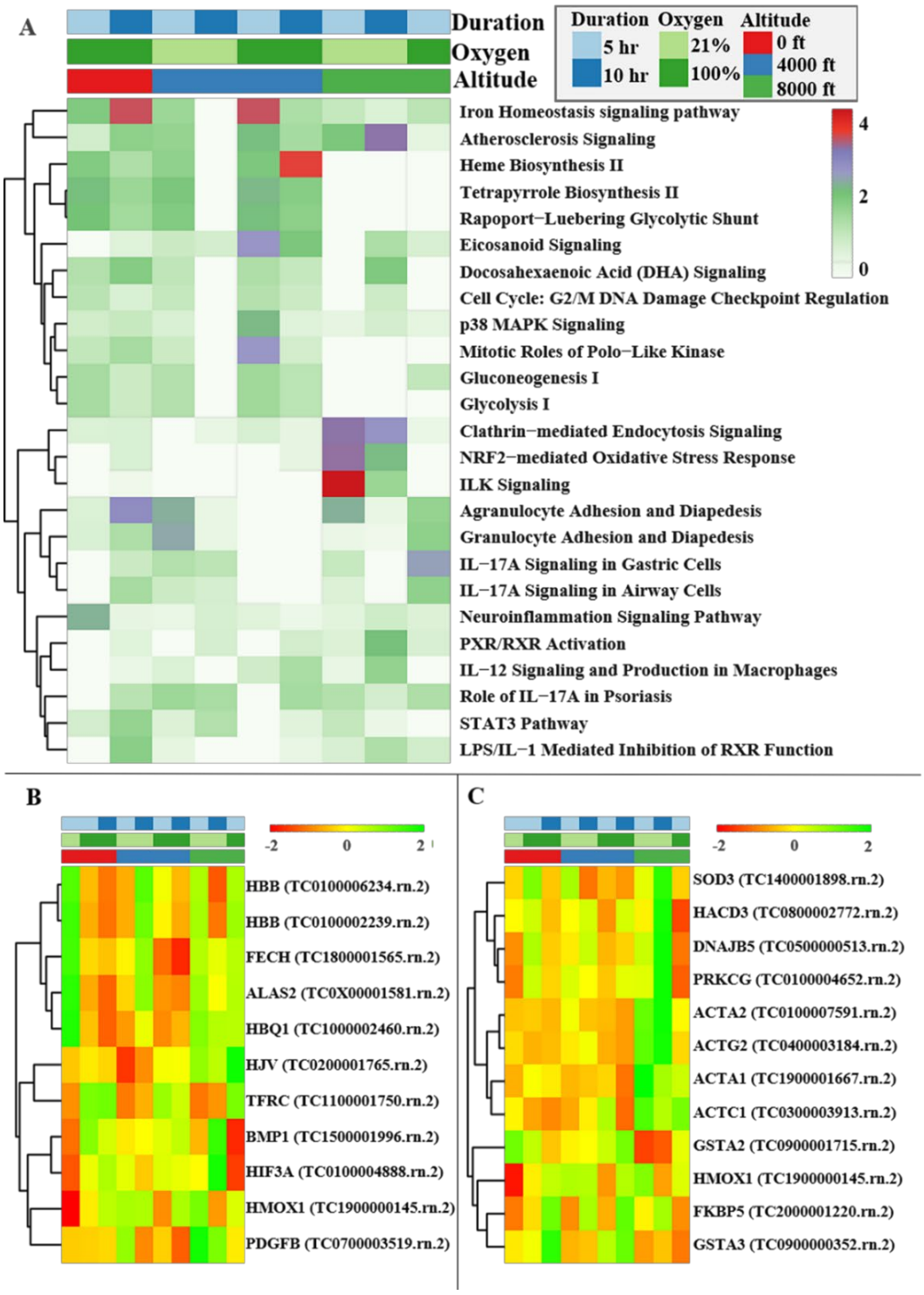


Figure 2: Heatmaps based on microarray analysis of lung RNA after indicated treatments. A) Canonical pathway enrichment based on differentially expressed transcripts from lung RNA. Treatment groups were compared to baseline (0 ft, 21% oxygen, 5 hr; not shown). Color gradient represents -$\log_{10}$(p-value). Individual heatmaps for B) Iron Homeostasis signaling pathway and C) NRF2-mediated oxidative stress response pathway. Transcripts are given on the right along with the probe-set ID corresponding to the specific isoform of that transcript. Color gradient represents gene expression.

