Urinary Metabolic modulation in human participants residing in Siachen base camp: A ^1H NMR Metabolomics Approach

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Abstract

The main physiological challenge in high altitude environment is hypoxia which affects the aerobic metabolism reducing the energy supply. These changes may further progress towards extreme environment related diseases. These are further reflected in changes in small molecule metabolites and metabolic pathways. In the present study, metabolic changes due to chronic environmental hypoxia were assessed using ¹H NMR metabolomics by comparing the urinary metabolic profile of 70 control soldiers residing at sea level with 40 soldiers residing in Siachen base camp (3700 m) for one year. Multivariate statistical analysis was carried out and PLSDA was able to detect 15 metabolites based on VIP score > 1. ROC analysis was able to detect cis-aconitate, Nicotinamide Mononucleotide, Tyrosine, Choline and Creatinine metabolites with high range of sensitivity and specificity. Pathway analysis revealed 16 pathways impact > 0.05 and phenylalanine tyrosine and tryptophan biosynthesis was the most prominent altered pathway indicating metabolic remodeling to meet the energy requirements. TCA cycle, Glycine serine and Threonine metabolism, Glutathione metabolism and Cysteine alterations were other metabolic pathways which were affected during long-term high-altitude hypoxia exposure. Present findings will help in unlocking new dimension for potential application of NMR metabolomics to address extreme environment related health problems, early detection and developing strategies to combat high altitude hypoxia.

Key Points

- ¹H NMR based metabolomics as a sensitive & early predictive platform to deduce Urinary Metabolic modulation in human participants residing in Siachen base camp & extreme environment.
- Cis-aconitate, Nicotinamide Mononucleotide, Tyrosine, Choline and Creatinine can be the fingerprint markers for early detection of high-altitude hypoxia related illnesses.
- Findings unveil the metabolic pathways which plays a crucial role in high altitude adaptations and needs to be monitored.
- Present findings open a new dimension for designing strategies to sustain high altitude exposure, medical management, and treatment related to high altitude maladies.

Introduction

High altitude environment main physiological challenge is characterised by low oxygen & low-pressure levels resulting in hypoxia^{1,2}. This causes reduced arterial partial pressure & oxygen saturation resulting in physiological hypoxic condition to tissues & organs leading to high-altitude pulmonary edema (HAPE) & High-altitude cerebral edema (HACE)³⁻⁶. This condition further reduces the energy supply & weakens aerobic metabolism². Various metabolic pathway changes are involved during oxygen deficiency, which is reflected in low molecular metabolites changes^{7,8}. In case of environmental hypoxia, the focus of attention is to reduce the resulting damage to the biological systems⁹. Present literature indicates lack of reactive oxygen species and oxidative stress resulting in alterations in metabolic pathways^{10,11}. Studies

are being carried out to understand the pathophysiology of health-related issues due to hypoxia but its early diagnosis still remain a challenge leading to delay in treatment and worsening of the disease^{12,13}. Early predictive biomarkers can help in identifying military personnel and people who are sensitive to environmental hypoxia thereby helping in initial screening and deciding the line of treatment^{14,15}.

Metabolomics is the technique which focuses on changes in the metabolites level under any kind of stress or diseased state and has been widely used to identify early predictive markers for hypoxia, pharmacology, medical sciences, and various other fields^{16–21}. It is a top-down approach which provides systemic understanding of biochemical and physiological status of individual or population. At global scale, metabolic profiling can identify metabolic changes in response to genetic differences, environmental influences and disease or drug perturbations. NMR spectroscopy has been extensively used to detect a wide range of low molecular weight endogenous metabolites in body-fluids and tissue extracts as it is rapid, non-destructive, rich in quantitative information and allows simultaneous investigation of the predefined set of metabolites^{22,23}.

Present studies are aimed at identifying early predictive metabolic biomarkers and understanding the pathophysiological mechanisms involved in acclimatization to high altitude hypoxia^{24,25}. This will open a metabolomics window for future studies, new drug development and personalized medicine for environmental hypoxia.

Materials & Methods

Participants. The study protocol was approved by institutional human ethics committee (IEC Ref No. IEC/DIPAS/C-11A/2) and all methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all the participants prior to study. Total of 70 controls soldiers residing in sea level and 40 soldiers (after normalizing & filtering the data) residing in Siachen base camp (3700 m) for one year were taken for the study. Urine samples were collected from control and Siachen group after 12hrs fasting and stores at -80°C for further NMR acquisition and analysis.

¹H-NMR spectroscopic analysis. Human urine samples were thawed, and 400 mL were mixed with 200 mL of deuterated 0.2 M sodium phosphate buffer solution (pH 7.4 containing 1 mM TSP prepared in D₂O). The TSP acts as a chemical shift reference ($\delta = 0$) and D₂O as locking agent. The samples were then centrifuged at 5000 rpm for 5 min to remove particulate contaminants. The supernatant was poured into NMR tube and NMR spectral data were acquired on a Bruker Avance III HD spectrometer (Bruker, Germany) with TXI probe operating at a frequency of 600.1 MHz at 298 K. A standard one-dimensional water pre-saturation pulse sequence (NOESYPR1D) was used to suppress water signal. A total 64 scans (acquisition time 1.7 s/scan) were collected into 64 k data points and the spectral width of 9615.4 Hz with a relaxation delay of 2 s. The raw FIDs were weighted by an exponential function with a 0.3 Hz line broadening factor, followed by Fourier transformation. The resulted NMR spectra of each sample were manually corrected for phase and baseline using Bruker Topspin 3.6.4 (Bruker, Germany) and the

reference peak (TSP) was set to $\delta = 0$, followed by visual inspection of the spectra from control and high-altitude Siachen group. The data was pre-processed using topspin software and individual metabolites were identified with the help of published literature using Human Metabolome Database (HMDB).

