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Urinary Metabolic Profiling of 'MARS-500 Project' with Nuclear Magnetic Resonance Spectroscopy

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Abstract

Human sustainable space exploration could go further with the development of strategies and preparatory activities. Enormous scientific work has been done during the 21st century. MARS-500 project is one of unique preparatory activities, and was finished within 520 day isolation on the Earth. The full 520 day MARS mission simulation provided an opportunity to conduct a long-term, isolated experiment to observe metabolic changes due to people differences and its stability to duration variation. Six volunteers were selected from Russia, France, Italy, and China, and their urine specimens were collected in six periods. Then the subtle metabolic variations were investigated by nuclear magnetic resonance (NMR) at six different periods. We found that the metabotypes of six crews drifted out from the first period to the farthest third period, then reverted to the nearest sixth period in metabolic space, suggesting a property of resilience for metabolic phenotype. Furthermore, a set of 20 biomarkers, discriminating great changes in metabolic phenotypes well, were associated with emotion, muscle turnover, circadian rhythms, energy expenditure, lipid metabolism, oxidative activity, cognitive performance, and gut-microbial activities. In deeps, to analysis the data from MARS-500 project with two-way ANOVA, an interaction between factor of people differences and long-term isolation showed that the six crews each had a specific response to 520 days' isolation. In this picture, accurate healthy management during future human space mission should aim at promoting his or her ability to revert to his or her stable metabotype under a defined and isolated environment.

Keywords: MARS-500 project; Metabotypes; Metabolic response; NMR

Introduction

Space exploration has received more and more attention in the world. Meanwhile, humans play the most important role in each space exploration mission. MARS-500 project is a simulation of a manned space flight to the red planet, which is a preliminary investigation to the impact of special space environment on crews in future flight. This project began on June 3, 2010 and ended on November 4, 2011. The full 520-day experimental paradigm simulated different phases of a mission to MARS: a 250-day interplanetary flight from Earth to MARS, a 30-day orbital stay that includes the MARS landing and a 240-day interplanetary flight from MARS to Earth. This special simulation project provided a long-duration social isolation and spatial restriction. Six volunteers (three Russians, two Europeans and one Chinese) are involved in this study. So far, several studies related to this project have been reported. For example, an ethological study was conducted to investigate the MARS-500 crews in daily life activities [1]. Another study was aimed to test the effect of different exercise protocols (endurance/strength orientated) on brain cortical activity and cognitive performance [2]. Moreover, Natalia Rakova introduced the reverse experimental approach to reveals infradian rhythmicity in human Na^+ balance [3]. Elena Feichtinger had reported the psychological support programme [4], while A.V. Mardanov conducted the metagenomic analysis of the dynamic changes in the gut microbiome of the participants of the MARS-500 experiment [5]. However, to the best of our knowledge, there has been no report on urinary metabonomics of these six volunteers which can reveal the metabolic response to a long isolated environment.

Metabonomics is defined as the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification [6]. Recent studies have showed that metabonomics can offer a powerful new means for discovering molecular biomarkers and metabolic pathways that underlie disease risk [7]. Moreover, obtaining comprehensive, untargeted metabolic profiles through analyzing complex biofluids (e.g., urine and plasm) can provide important diagnostic and prognostic information [8]. Among the biofluids most commonly analyzed in metabolomic studies, urine appears to be particularly useful, because it is abundant, readily available, easily stored and can be collected by simple, noninvasive techniques [9]. Although, persistent alterations induced by dietary or chronic interventions may also be detected from plasma, but it just provides a description of the metabolic system at the time of sampling, and yet some biochemical or pathological changes may not be specific to external stimuli or genetic modification. By contrast, information from urine is time-averaged because of its collection and storage in the bladder [8]. Therefore, urine was selected to conduct metabolic mapping in many publications [10-12]. Among analytical techniques used in metabonomics, nuclear magnetic resonance (NMR) [13] and mass spectroscopy (MS) [14] possessed the dominant status. As known, NMR has the advantage of being rapid, high reproducibility, nondestructive to samples, applicable to intact biomaterials and rich in chemical structural information. In addition, unlike GC–MS and certain liquid chromatography–mass spectrometry (LC–MS) methods,

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no chemical derivatization or ionization are necessary. NMR is particularly amenable to detecting polar and uncharged compounds, such as sugars, amines or relatively small, volatile compounds (such as formic acid, formaldehyde, acetone, etc.) which are often undetectable by LC–MS methods.