Figure 3: Heatmap of canonical pathway enrichment based on differentially expressed transcripts in heart RNA. Treatment groups were compared to baseline (0 ft, 21% oxygen, 5 hr; not shown). Darker shading indicates greater significance.

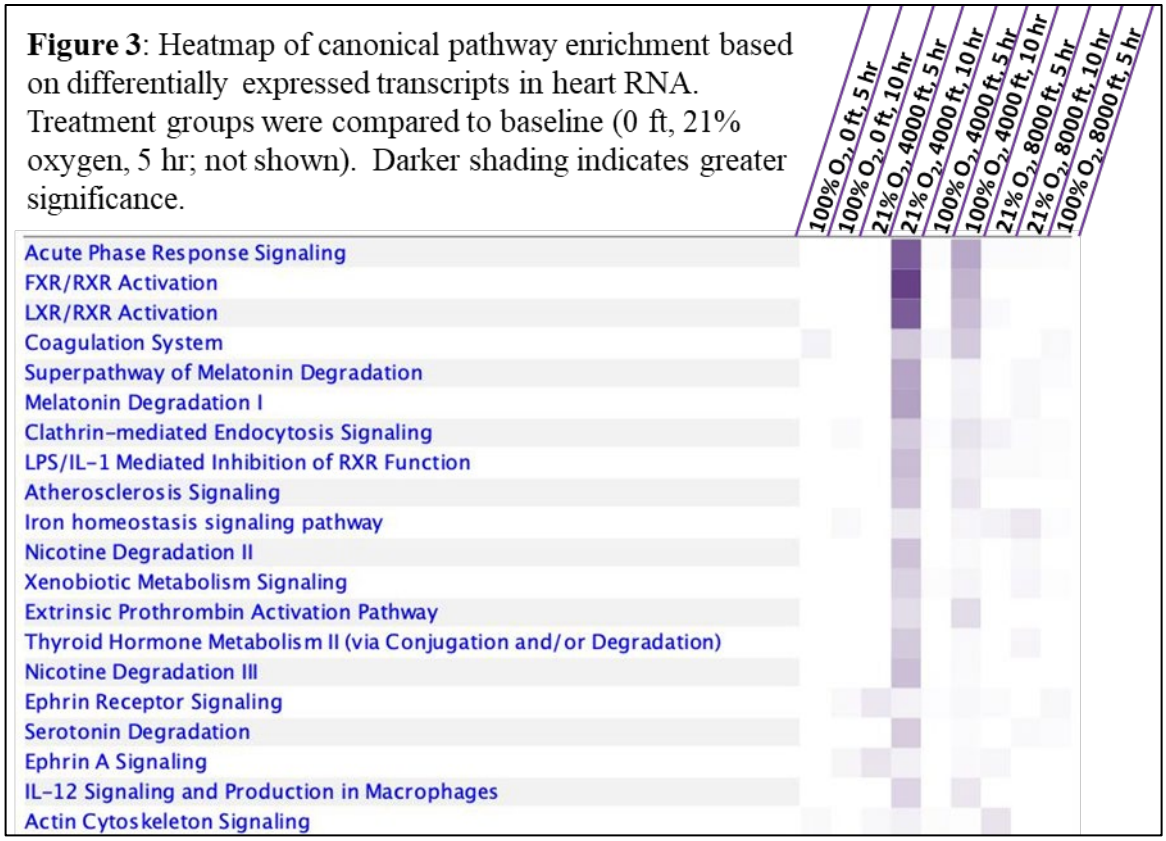