Data reduction and pattern recognition (PR) analysis. The statistical expression of different metabolites in control and Siachen group were analyzed using multivariate statistical analysis. The multivariate analysis was performed in a total of 110 spectra of which 70 are from the control sea level group and 40 from the Siachen group after normalizing & filtering the data. All spectra were phased and corrected manually for baseline using TOPSPIN 3.6.4 (Bruker, Germany). The corrected NMR spectra with spectral range of δ 0.5–9.5 were binned in AMIX (Bruker, Biospin, Germany) and all the spectra were segmented into binned region of equal width of 0.01 ppm. Prior to any statistical analysis, regions for water (4.5–5.0 ppm) and urea (5–6.0 ppm) were eliminated. Normalization by sum was carried out to reduce the possible differences in concentration between samples. Following normalization, auto-scaling (mean-centering and division by the square root of the standard deviation of each variable) was carried out to give all variables equal weight. The pre-processing is done to eliminate the possible bias arising either due to sample variability or handling. The binned data were saved in .csv format and uploaded to the online tool metaboanalyst 5.0. After data pre-processing, an unsupervised pattern recognition (PR) method PCA was carried out to detect systemic variation and intrinsic clusters within the data set. Based on PCA, the spectral region & bins differentiating between controls and Siachen group were identified, integrated and the relative intensity of each of the identified metabolites was calculated. Relative intensities were then further used to perform the univariate and supervised multivariate analysis. The fitting was assessed by R^2 (explained variance) and Q^2 (predictive ability) using leave one-out cross validation procedure. The class dependent clusters were studied by PLS-DA score plots. The weighted sum of PLS regression coefficients were calculated to identify top 30 important buckets that had a maximum contribution to the separation of clusters in PLS-DA and assigned with corresponding metabolite. The concentrations of metabolites, corresponding to top 30 important buckets, from samples of controls and Siachen group were calculated by integration of individual peaks normalized by total spectra, which were plotted as violin plots. A supervised PR method sPLS-DA and OPLS-DA was done to achieve maximum separation of control and Siachen group. Additionally, univariate analysis such as for fold change and correlation analysis was also carried out for the identified metabolites. Statistical t-test was performed and p values and FDR values for significantly different metabolites was calculated. Finally, based on t-test, p value, False discovery rate (FDR), correlation coefficient, VIP and ROC plot, the specific metabolite responsible for differentiation of control and Siachen group were identified. The methodology is part of standard protocols followed by our group and has been mentioned in earlier work Koundal et al.^{25,54}

Results

NMR Metabolomics for urine of high-altitude Siachen subjects. Comparative ^1H NMR spectra for urine of control and Siachen group are shown in Fig. 1. Various multivariate statistical analysis was carried out to

differentiate control and high-altitude hypoxia group which further assessed the capability of ^1H NMR based metabolomics approach for early prediction in such scenarios. Principal component analysis (PCA) score plot showed clear separation between control & Siachen group (Fig. 2). Loading plots from PCA were generated to identify the metabolites which lied far away from the origin and were responsible for the separation between the two groups. Statistical t-test was performed, and it was identified that 23 metabolites (Fig. 3) were found to be significantly different (Table 1). These data were further used to perform supervised clustering, orthogonal partial least square discriminant analysis (OPLSDA) (Fig. 4A) and sparse partial least square discriminant analysis (sPLSDA) (Fig. 4B). By comparing the scores & loading plots, biplot was generated which identified the relationship between samples and metabolites. Biplot for control and Siachen group (Fig. 5) gave an overview between samples and metabolites where samples were displayed as points and metabolites were displayed as vectors. Scree plot as showed in Fig. 6 was generated for the first five Principal components (PCs) to identify the contribution of each of the principal components to the total variation in the data set. The green line showed the cumulative variance explained by the first five components which accounted for 54% whereas blue line indicated the individual variance for each principal component. PLS-DA revealed the most discriminate variables which were responsible for clustering pattern (Fig. 7A). PLS-DA model quality parameters; R^2 and Q^2 , greater than 0.5 indicates a model with reasonable fit with good predictive power. The cross-validation analysis with first five components produced a strong model with high validated predictability ($Q^2 = 0.91$), goodness of fit value ($R^2 = 0.78$) and accuracy (accuracy = 0.96) (Fig. 7B).

Table 1

List of 23 Key metabolites obtained from ^at-test responsible for discriminating sea level control group and Siachen group. ^bp values were derived from two-tailed Student's t test. ^cFDR values were calculated which indicates false discovery rates.