Initially, genomics and proteomics have shown their potential as innovative approaches in uncovering the exact pathophysiology, e.g. Single Nucleotide Polymorphism (SNP) [15]. However, they only tell us what might have happened, as there are several downstream regulatory mechanisms. Metabonomics, on the other hand, tells us what actually did happen [16]. Recently, progresses in analytical strategies permit us relating metabolic readout of the physiological state of the human body with genotypes [17,18]. These genetically determined metabotypes may subscribe the risk for a certain medical phenotype, the response to a given drug treatment, or the reaction to a nutritional intervention or environmental challenge [15]. In the present study, urine from six crews was collected in six periods. Subsequently, we used ^{1}H-NMR profiles coupled with multivariate statistical analysis to ascertain and investigate those physiological changes that occurred in response to stimuli of 520-days isolation which is only conducted once during human history. To this aim, impactions of the 520-days isolation on human metabolic characterization were analyzed with pattern recognition analysis. Furthermore, people differences and its interactions with 520-days isolation were also interpreted using two-way ANOVA. The longest longitudinal study (up to 9 years) of the metabolic phenotype told us that each individual occupies a well-defined region in the broad metabolic space, within which a limited degree of allostasis is permitted. Meanwhile, the insurgence of significant stressful conditions causes a shift of the metabotype to another distinct region [12]. So an interesting question stands out which ask "how long will they be able to maintain their original metabotypes and where a new metabolic space will they drift toward when the 6 volunteers are subjected into the isolated environment over 520 days.

Materials and Method

MARS-500 experiment

MARS-500 experiment was carried out in the MARS-500 experimental unit, the structure of which contains EU-50, EU-100, EU-150, EU-250, and simulator of the Martian surface (http://MARS500.imbp.ru/en/nek.html). The MARS-500 international crews was composed of three Russians, two Europeans and one Chinese (n=6). The subjects were all males, aged between 26 years and 38 years. Their functions during the experiment were commander, physician, on-board engineer and investigator. They were selected on the basis of motivation, health and professional background. They were trained on payloads, simulated space craft systems, coping techniques and baseline data collection. Food testing and team-building activities were also an important part of training. This research was conducted in accordance with the principles expressed in the Declaration of Helsinki and approved from the local University ethics committee, the ethic committee of the European Space Agency and the Institutional Review Board of the Institute for Biomedical Problems (IBMP), and all Mars500 crew members underwent a thorough clinical examination prior to participation and gave written informed consent.

Urine collection and storage

The urine specimens were collected in six periods. The first period corresponds to 10 days before entering the MARS-500 experimental unit, and this is a preliminary period. The second period is 60-70 days after entering MARS-500 experimental unit, which is the simulation period of a flight on the heliocentric orbit up to the Martian vicinity. The third period is over a range of 159-169 days after entering the unit-a period keeping on flight after period two. The fourth period corresponds to 359-369 days after entering the unit, and this period is a simulation of a flight on the heliocentric orbit up to the Earth vicinity after the MARS missions was finished. The fifth period corresponds to 439-449 days after entering the unit, and during this period a flight is simulated close to the gravitational field of the Earth. The sixth period is over a range of 511-520 days after entering the unit, and this period is a simulation process of a flight along the spiral path in the gravitational field of the Earth. In details, the procedure of sampling is shown in Figure S1. All these information can be gained freely in detail on the website (http://mars500.imbp.ru/en/ 520_dates.html).

All the urine specimens were collected into sterile tubes (Sterilin, U.K.) in three excessive mornings and stored at -80 °C immediately for later analyses. The urine samples (during a period) from different crew members were collected at different times but all within ten days. The urine samples from the fourth crew at the first sampling time in period one and from the sixth crew at the first sampling in period five were not available. Finally, 106 urine samples were collected. Furthermore, quality control (QC) aliquots for NMR analysis were prepared by combining aliquots of urine from randomly selected subgroups of individuals and had been divided into six batches of the specimens (each batch has five QC).

NMR experiments

Before NMR analysis, urine specimens were prepared by the addition of phosphate buffer made up in deuterium oxide containing 1 mM 3-(trimethylsilyl)-[2,2,3,3-$^{2}H_{4}$]-propionic acid sodium salt (TSP) as an external reference and 2 mM sodium azide as a bacteriocide. 1-dimensional (1-D) NMR spectra were performed at 298 K on a Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz, and a standard one-dimensional (1D) pulse sequence [recycle delay (RD)-90°-t_1-90-t_m-90°-acquire] free induction decay (FID) was used with 32 times superposition. Meanwhile, water peak suppression was conducted during recycle delay (RD) of 2 *s*, and the mixing time (t_m) was set to 100 *ms*. Furthermore, the 90° pulse length was approximately 10 μs and the t_1 time was set to 4 μs. For each specimen, 8 dummy scans were followed by 128 scans. The spectra were collected into 64 K data points using a spectral width of 20 ppm. For spectral processing, FIDs were multiplied by an exponential function corresponding to a line broadening of 0.3 Hz before Fourier transformation. To solve overlap in 1-dimensional (1-D) NMR spectrum, JRES, ^{1}H *J*-resolved spectrum; COSY, chemical shift correlated spectroscopy; TOCSY, total correlation spectroscopy; HSQC, ^{1}H-^{13}C hetero nuclear single-quantum correlation spectra; HMBC, ^{1}H-^{13}C heteronuclear multiple bond correlation spectrum. For the 2D-NMR spectra, water suppression was achieved using presaturation during the relaxation delay. The TOCSY spectra was acquired with the spectral widths set to 10.5 ppm in the proton and carbon dimensions, 2048 × 512 data points and 64 scans per increment was collected, MLEV-17 pulse for spin locked, and the mixing time (t_m) was set to 100 *ms*, For the ^{1}H-^{13}C HSQC spectra, the spectral widths were set to 10 and 175 ppm in the proton and carbon dimensions, with 2048 × 512 data points and 320 scans per spectrum.