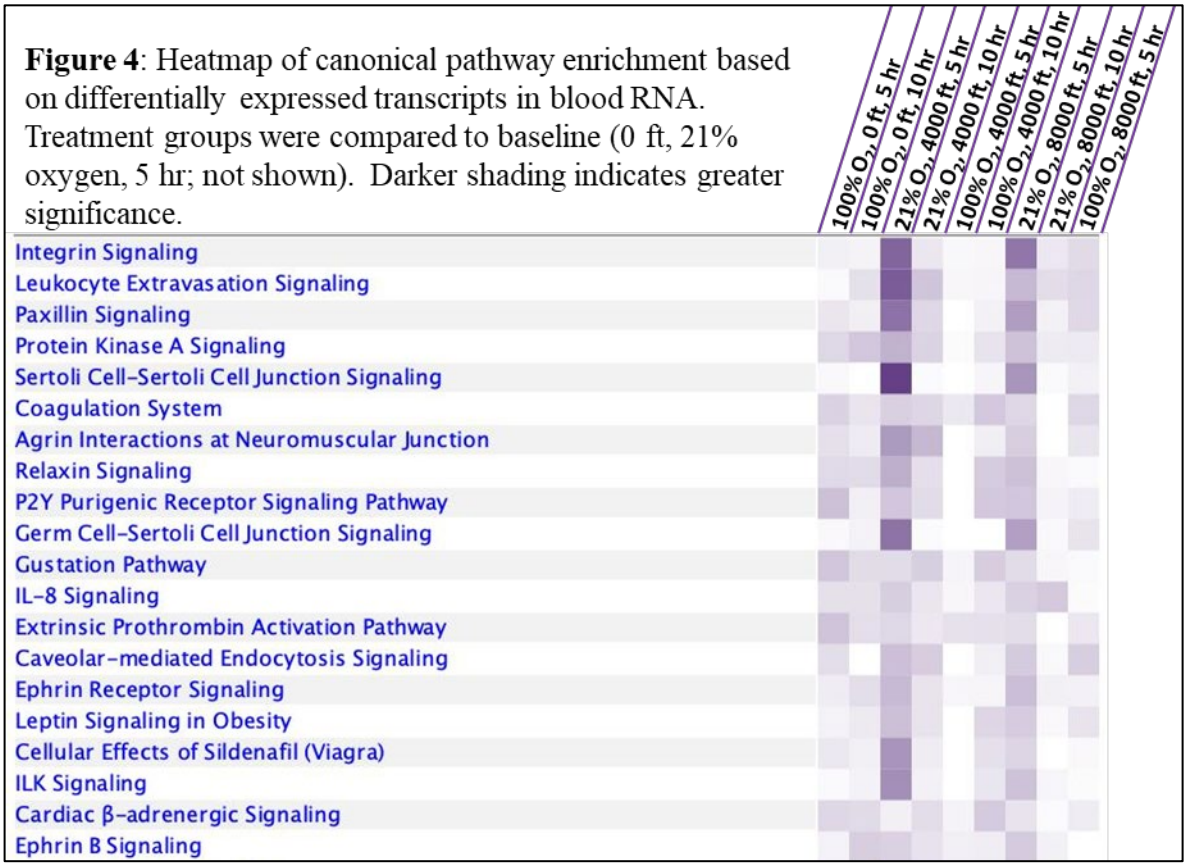


Figure 4: Heatmap of canonical pathway enrichment based on differentially expressed transcripts in blood RNA. Treatment groups were compared to baseline (0 ft, 21% oxygen, 5 hr; not shown). Darker shading indicates greater significance.