	^a t.stat	^b p.value	-log ₁₀ (p)	^c FDR
Cisaconitate	-14.556	6.19E-27	26.208	2.23E-25
NMN	-12.544	1.32E-22	21.88	2.37E-21
Tyrosine	-9.4024	1.34E-15	14.872	1.61E-14
Choline	-9.1794	4.24E-15	14.372	3.82E-14
Creatinine	7.6818	8.65E-12	11.063	6.23E-11
Isoleucine	-7.5397	1.76E-11	10.755	1.05E-10
Alanine	-6.6677	1.24E-09	8.9054	6.39E-09
Cysteine	6.2969	7.19E-09	8.1432	3.24E-08
dihydroxymandalate	-5.8819	4.88E-08	7.3118	1.95E-07
Allantoin	-4.9027	3.46E-06	5.4609	1.25E-05
Acetoacetate	-4.0759	8.93E-05	4.0491	0.000292
Lactate	-3.8325	0.000216	3.6647	0.000649
Sarcosine	-3.5301	0.000618	3.2089	0.001594
TMAO	3.5292	0.00062	3.2076	0.001594
Succinate	3.4421	0.00083	3.0809	0.001992
indoxylsulphate	3.1382	0.002207	2.6563	0.004965
methylhistidine	2.8163	0.005804	2.2363	0.01229
dimethylamine	2.6066	0.010473	1.9799	0.020946
Glycine	-2.5009	0.013932	1.856	0.026398
Acetate	2.4159	0.017424	1.7589	0.031363
dimethylglycine	-2.3599	0.020127	1.6962	0.034503
hydroxybutyrate	-2.283	0.024446	1.6118	0.040002
urocanate	2.2182	0.028696	1.5422	0.044915

There were 23 metabolites that were identified by PCA as important metabolites. PLS-DA was able to detect 15 metabolites which were significantly different based on VIP score > 1 (Fig. 7C). Violin plots

(Fig. 8) for these 15 metabolites between control & Siachen group showed increase & decrease of various metabolites as depicted in Table 2. Further Receiver Operating characteristics (ROC) curve analysis was carried out to evaluate the performance of metabolites as biomarkers. Five metabolites namely cis-aconitate, Nicotinamide mononucleotide, tyrosine, choline & creatinine had area under the curve (AUC) > 0.90 indicating high range of sensitivity and specificity (Table 2).

Table 2

List of 15 Key metabolites responsible for discriminating discriminating sea level control group and Siachen group. ^aVariable Importance in the projection (VIP) was obtained from OPLS DA with a threshold of 1.0 ^bAUC is the area under the curve indicating sensitivity & specificity of each metabolite. Positive values (↑) indicate higher levels in Siachen group and negative values (↓) indicate lower levels in Siachen group.

	^a VIP	HMDB	^b AUC	increase ↑
Cisaconitate	2.1597	HMDB0000072	0.93478	↑
NMN	1.8782	HMDB0000229	0.96911	↓
Tyrosine	1.7805	HMDB0000158	0.89436	↑
Choline	1.756	HMDB0000097	0.90656	↓
Creatinine	1.5431	HMDB0000562	0.91037	↓
Isoleucine	1.5348	HMDB0000172	0.81236	↑
Cysteine	1.4294	HMDB0000574	0.86384	↓
Alanine	1.4138	HMDB0000161	0.85126	↓
dihydroxymandalate	1.3182	HMDB0001866	0.82914	↓
Allantoin	1.2858	HMDB0000462	0.81808	↓
Lactate	1.0761	HMDB0000190	0.84249	↓
Acetoacetate	1.065	HMDB0000060	0.79291	↓
Succinate	0.84182	HMDB0000254	0.69908	↓
Sarcosine	0.83407	HMDB0000271	0.75286	↓
TMAO	0.80072	HMDB0000925	0.65294	↓

Metabolic pathway analysis for differentially expressed metabolites. To get an insight of metabolic pathways getting altered due to extreme environment, pathway analysis was carried out. Out of 29 metabolic pathways analysed, 16 pathways with pathway impact > 0.05 were considered. Succinate, cis-aconitate, citrate, pyruvate and fumarate were the metabolites of TCA cycle out of 20 metabolites found to be altered between control and Siachen group (Fig. 9). Phenylalanine, tyrosine and tryptophan

biosynthesis was the most prominent pathways found to be altered at extreme environment with impact value of 1.

KEGG databases from differential metabolites revealed 16 metabolic pathways between sea level and Siachen group which were distinctly different. These metabolites were found to be primarily involved in Phenylalanine, tyrosine and tryptophan biosynthesis, Glycine, serine and threonine metabolism, TCA cycle, Tyrosine metabolism, Glyoxylate and dicarboxylate metabolism, Glutathione metabolism, Nicotinate and nicotinamide metabolism, Phenylalanine metabolism, Cysteine and methionine metabolism, Taurine and hypotaurine metabolism, Butanoate metabolism, Pyruvate metabolism, Synthesis and degradation of ketone bodies, Glycolysis / Gluconeogenesis, Histidine metabolism, Arginine biosynthesis (Table 3). Debiased Sparse Partial Correlation Network Modeling (DSPC) and Visualization using the Network Explorer Module was carried out to visualize partial correlation networks using a data-driven network approach, DSPC reconstructs a network and calculates partial correlation coefficients and P-values for each pair of metabolic features. Figure 10 shows the results which can be visualized as weighted networks, where nodes represent the metabolic features, and the edges depict the correlations among them²⁶. The results are visualized as a weighted network and the thicker the edges, the stronger the correlation between various metabolites and pathways (The red edges represent positive correlations, while the blue edges represent negative correlations).