Data preprocessing

All 1D-NMR spectra were batch preprocessed using MesReNova 10.0.1 (Mestrelab research) professional software (commercial

available), including automatically phasing with deconvolution method, baseline correction with polynomial fit and calibration using 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP; δ 0.00) signal. The peaks corresponding to water (δ 4.62-5.17) and urea (δ 5.57-6.15) signals were removed from spectra before further analysis. Then all the 1D-NMR spectra were integrated with 0.004 ppm interval from the region 0.5 ppm to 9.5 ppm. The spectral data were further digitalized onto 106 ^{x} 2250 data set with all samples in row and chemical shift (δ, metabolites) in column and finally saved as excel format (xls). The peak assignment of 1D-NMR spectra were carried out referring to previous reports [6], which was further conducted by searching the Human Metabolome Database (HMDB). For ambiguous peak assignment and overlapped chemical shift of 1D-NMR spectra, 2D-NMR spectra were used to confirm the possible identification.

2D spectra were semi-automatically processed using MesReNova 10.0.1, employing a 90° shifted squared sine-bell window function in both dimensions. The number of data points was set to 2048 (2 k) and zero filled prior to Fourier transform using complex forward linear prediction. All 2D spectra were manually phase corrected, and a polynomial baseline correction was applied excluding the region around the water artifact. All the processed 2D spectra were imported into MetaboMiner (http://wishart.biology.ualberta.ca/metabominer) for peak assignment and possible metabolites were confirmed by literature report and HMDB.

Data analysis

The data matrix was imported into MatLab software (2014a version, the Mathworks Inc., Natwick, MA) for preprocessing, normalized by constant sum, Pareto scaling and cubic root transformation. The data set were further analyzed using SIMCA P+ (V14.0, Umetrics AB, Umea, Sweden), a widely used statistical NMR data analysis software. Afterwards, a PCA model was used for overviewing and explaining 'clustering' and trends as well as outliers' recognition based on the normalized data set. While all the outliers were excluded, an orthogonal partial least squares discriminant analysis (O2PLS-DA) model was built to exclude noises from the inter-individual variation. The goodness of fit of O2PLS-DA model was further validated by permutation test with 200 iterations as well as 7 fold cross-validation. Then VIP value derived from O2PLS-DA model was used to screen candidate biomarkers which had significantly changed according to the periods. For metabolites with VIP>1 in the O2PLS-DA model was further analyzed by the statistical one-way ANOVA analysis with the significant level *alpha* being 0.05.

To investigate the metabolic responses to people differences, data set derived from 1D-NMR were finally imported into MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst) for two-way analysis. For metabolic biomarkers identified by the above 1D- and 2D-NMR, their data set was finally imported into R programming language (version 3.2.1) for correlation structure analysis.

Results and Discussion

^{1}H-NMR peak assignment

Peak assignment is the base of metabotypling, and a total of 43 metabolites were identified as depicted in Figure 1a and 1b. As viewed from the spectra, several metabolites, e.g., taurine and trimethylamine *N*-oxide have coincident peak assignment at partial chemical shift. Meanwhile, signals of some low abundant metabolites such as glucose and *o*-acetylcarnitine were covered by urea and solvent. These challenges can be partially solved by high-resolution 2D-NMR spectrum as displayed in Figure S2. a, b, c, d, and e (including: JRES, ^{1}H *J*-resolved spectrum, COSY, chemical shift correlated spectroscopy, TOCSY, total correlation spectroscopy, HSQC, ^{1}H-^{13}C hetero nuclear single-quantum correlation spectra, HMBC, ^{1}H-^{13}C heteronuclear multiple bond correlation spectrum).

Metabolic responses to 520 days' isolation

Biological changes due to long-term isolation: Data normalization before modeling is very important in metabolomics studies, because normalization procedures can remove unwanted sample-to-sample variation and make features more comparable. Urine ^{1}H-NMR spectral data from the six periods was normalized by different methods, and then a combination of following methods produced a good data set: Normalization by peak intensity of creatinine, transformation by logarithmic function, and Pareto Scaling. The data quality promotion can be seen by a box plots conjunct with kernel density plots (Figure S3). As seen by comparing the linear curve on the left which has an exponential decay character to the transformed curve on the right which looks reasonably Gaussian, which will exert significant elevation to the goodness of subsequent modeling (PCA and O2PLS-DA models).