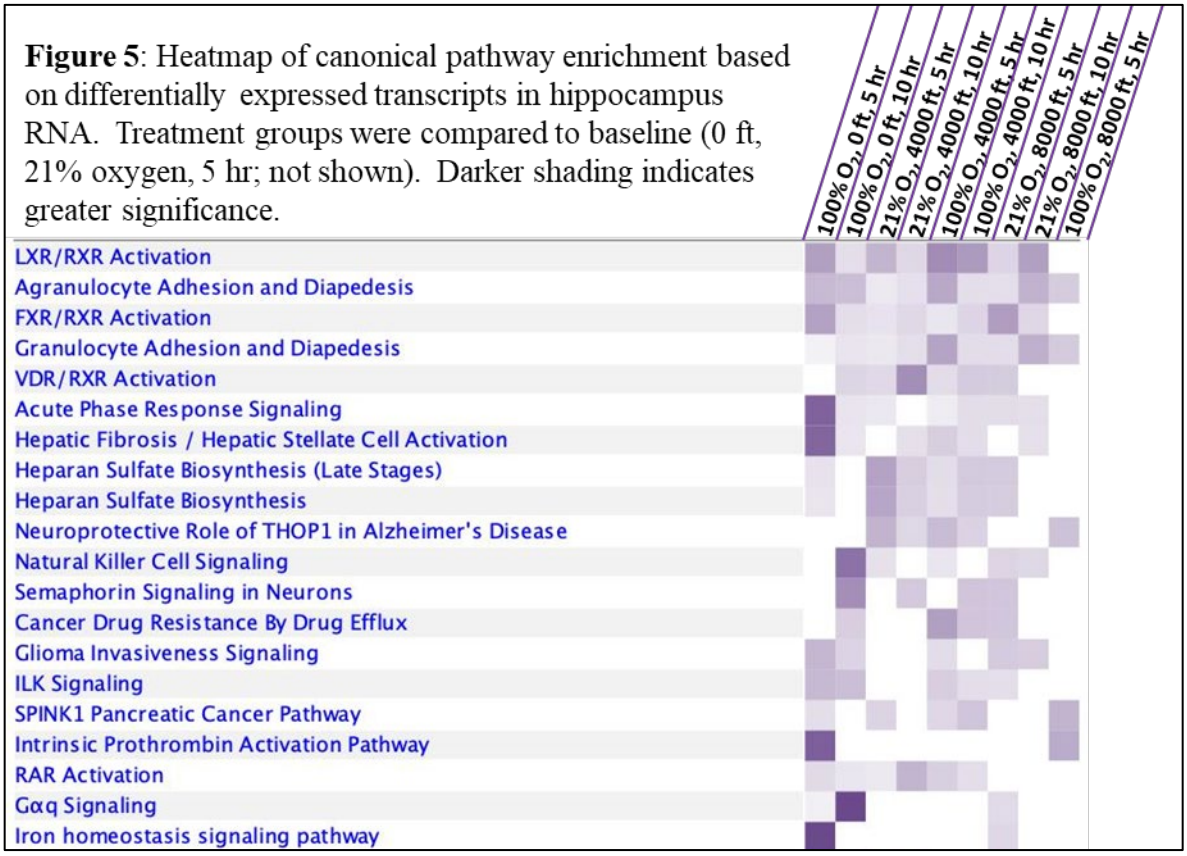


Figure 5: Heatmap of canonical pathway enrichment based on differentially expressed transcripts in hippocampus RNA. Treatment groups were compared to baseline (0 ft, 21% oxygen, 5 hr; not shown). Darker shading indicates greater significance.