Table 3
KEGG Pathways of significantly different metabolites between sea level group & high-altitude Siachen group.

Id	Pathway Name	Compound name	KEGG ID	HMDB
1	Phenylalanine, tyrosine and tryptophan biosynthesis	L-Phenylalanine	C00079	HMDB0000159
		L-Tyrosine	C00082	HMDB0000158
2	Glycine, serine and threonine metabolism	Choline	C00114	HMDB0000097
		N,N-Dimethylglycine	C01026	HMDB0000092
		Sarcosine	C00213	HMDB0000271
		Glycine	C00037	HMDB0000123
		Cysteine	C00097	HMDB0000574
		Pyruvate	C00022	HMDB0000243
3	Citrate cycle (TCA cycle)	Succinate	C00042	HMDB0000254
		Citrate	C00158	HMDB0000094
		cis-Aconitate	C00417	HMDB0000072
		Pyruvate	C00022	HMDB0000243
		Fumarate	C00122	HMDB0000134
4	Tyrosine metabolism	3,4-Dihydroxymandelate	C05580	HMDB0001866
		L-Tyrosine	C00082	HMDB0000158
		Fumarate	C00122	HMDB0000134
		Pyruvate	C00022	HMDB0000243
		Acetoacetate	C00164	HMDB0000060
5	Glyoxylate and dicarboxylate metabolism	cis-Aconitate	C00417	HMDB0000072
		Citrate	C00158	HMDB0000094
		Glycine	C00037	HMDB0000123
		Acetate	C00033	HMDB0000042
		Pyruvate	C00022	HMDB0000243
		Formate	C00058	HMDB0000142

Id	Pathway Name	Compound name	KEGG ID	HMDB
6	Glutathione metabolism	Glycine	C00037	HMDB0000123
		L-Cysteine	C00097	HMDB0000574
7	Nicotinate and nicotinamide metabolism	Nicotinamide D-ribonucleotide	C00455	HMDB0000229
8	Phenylalanine metabolism	L-Phenylalanine	C00079	HMDB0000159
		Hippurate	C01586	HMDB0000714
		L-Tyrosine	C00082	HMDB0000158
9	Cysteine and methionine metabolism	Cysteine	C00097	HMDB0000574
		Pyruvate	C00022	HMDB0000243
10	Taurine and hypotaurine metabolism	L-Cysteine	C00097	HMDB0000574
		Taurine	C00245	HMDB0000251
11	Butanoate metabolism	Acetoacetate	C00164	HMDB0000060
		Succinate	C00042	HMDB0000254
12	Pyruvate metabolism	Pyruvate	C00022	HMDB0000243
		(S)-Lactate	C00186	HMDB0000190
		Acetate	C00033	HMDB0000042
		Fumarate	C00122	HMDB0000134
		Acetoacetate	C00164	HMDB0000060
14	Glycolysis / Gluconeogenesis	Pyruvate	C00022	HMDB0000243
		(S)-Lactate	C00186	HMDB0000190
		beta-D-Glucose	C00221	HMDB0000122
		Acetate	C00033	HMDB0000042
15	Histidine metabolism	Urocanate	C00785	HMDB0000301
		N(pi)-Methyl-L-histidine	-	-
16	Arginine biosynthesis	L-Citrulline	C00327	HMDB0000904
		Fumarate	C00122	HMDB0000134

Discussion

In current study urinary metabolic profiling was carried out between sea level soldiers and soldiers stationed at Siachen base camp (3700 m). It is a well-known fact that travelling to elevation is associated with risk of developing AMS, HACE and HAPE^{27,28}. Hypobaric hypoxia elicits series of physiological responses that are highly variable in humans²⁹⁻³¹. For military personnel, high altitude sickness such as AMS HAPE HACE can compromise occupational performance and pose serious health risks. Current method of screening for these illnesses relies on physical examination, oxygen saturation, evaluation of symptoms etc. No reliable technique for early diagnosis is currently being used. Study of metabolic markers will be effective in understanding of pathophysiological mechanism, early diagnosis, prognosis, and effective treatment. Out of various Omics techniques, NMR based metabolomics have potential of identifying fingerprint markers for early diagnosis. The finding of the study suggests levels of wide variety of amino acids being altered during high altitude adaptation.

Phenylalanine, Tyrosine & Tryptophan biosynthesis/ Tyrosine metabolism

Changes in amino acid were observed reflecting metabolic remodeling to meet the energy requirements. Amino acid concentration alteration might reflect utilization of glucogenic substrates. Altitude exposure results in stress on the body to maintain oxygen driven energetic pathways and redox homeostasis. Tyrosine metabolism was found to be affected during high altitude adaptation. Reduction in ATP production due to inhibition of citrate cycle induced by high altitude hypoxia could lead to utilization of branched chain amino acids such as tyrosine as energy compensation. Phenylalanine, Tyrosine & Tryptophan biosynthetic signalling pathways was affected as a response to high altitude adaptation. Levels of Tyrosine increased which might be due to replenishment of insufficient energy supply to adapt to hypoxic stress. Phenylalanine metabolism was affected as it needs to be converted to Tyrosine which further helps in regulating oxidative stress, immune response & inflammation to protect damage³²⁻³⁴.