An NMR sample can be considered to be an *n*-dimensional object, the coordinate along each dimension being the concentrations of individual measurable metabolites or more simply the NMR spectral intensity at each data point or collection of data points. Thus the NMR spectrum of the urine provides an *n*-dimensional metabolic fingerprint of the crews who were restricted in an isolated and predefined environment, and this metabolic profile is characteristically changed according to the physiological or psychologic state. Hence we have turned to computer-based pattern recognition analysis (PR) approaches to interpret the NMR data obtained in MARS-500 project. One of the most useful and easily applied PR techniques is principal components analysis (PCA). Principal components (PCs) are new variables created from linear combinations of the starting variables with appropriate weighting coefficients. The properties of these PCs are such that (i) each PC is orthogonal (uncorrelated) with all other PCs and (ii) the first PC contains the largest part of the variance of the data set (information content) with subsequent PCs containing correspondingly smaller amounts of variance [13]. Thus a plot of the first two or three PCs gives the "best" representation, and PC maps can be used to visualize inherent clustering behavior for physiological or psychologic changes in different periods. As shown in the PCA score plot (Figure S4), no obvious separation among different period was observed other than a big variation displayed in samples from the same period, suggesting inter-individual differences were big to an extent to result incomplete separation of different period. However, unrelated variation can withdraw in an orthogonal partial least squares- discriminant analysis (O2PLS-DA).

O2PLS-DA are extensions to the PLS algorithm, which work by splitting the variation of the predictor variables into two parts: variation orthogonal (uncorrelated) to the response and variation correlated to the response. While the predictive accuracy remains the same as for conventional PLS, by separating the variation in this way, interpretation of the model can be improved. In PCA, the model describes the space corresponding to the highest variance of the data, while in PLS the space corresponds to that with the highest covariance between the NMR data and the response variable. This is most easily explained by O2PLS-DA score plot (Figure 2a), which can be well manifested by the clear separation among the first four periods. Nonetheless, sample points from the fourth and fifth period overlaid on each other, and the sixth period lied very close or even mixed to

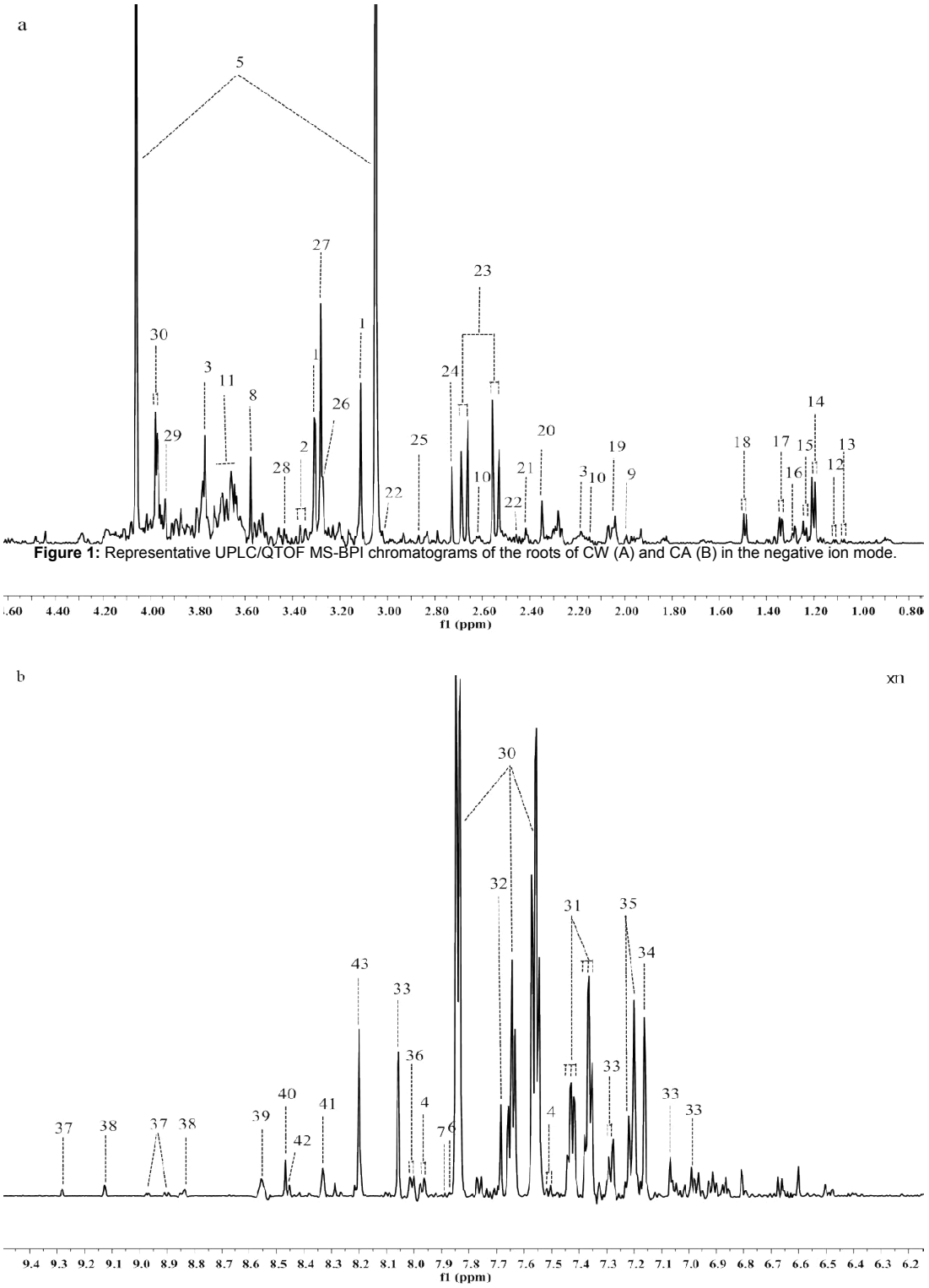
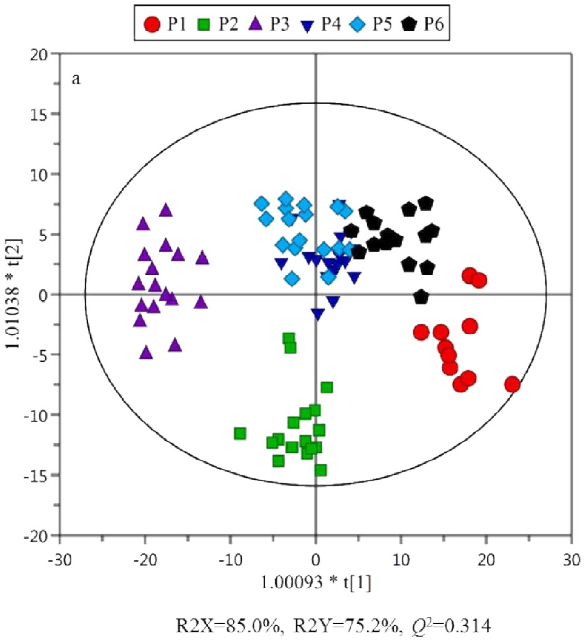