5.0 DISCUSSION

During Operations IRAQI FREEDOM and ENDURING FREEDOM, explosive blasts were the primary cause of injury requiring CCAT evacuation (2), with over 80% of all sustained injuries resulting from explosive blasts (22). TBI was associated with 60-70% of these casualties (23, 24) and was a leading cause of death and disability. Over half of blast TBI patients requiring AE also suffered other forms of trauma (24). Lungs are particularly sensitive to concussive blasts (9, 25, 26), and so any further disruption to cellular processes would have added consequences. The lungs are an important aspect of regulating early inflammation. Through cellular crosstalk, alveolar epithelial cells regulate the activity of macrophages and neutrophils (27). Dysregulation of lung epithelial cells through oxidative damage exacerbates the inflammatory response, leading to the release of danger-associated molecular patterns that circulate throughout the body (28). Atmospheric conditions have an acute effect on lung reactive oxygen species (ROS) generation, which increases linearly with oxygen concentration (29) and may be similarly affected by decreased pressure (7, 9). Indeed, several of the pathways in Figure 2A have a connection to oxidative stress. The iron homeostasis signaling pathway is essential for redox homeostasis, and transcriptional expression of genes in this pathway shows down-regulation of hemoglobin subunit β (HBB), ferrochelatase (FECH), 5'-aminolevulinate synthase 2 (ALAS2), and hemoglobin subunit theta 1 (HBQ1), and upregulation of bone morphogenic protein 1 (BMP1), TFRC, and HIF3A under hyperoxia (Figure 2B). Injury-induced dysfunction

could cause a disruption in iron homeostasis, resulting in vulnerability to oxidative damage, inflammation, and even cell death (30).

The NRF2-mediated activation of activation of gene expression is a primary pathway responsible for elevating antioxidant defense activities in response to increased net production of ROS (Figure 2C). Atmosphere-induced lung ROS generation often occurs more quickly than in other tissues (26) and can lead to lung selective inflammation. Dayani et al (9) reported that rats exposed to 12 hr of 8000 ft hypobaria 48 hr after blast injury exhibited aggravated lung damage compared to rats maintained under normobaria. This effect of hypobaria was attributed to increased oxidative and nitrosative stress. Moreover, hyperoxia promotes ROS generation in a wide variety of trauma and acute care scenarios (15, 16). Even short exposures to hyperoxia can cause changes in protein levels that last for days (31). In a rat global cerebral ischemia model, hyperoxic reperfusion resulted in a greater neuronal death and cellular inflammatory reactions and behavioral deficits compared to normoxic reperfusion (32). These effects of hyperoxia are also observed in humans and are associated with higher mortality in TBI patients (33). The increase in NRF2-mediated gene expression in the lungs observed following hypobaria or hyperoxia indicates that these seemingly mild physiological stressors are sensed by the lung transcriptome. These responses also provide confidence that pharmacological activation of the NRF2 pathway by agents such as sulforaphane could protect the lungs from damage, as we have shown in the brain (34).

Although we have identified several transcripts that showed significant differential expressed in the analyzed tissues as a consequence of the relatively mild environmental exposures, our observation of no significant pathological trend in gene expression caused by hypobaria is similar to recent observations made by other groups. Arnaud et al (35) observed no difference in metabolic parameters caused by hypobaria in the blood of rats absent an initial injury. Meanwhile, although Dayani et al (9) did not observe significant differences in pro-inflammatory blood cytokines between sham and injured hypobaric rats, the level of anti-inflammatory cytokine interleukin 4 (IL-4) was significantly lower in rats that were injured. These observations point to the theory of the "second hit" of injury, in which the body is primed for an increased chance of secondary injury after an initial trauma (4, 36, 37) and that delayed evacuation could improve outcomes (5, 6, 8, 24, 38). Effects of this secondary hit can be noticed for days after hypobaric exposure or hyperoxia and have lasting implications for cognition (6, 8) and protein expression (31, 39). A recent comprehensive 7-year study showed that patients that suffered TBI and had delayed AE resulted in better outcomes and discharge characteristics than those evacuated more quickly (24). However, the standard of care for combatants that have suffered blast-induced TBI has been evacuation as soon as is feasible (2), so these observations stress the need to balance the timing of evacuation with the necessities of critical care.

This study has several limitations. The experiments only included young male Sprague Dawley rats. Future experiments should include additional species and direct comparisons between females and males. Analysis of the effects of hypobaria and hyperoxia on other tissues, e.g., liver, kidney, cerebral cortex, should also be conducted. The current study was limited to 2 exposure durations. Although gene expression was altered acutely through exposure to hypobaria and/or hyperoxia, later time points could further elucidate the effects these environments induce in the longer term as well as create a longer timeline in which to identify trends. Cho et al (40, 41) have shown that hyperoxia significantly altered lung transcriptomics in

mice, but after much longer exposures of at least 24 h. 10 hours is likely not long enough to observe all downstream effects of some genetic cascades, such as the cellular hypoxia response. Additionally, this study only analyzed the expression of genes immediately after the period of exposure to hypobaria / hyperoxia. Extractions of RNA and protein at periods up to one day after the exposure would provide further insight into the transcriptome and proteome generated by these comparisons.