TCA cycle

It is a known fact that hypoxia affects the cellular ATP production through downregulation of several TCA cycle enzymes and compromising electron transport chain complexes. Reduction in ATP production due to inhibition of TCA cycle induced by high altitude hypoxia could lead to utilization of branched chain amino acids as energy resource. Cis-aconitate was altered which is an intermediate in TCA cycle. In TCA cycle, citrate undergoes stereospecific isomerization to isocitrate by the enzyme aconitase hydratase and intermediate cis-aconitate. Aconitase affects the conversion of citrate to iso-citrate through an intermediate cis-aconitate. Itaconate metabolite is derived from TCA cycle intermediate cis-aconitate which is identified as anti-inflammatory signal and shown to upregulate cellular antioxidant defenses via induction of Nrf2³⁵⁻³⁸.

Glycine, serine & Threonine metabolism

Glycine, serine & Threonine are important amino acids for various amino acid metabolism pathways. Serine is made from phosphoglycerate and degraded to pyruvate whereas glycine is made from serine with multiple pathways of degradation. Both TCA cycle and amino acid metabolism are linked as there is

reduction in ATP production due to inhibition of TCA cycle induced by high altitude hypoxia which leads to utilization of branched chain amino acids as energy resource^{2,39,40}.

The alterations in the choline and macromolecules level may indicate compromised cell membrane metabolism or cellular damage. The large part of cholines in biological systems occur in the form of immobile phospholipids and cell membranes. Choline levels were decreased in response to high altitude adaptation, which gives insights of altered metabolism of cholines and high choline demands in the cells and brain, as it forms the major precursor of neurotransmitters and cellular membranes^{41,42}.

Creatinine is a waste product that the muscles produce at a steady rate as part of normal daily activity. The bloodstream carries creatinine to the kidneys, which filter it out of the blood through urine. The kidneys play an important role in human adaption to high altitude, during acclimatization and in mountain sickness illnesses by helping in regulating body fluids, electrolyte and acid–base homeostasis. Creatinine levels were found to be decreased at high altitude due to decreased glomerular filtration rate which in turn was the result of changes in tissue oxygenation, renal blood flow and adaptive response of renal tissue. At chronic high-altitude exposure, vascular remodelling or enlargement was observed which resulted in increased erythropoietin production thereby increasing the red blood cell mass and haemoglobin to improve the oxygen carrying capacity of blood. The magnitude and duration of exposure to high altitude affects glomerular filtration rate, renal blood flow, renal plasma flow, and filtration fraction^{43,44}.

Isoleucine which is a branched chain amino acid (BCAA) and primary mediator of alanine and glutamine biosynthesis was increased during high altitude adaptation. BCAA catabolism begins in skeletal muscle with transamination of α -ketoglutarate yielding branched chain ketoacids which are further oxidised as succinyl co-A in TCA cycle. BCAA plays a vital role in energy metabolism and its increase is an indication of impaired energy metabolism resulting from oxidative stress and impaired mitochondrial respiration. During high altitude exposure it is known that there is loss of fat free mass due to decreased physical activity, sleep cycle disruption, cold exposure, hypoxia, and changes in protein metabolism. As an adaptation to maintain muscle mass, there is an increase in BCAA isoleucine to compensate the increased energy requirements at high altitude^{39,45,46}.

Alteration in alanine metabolite was observed which is a critical gluconeogenic α -amino acid delivering carbon from amino acid degradation in peripheral tissues and skeletal muscles^{47,48}. Alanine is constituent of all proteins/peptides and it can act as energy fuel via pyruvate oxidation released by transamination of alanine. Alanine gets converted to biomolecules such as pyruvate, 2-oxoglutarate and fumarate which enters the TCA cycle to meet the ATP depletion and energy requirements which further promotes high altitude acclimatization.

Glutathione metabolism and cysteine alterations

Glutathione is most important component in antioxidant defence systems and gives a measure of level of oxidative stress. It acts as a scavenger for free radicals. Regulation of Glutathione synthesis is highly dependent on intra cellular availability of L-cysteine. During high altitude exposure there is an increase in free radical production which might require more glutathione which in turn is generated by cysteine thereby resulting in decreased level of cysteine^{49,50}.

There was a decrease in lactate concentration that was observed with acclimatization to high altitude. This might be explained by lactate paradox phenomena which is linked to muscle oxygen delivery and energy demand at high altitude. This in turn is linked to upregulated control contributions from cellular ATP demand and supply pathways^{51,52}.

Trimethylamine N-oxide (TMAO) is an important gut microbe dependant metabolite generated from choline, betaine and L-carnitine, metabolized to trimethylamine (TMA) through gut microbiota metabolism. Alterations in TMAO indicates disturbed gut microbiota at high altitude⁵³.