Figure 1: Representative UPLC/QTOF MS-BPI chromatograms of the roots of CW (A) and CA (B) in the negative ion mode.

Figure 1: Typical 600 MHz ^{1}H-NMR Spectra of Urine from Crew of MARS-500 Project. Numbers indicates the following metabolites: 1: serotonin (Ser); 2: *trans*-4-hydroxy-L-proline (Hyp); 3: L-glutamine (Gln); 4: indole-3-acetate (IAA); 5: creatinine (Cn); 6: xanthine (Xa); 7: xanthosine (Xan); 8: glycine (Gly); 9: isovaleric acid (IVA); 10: *o*-acetylcarnitine (OAC); 11: threonine (Thr); 12: 3-methyl-2-oxovalerate (MOV); 13: isobutyrate (IB); 14: 3-hydroxybutyrate (3-HB); 15: methylmalonate (MMA); 16: isoleucine (ile); 17: lactate (Lac); 18: alanine (Ala); 19: *N*-acetylglutamate (NAG); 20: pyruvate (Py);21: succinate (Suc); 22: α-ketoglutarate (Kg); 23: citrate (Cit); 24: dimethylamine (DMA); 25: trimethylamine (TMA); 26: glycerophosphorylcholine (GPC); 27: trimethylamine N-oxide (TMAO); 28: taurine (Tau); 29: creatine (Cr); 30: hippurate (Hip); 31: phenylacetylglycine (PAG); 32: 3-Methylhistidine (3-MH); 33: 3-hydroxymandelate (3-HM); 34: Methylhistidine (MH); 35: melatonin (Mel); 36: salicylurate (Sal); 37: *N*-methylnicotinamide (NMND); 38: trigonelline (Tri); 39: IMP; 40: NADH; 41: imidazole (Imi); 42: formic acid (For); 43: oxypurinol (Oxy).