Although a similar study with human subjects would be for the most part untenable, drawing blood from human passengers during flight and assaying gene expression from different time points would be clinically relevant. It would be informative to compare the human blood genomics results to the rat blood results, and these results may give insight into not only complications in injured patients during AE, but also the physiological responses of flight crews or medical personnel during flight-mediated hypobaria.

6.0 CONCLUSIONS AND WAY FORWARD

1. Exposure of rats to flight cabin relevant hypobaria as mild as 4000 ft for 5 hr elicits both an increase and a decrease in gene transcripts present in blood and vital organs.
2. The baseline transcriptome is blood and organ type specific.
3. The effects of ambient pressure, ambient O_2 concentration, and duration of exposure are blood and organ type specific.
4. The effects of hypobaria, hypoxia, and hyperoxia on the tissue-selective transcriptomes are generally greatest for blood and the least for the heart.
5. Transcriptomics should be expanded to cover the kidney, skeletal muscle, and cerebral cortex, which were obtained together with the other samples in this project.
6. Future research should focus on the effects of hypobaria on the transcriptomes present at 24 hr post-exposure, when more changes in gene expression occur compared to those at 5 or 10 hr post-exposure.
7. Proteomics should be used to identify proteins that are increased or decreased in association with similar changes in gene expression.
8. Transcriptomics and proteomics should be used in conjunction with our animal models of traumatic brain injury and polytrauma to better understand the molecular mechanisms responsible for the interplay between multiple organ dysfunction and neuropathology.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

↑	increased expression
↓	decreased expression
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACTB	Actin, β
AE	aeromedical evacuation
ALAS2	5'-aminolevulinate synthase 2
BMP1	bone morphogenic protein 1
CCAT	Critical Care Air Transport
cDNA	complementary DNA
CIBR	Center for Innovative Biomedical Resources
cRNA	complementary RNA
DE	differentially expressed
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
°C	degrees centigrade
ft	feet
FECH	ferrochelatase
g	gram
HBB	hemoglobin subunit β
HBQ1	hemoglobin subunit theta 1
HJV	hemojuvelin bone morphogenic protein co-receptor
HIF-3α	hypoxia inducible factor-3α
HMOX1	heme oxygenase 1
hr	hour
IDT	Integrated DNA Technologies, Inc.
IGS	Institute for Genome Sciences
IL4	interleukin 4
ILK	integrin linked kinase
IPA	Ingenuity Pathway Analysis
min	minute

mRNA	messenger ribonucleic acid
µg	microgram
NCBI	National Center for Biotechnology Information
ng	nanogram
NPR3	natriuretic peptide receptor 3
NRF2	nuclear factor erythroid 2-related factor 2
NTRK2	neurotrophic tyrosine kinase, receptor, type 2
O_2	oxygen
OSGIN1	oxidative stress induced growth inhibitor 1
PCA	Principal Component Analysis
%	percent
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity number
RMA	Robust Multichip Average
RNA	ribonucleic acid
RNase H	ribonuclease H
ROS	reactive oxygen species
s	second
SLC4A1	solute carrier family 4 (anion exchanger)
ss-cDNA	single-stranded complementary DNA
SOD3	superoxide dismutase 3
T_a	annealing temperature
TBI	traumatic brain injury
TFRC	transferrin receptor
TTPA	tocopherol (alpha) transfer protein
UMB	University of Maryland Baltimore
ZBTB16	zinc finger and BTB domain containing 16

**Note that all abbreviations for specific genes and genomic pathways can be found in the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/*