Nicotinamide adenine dinucleotide (NAD⁺) levels in the body is associated with downregulation of energy production in mitochondria, oxidative stress, DNA damage, cognitive impairment and inflammatory conditions. NMN is the precursor of NAD⁺ and under hypoxic conditions, NMN can slow down this process by elevating NAD⁺ levels in the body. Hence, a decrease in NMN levels were observed under long-term high-altitude hypoxia to prevent the damage.

Conclusion

Present study is first to profile the systemic metabolic alterations due to chronic high-altitude hypoxia in humans using NMR based Metabolomics. The outcome of the study demonstrates NMR metabolomics as powerful platform to understand metabolic adaptations and maladaptation due to progressive altitude exposure. It is evident from the findings that cis-aconitate, Nicotinamide Mononucleotide, Tyrosine, Choline and Creatinine can be the fingerprint biomarkers for early detection of high-altitude hypoxia related illnesses. Findings unveil the metabolic pathways which plays a crucial role in high altitude adaptations and needs to be monitored. The outcome and findings of current study demonstrates the potential of metabolomics to provide detailed information on physiological adaptations, early diagnosis, prognosis, and effective treatment for high altitude maladies.

Abbreviations

¹H NMR Proton Nuclear Magnetic Resonance Spectroscopy; HH Hypobaric Hypoxia; HAPE high-altitude pulmonary edema; HACE High-altitude cerebral edema; AMS Acute Mountain Sickness; TSP Trimethylsilyl-2,2,3,3- tetra-deuteriopropionic acid; D₂O deuterium oxide; TXI Triple Resonance Probe; FID Free Induction Decay; HMDB Human Metabolome Database; PR Pattern Recognition; PCA Principle Component Analysis; PLS-DA partial least squares discriminant analysis; OPLS-DA Orthogonal Projections to Latent Structures Discriminant Analysis; VIP Variable importance in projection; ROC

Receiver Operating Characteristic; sPLSDA sparse partial least square discriminant analysis; PC Principle Component; AUC Area under the curve; TCA Tri carboxylic acid; ATP Adenosine Triphosphate; BCAA branched chain amino acid; TMAO Trimethylamine N-oxide; HMDB Human metabolome database; NOESY Nuclear Overhauser Effect Spectroscopy

Declarations

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The authors report no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author Dr. Sonia Gandhi. The data are not publicly available due to DRDO being part of Ministry of Defence, Government of India, sharing of data through FTP server is not allowed due to security reasons.

Author contributions

S.G. designed the study, involved in experimentation, analysis, interpretation of data and writing of manuscript. K.B. conceived the project & designed the study. I.G. involved in experimentation and analysis of data. R.S.K. conceived the project and evaluated the manuscript critically and all the authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

References

1. West, J. B. Recent advances in high altitude medicine and biology. *High Alt Med Biol* **16**, 73 (2015).
2. Chang, Y. *et al.* Metabonomics window into plateau hypoxia. *Journal of International Medical Research* **47**, 5441–5452 (2019).
3. Luks, A. M., Swenson, E. R. & Bartsch, P. Acute high-altitude sickness. *Eur Respir Rev.* **26**, 143 (2017).
4. Luks, A. M. *et al.* Wilderness Medical Society consensus guidelines for the prevention and treatment of acute altitude illness. *Wilderness Environ Med.* **21**, 146–155 (2010).
5. Richalet, J. P. *et al.* Physiological risk factors for severe high-altitude illness: a prospective cohort study. *Am J Respir Crit Care Med* **185**, 192–198 (2012).

6. Joyce, K. E. *et al.* Advances in the available non-biological pharmacotherapy prevention and treatment of acute mountain sickness and high altitude cerebral and pulmonary oedema. *Expert Opin Pharmacother* **19**, 1891–1902 (2018).
7. Johnson, C. H., Ivanisevic, J. & Siuzdak, G. Metabolomics: beyond biomarkers and towards mechanisms. *Nat Rev Mol Cell Biol* **17**, 451 (2016).
8. Nicholson, J. K. & Wilson, I. D. Understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* **2**, 668–676 (2003).
9. Grocott, M. P. *et al.* Arterial blood gases and oxygen content in climbers on Mount Everest. *N Engl J Med* **360**, 140–149 (2009).
10. Forrester, S. J., Kikuchi, D. S., Hernandez, M. S., Xu, Q. & Griendling, K. K. Reactive Oxygen Species in Metabolic and Inflammatory Signaling. *Circulation Research* **122**, 877–902 (2018).
11. Schieber, M. & Chandel, S. N. ROS Function in Redox Signaling and Oxidative Stress. *Current biology* **24**, R453-R462 (2014).
12. Michiels, C. Physiological and Pathological Responses to Hypoxia. *Am J Pathol.* **164**, 1875–1882 (2004).
13. Chen, P. S. *et al.* Pathophysiological implications of hypoxia in human diseases. *Journal of Biomedical Science* volume **27**, 63 (2020).
14. Liao, W. T. *et al.* Metabolite Modulation in Human Plasma in the Early Phase of Acclimatization to Hypobaric Hypoxia. *Scientific Reports* **6**, 22589 (2016).
15. Dzhalilova, D. & Makarova, O. Differences in Tolerance to Hypoxia: Physiological, Biochemical, and Molecular-Biological Characteristics. *Biomedicines* **8**, 428–432 (2015).
16. Clish, C.B. Metabolomics: an emerging but powerful tool for precision medicine. *Cold Spring Harb Mol Case Stud.* **1**, a000588 (2015).
17. Yan, M. & Xu, G. Current and future perspectives of functional metabolomics in disease studies—A review. *Analytica Chimica Acta.* **1037**, 41–54 (2018).
18. Mussap, M., Loddo, C., Fanni, C. & Fanos, V. Metabolomics in pharmacology – a delve into the novel field of pharmacometabolomics. *Expert review of clinical pharmacology.* **13**, 115–134 (2020).
19. Jacob, M. *et al.* A targeted metabolomics approach for clinical diagnosis of inborn errors of metabolism. *Anal Chim Acta* **26**, 141–153 (2018).
20. Kennedy, A. D. *et al.* Metabolomics in the clinic: A review of the shared and unique features of untargeted metabolomics for clinical research and clinical testing. *Journal of mass spectrometry* **53**, 1143–1154 (2018).
21. Davison, G. *et al.* Metabolomic Response to Acute Hypoxic Exercise and Recovery in Adult Males. *Front Physiol.* **26**, 1682 (2018).
22. Wishart, D. S. Metabolomics for investigating physiological and pathophysiological processes. *Physiol Rev* **99**, 1819 – 1875 (2019).