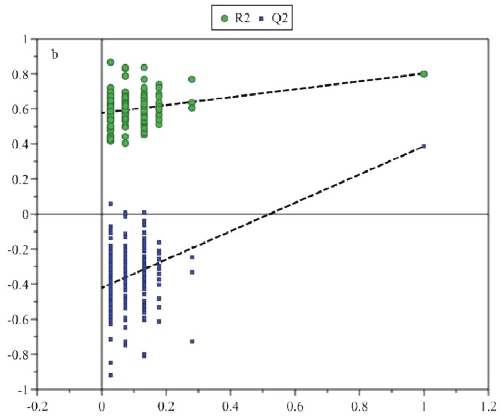


Figure 2: OPLS-DA Score Plot of the Six Collections over 520 Days. a, data from different period were marked by varied symbol styles and color codes: 1st period (red circle), 2nd period (blue box), 3rd period (turquoise triangle), 4th period (purple inverted triangle), 5th period (blue diamond), and 6th period (black pentagon); b, Permutations plot for O2PLS-DA Model with 200 iterations. The green and blue line each represents regression line of R^2 and Q^2 value, then all blue Q^2-values to the left are lower than the original points to the right and the blue regression line of the Q^2-points intersects the vertical axis (on the left) below zero, thus strongly indicating that the original model was valid.

the fourth or fifth period. On the other hand, a global observation showed that sample points derived from different periods seemed to drift out from the first period to the farthest third period step by step, then restored from the third period to the sixth period which was very close to the first period but yet clearly separated from each other. The good validity of O2PLS-DA model was further proved by the plot of permutation test (Figure 2b) and CV-ANOVA table of 7 fold cross-validation (Table 1). For instance, viewed from Figure 2b, all blue Q^2-values to the left were lower than the original points to the right and the blue regression line of the Q^2-points intersects the vertical axis (on the left) below zero. Moreover, cross-validation demonstrated that the O2PLS-DA model had a p-value of 0.0177764 which is less than 0.05. These strongly indicating that the original model was valid.

Biomarkers responsible for the big changes caused by 520 days' isolation: Metabolites associated with the biological changes were initially screened referring to the VIP value which was derived from O2PLS-DA model, and these metabolites were further tested by one-way analysis of variance (ANOVA), followed by post-hoc analyses. Afterwards, a total of 20 metabolites, as displayed in Table 2, were significantly changed in their relative concentration and contributed to discriminate the big biological changes during 520 days' isolation. The trends of the 27 metabolites in six periods were therefore performed as box plots with a significant level adjusted by a p value (Figure 3).

Loadings of O2PLS-DA model and one-way ANOVA analysis (Table 2) showed that the most contributive and discriminating biomarker was serotonin ($p=1.60\times10^{-9}$, VIP=3.998), and serotonin had

O2PLS-DA model	SS	DF	MS	F	p	SD
Total corr.[1]	470	470	1	-	-	1
Regression[2]	159.732	130	1.2287	1.34645	0.01778	1.10847
Residual[3]	310.268	340	0.91255	-	-	0.95528

1, Total corrected: SS of the Y of the training set corrected for the mean. 2, Regression: fraction of total corrected SS accounted for by the PLS or OPLS model, here estimated by CV. 3, Residual: difference between total corrected and regression SS, that is, the fraction of total corrected unaccounted for by the PLS or OPLS model.

Abbreviations: SS: the sum of squares (SS(d)) and (SS(e)), DF: degree of freedom, MS: the corresponding mean squares

Table 1: CV-ANOVA in the 7-fold Cross-Validated Residuals of Y-Variable.

Biomarkers	Chemical shift δ/ppm	PLS-DA VIP	one-way ANOVA[a]	
			p.value	FDR[b]
Serotonin (Ser)	3.308 (t)	3.998	6.62E-17	1.79E-15
Melatonin(Mel)	7.192 (s)	1.217	>0.05	-
Glycerophosphorylcholine (GPC)	3.268 (s)	1.611	>0.05	-
4-Hydroxyproline (Hyp)	3.344 (d)	2.651	8.83E-05	2.17E-04
L-Glutamine (Gln)	2.156 (m)	2.507	8.79E-14	1.19E-12
N-Acetylglutamate (NAG)	2.048 (s)	1.27	2.59E-03	5.00E-03
Creatine (Cr)	3.936 (s)	1.191	1.17E-05	3.93E-05
O-Acetylcarnitine (OAC)	2.148 (s)	2.09	1.53E-07	8.24E-07
3-Methyl-2-oxovalerate (MOV)	0.832 (d)	1.223	2.25E-02	2.76E-02
Methylmalonate (MMA)	1.120 (d)	1.52	1.19E-02	1.61E-02
Xanthine (Xa)	7.876 (s)	2.387	3.26E-03	5.86E-03
Isovaleric acid (IVA)	2.200 (d)	2.104	1.82E-10	1.64E-09
Succinate (Suc)	2.410 (s)	1.01	1.50E-02	1.93E-02
Citrate (Cit)	2.532 (t)	1.156	2.25E-03	4.67E-03
α-Ketoglutarate (Kg)	2.460 (t)	1.445	4.77E-03	8.05E-03
Glycine (Gly)	3.560 (s)	2.248	4.94E-08	3.33E-07
Alanine (Ala)	1.484 (d)	1.225	>0.05	-
Threonine (Thr)	3.724 (m)	2.042	1.13E-06	5.07E-06
N-methylnicotinamide (NMND)	8.936 (d)	1.965	7.75E-03	1.16E-02
3-hydroxymandelate (Hym)	7.280 (d)	1.238	7.86E-05	2.17E-04
Taurine (Tau)	3.344 (t)	2.651	8.83E-05	2.17E-04
Hippurate (Hip)	3.980 (d)	1.115	6.66E-03	1.06E-02
Indole-3-acetate (IAA)	7.492 (d)	2.633	1.56E-03	3.51E-03
4-Hydroxybenzoic acid (HBA)	7.772 (d)	1.717	>0.05	-
Imidazole (Imi)	8.340 (s)	1.226	>0.05	-