23. Yang, Q. *et al.* Metabolomics biotechnology, applications, and future trends: a systematic review. *RSC Adv.* **9**, 37245–37257 (2019).
24. Nemkov, T. *et al.* Metabolism of citrate and other carboxylic acids in erythrocytes as a function of oxygen saturation and refrigerated storage. *Front Med (Lausanne)* **4**, 175 (2017).
25. Koundal, S. *et al.* “Omics” of high altitude biology: a urinary metabolomics biomarker study of rats under hypobaric hypoxia. *OMICS* **19**, 757–765 (2015).
26. Basu, S. *et al.* Sparse network modeling and metscape-based visualization methods for the analysis of large-scale metabolomics data. *Bioinformatics* **15**, 1545–1553 (2017).
27. Gonggalanzi *et al.* Acute mountain sickness among tourists visiting the high-altitude city of Lhasa at 3658 m above sea level: a cross-sectional study. *Archives of Public Health* **74**, 23 (2016).
28. Taylor, A. T. High-Altitude Illnesses: Physiology, Risk Factors, Prevention, and Treatment. *Rambam Maimonides Med J.* **2**, e0022 (2011).
29. Viscor, G. *et al.* Physiological and Biological Responses to Short-Term Intermittent Hypobaric Hypoxia Exposure: From Sports and Mountain Medicine to New Biomedical Applications. *Front Physiol.* **9**, 814 (2018).
30. Coppel, J., Hennis, P., Kawai, E. G., & Grocott, M. P. W. The physiological effects of hypobaric hypoxia versus normobaric hypoxia: a systematic review of crossover trials. *Extrem Physiol Med.* **4**, 2–6 (2015).
31. Norboo, T. *et al.* Mini review of high altitude health problems in Ladakh. *Biomedicine & Pharmacotherapy* **58**, 220–225 (2004).
32. Chicco, A. J. *et al.* Adaptive remodeling of skeletal muscle energy metabolism in high-altitude hypoxia: Lessons from AltitudeOmics. *J Biol Chem.* **293**, 6659–6671 (2018).
33. Niu, Y. *et al.* Metabolic responses of plasma to extreme environments in overwintering Tibetan frogs *Nanorana parkeri*: a metabolome integrated analysis. *Front Zool* **18**, 41–45 (2021).
34. Liu, J. *et al.* UPLCQTOFMSbased metabolomic analysis of the serum of hypoxic preconditioning mice. *Molecular medicine reports* **16**, 6828–6836 (2017).
35. St-Pierre, J. & Boutilier, R. G. Aerobic capacity of frog skeletal muscle during hibernation. *Physiol Biochem Zool.* **74**, 390–397 (2001).
36. Storey, K. B. & Storey, J. M. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q Rev Biol.* **65**, 145–74 (1990).
37. Flanigan, J., Withers, P., Storey, K. & Guppy, M. Changes in enzyme binding and activity during aestivation in the frog *Neobatrachus pelobatoides*. *Comp Biochem Physiol B.* **96**, 67–71 (1990).
38. Reyes, I. M. & Chandel, N. S. Mitochondrial TCA cycle metabolites control physiology and disease. *Nature Communications* **11**, 102–105 (2020).
39. O'Brien, K. A. *et al.* Metabolomic and lipidomic plasma profile changes in human participants ascending to Everest Base Camp. *Scientific Reports* **9**, 2297 (2019).