Table 2: Summary of Metabolites with VIP > 1 in the PLS-DA Model of the Six Crews over Different Periods.

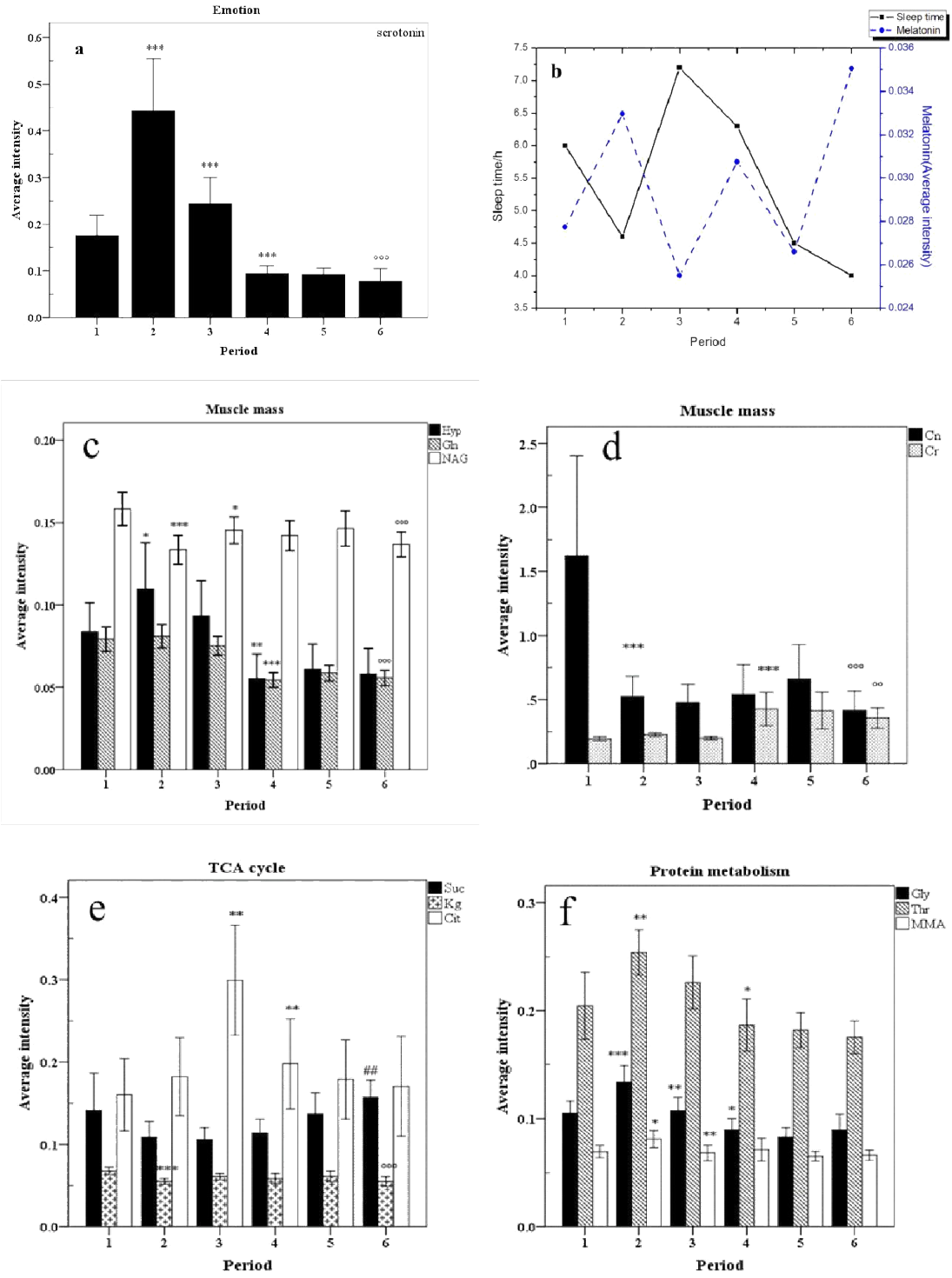


Figure 3: Line and Bar Plots of Different Biomarkers Conjunction with a Significant Level. Significant level between two continuous period: *p<0.05, **p<0.01 and ***p<0.001; comparing with period two: $^{\#}p$<0.05, $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001; and comparing period six with period one: °p<0.05, °p<0.01 and °p<0.001. One-way ANOVA with posthoc Bonferroni test). Data of sleep time was derived from the first volunteer and this data was provided by Ke Lv, et al.