40. Zhu, G. *et al.* Metabolomic analysis of plasma from patients with acute mountain sickness using chromatography mass spectrometry. *Medicine (Baltimore)* **94**, e1988 (2015).
41. Freeman, J. J. Regulatory mechanisms of choline production. *Life Sci* **58**, 1921–1927 (1996).
42. Zeisel, S. H. *et al.* Choline, an essential nutrient for humans. *FASEB J* **5**, 2093–2098 (1991).
43. Goldfarb-Rumyantzev, A. S. & Alper, S. L. Short-term responses of the kidney to high altitude in mountain climbers. *Nephrol Dial Transplant*. **29**, 497–506 (2014).
44. Palubiski, L. M., O'Halloran, K. D. & O'Neill, J. Renal Physiological Adaptation to High Altitude: A Systematic Review. *Front Physiol*. **11**, 75 (2020).
45. Luo, Y., Zhu, J. & Gao, Y. Metabolomic analysis of the plasma of patients with high-altitude pulmonary edema (HAPE) using ¹H NMR. *Mol Biosyst* **8**, 1783–1788 (2012).
46. Pichler, H. J. *et al.* Oxidative stress in hypobaric hypoxia and influence on vessel-tone modifying mediators. *High Alt Med Biol* **14**, 273–279 (2013).
47. D'Alessandro, A. *et al.* AltitudeOmics: Red Blood Cell metabolic adaptation to high altitude hypoxia. *J Proteome Res* **15**, 3883–3895 (2016).
48. Xuefeng, C. *et al.* Preliminary study of patients with chronic mountain sickness by GC-TOF-MS based serum metabolomics analysis. *Chinese Journal of Pathophysiology* **33**, 1676–1682 (2017).
49. Tissot van Patot, M. C. *et al.* Enhanced leukocyte HIF-1alpha and HIF-1 DNA binding in humans after rapid ascent to 4300 m. *Free Radic Biol Med* **46**, 1551–1557 (2009).
50. Maimaitiyimin, D. *et al.* Effects and mechanisms of acetyl-L-cysteine in rats with chronic mountain sickness with ¹H-NMR metabolomics methods. *Med Sci Monit* **20**, 767–773 (2014).
51. Binns, N., Wright, A. D., Singh, B. M., Coote, J. H. & Bradwell, A. R. Blood lactate changes during exercise at high altitude. *Postgrad Med J*. **63**, 177–178 (1987).
52. Hochachka, P. W. *et al.* The lactate paradox in human high-altitude physiological performance. *News Physiol Sci* **17**, 122–6 (2020).
53. Yang, S. *et al.* Gut Microbiota-Dependent Marker TMAO in Promoting Cardiovascular Disease: Inflammation Mechanism, Clinical Prognostic, and Potential as a Therapeutic Target. *Front Pharmacol* **19**, 1360–1365 (2019).
54. Tyagi, R., Maan, K., Khushu, S. & Rana, P. Urine metabolomics based prediction model approach for radiation exposure. *Scientific Reports* **10**, 16063–16070 (2020).

Figures

Figure 1

Representative & comparative ^1H -NMR spectra **(A)** δ 0- δ 10.0 **(B)** δ 0.5- δ 4.5 **(C)** δ 6.5- δ 9.5, of urine samples from sea level control group and Siachen group.

Figure 2

(A) PCA score plot between component 1 versus component 2 for ^1H -NMR urine spectra from sea level control group (o 0) and Siachen group (o 1) **(B)** PCA 3D score plot between component 1, component 2, and component 3 for ^1H -NMR spectra of urine from sea level control group (Red) and Siachen group (Green).

Figure 3

Important features selected by t-tests with threshold 0.1. The pink circles represent features above the threshold. Note the p values are transformed by $-\log_{10}$ so that the more significant features (With smaller p values) will be plotted higher on the graph.

Figure 4

(A) Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot of all metabolite features between sea level control group (o 0) and Siachen group (o 1). **(B)** Sparse partial least square discriminant analysis (sPLSDA) score plots between selected PCs showing supervised clustering and separation between sea level control group (o 0) and Siachen group (o 1).

Figure 5

PCA biplot between the selected PCs. Biplot for control and Siachen group identifying the relationship between samples and metabolites where samples were displayed as points and metabolites were displayed as vectors.

Figure 6

Scree plot was generated for the first five Principal components (PCs) to identify the contribution of each of the principal components to the total variation in the data set. The green line showed the cumulative variance explained by the first five components which accounted for 54% whereas blue line indicated the individual variance for each principal component.

Figure 7

(A) PLS score plot between component 1 versus component 2 for ^1H -NMR urine spectra from sea level control group (o 0) and Siachen group (o 1). **(B)** PLS-DA classification using different number of components. The red star indicates the best classifier. R^2 and Q^2 , greater than 0.5 indicates a model with reasonable fit with good predictive power. The cross-validation analysis with first five components produced a strong model with high validated predictability ($Q^2 = 0.91$), goodness of fit value ($R^2 = 0.78$) and accuracy (accuracy = 0.96). **(C)** Based on the weighted sum of PLS-regression coefficients, the top 30 bins that contributed the maximum to separation of clusters in PLS-DA are shown. The coloured boxes on right show the relative concentrations of the corresponding metabolite.

Figure 8

Violin plots for significantly different metabolites showing density & distribution of metabolite concentrations between sea level control group and Siachen group.

Figure 9

Summary of altered metabolic pathways in response to high altitude hypoxia environment.

Figure 10

Debiased Sparse Partial Correlation Network Modeling (DSPC) indicating correlation between various metabolic features.