been proved to be a biochemical messenger and regulator. Nonetheless, serotonin in the nervous system acts as a local transmitter at synapses, and as a paracrine or hormonal modulator of circuits upon diffusion, allowing a wide variety of "state-dependent" behavioral responses to different stimuli, for instant, tension, aggressive behavior and rhythmic motor patterns [19]. In addition, the trends of serotonin had been described by a boxplot (Figure 3a) with the six periods being the time axis. Viewed from Figure 3a, the relative concentration of serotonin

markedly increased from the 1st period to the 2nd where it reached the largest, resulting in the appearance of inflection point. Practically, in the MARS-500 simulation programme, the six crews were sent from outside to the MARS-500 experiment unit from the first period to the second. Thus, it is reasonable to believe that such a large change in their living environment could cause a great increase in the concentration of serotonin. Subsequently, the relative concentration of serotonin went through a rapid decrease from the second to the end. Thus may be a flexibility of the subjects. Particularly, Wang Y, et al. [20], had conducted a questionnaire-based investigation of emotional responses and psychological adaptation over 520 days' isolation, and results had demonstrated an obvious bias on valence rating for unpleasant stimuli with time ($p<0.05$), thus may explain the elevation of serotonin at period two. Furthermore, the presence of increased psychosocial issues caused by long-term isolation had been proved by many researches [21-26]. Thus these results suggested that physiological problems are very serious in an isolated environment.

Melatonin is implicated in the regulation of the circadian rhythms of several biological functions, including the sleep-wake cycle. In particular, melatonin regulates the sleep-wake cycle by chemically causing drowsiness and lowering the body temperature [27]. A tendency chart demonstrates a negative association between melatonin and sleep time at each period and one exception-period five (Figure 3b), that is an increase in relative intensity of melatonin when the sleep time had deceased, and this might be an adaption of organism. In addition, a decrease in sleep time at period two may be caused by differences in living conditions and increase at period three maybe regard as an adaptation. Whereas, a sustaining decrease in the last three periods might be caused by bad physiological problems. Changing in circadian rhythms under an isolated environment has also been reported in many publications [28-30]. It has been shown that sleep time had decreased for people when they were pooled into an isolated and predefined space. These changing in circadian rhythms may account for bad emotion, such as lonely, tension, and depression.

Variation in the relative concentration of metabolites including 4-hydroxyproline, *L*-glutamine, creatinine, and *N*-acetylglutamate might reflect a change in muscle mass. 4-hydroxyproline is a major component of the protein collagen and declining levels of urinary 4-hydroxyproline are indicative of muscle loss [31]. *L*-glutamine occupies a major proportion of free intracellular amino acids in the total pool of muscle [32], and degradation level of muscle protein is associated with content of free amino acids like *L*-glutamine. The catabolism of amino acids results in the production of ammonia (NH^3), which is converted into urea in the liver via the urea cycle. Whereas, carbamyl phosphate synthetase 1 (GPS) catalyzes the initial and rate limiting step of the urea cycle and is vital for the regulation of the pathway [33], but its allosteric activator *N*-acetylglutamate (NAG), is essential for the function of CPS in the urea cycle [34]. All in all, decreasing in urinary *N*-acetylglutamate was an indicative of low activity of urea cycle, and that might be caused by low muscle mass. So, decreasing in urinary *N*-acetylglutamate may be a signal of decline in total muscle protein. Creatinine or creatine is a breakdown product of creatine phosphate in muscle [35]. Viewed from Figure 3c and 3d, *L*-glutamine, creatinine, and *N*-acetylglutamate had a reduction in a holistic level comparing with the 1st period (Gln: $p=2.91 \times 10^{-9}$; Cn: $p=2.48 \times 10^{-6}$; NAG: $p=0.00097$), and this reduction exhibits extremely huge for 4-hydroxyproline ($p=0.0048$) and L-glutamine (1.13×10^{-8}) when comparing the fourth period with the third, suggesting a decrease in muscle mass after 520 days' isolation.

Altered excretion of urinary tricarboxylic acid cycle [36] intermediates, including citrate, succinate, and α-ketoglutarate, might indicate changes in energy expenditure during the isolation. Especially at the third point, a significant increase ($p=0.0064$) in citrate was observed comparing with the second point (Figure 3e). Increased urinary excretion of tricarboxylic acid cycle (TCA) intermediates indicates a decreased cellular utilization of these intermediates [37] in crews at period three. Nonetheless, α-ketoglutarate had significant decreased at period two ($p=0.0005$) and period six ($p=0.0005$), comparing with the initial point. Succinate exhibited no significant differences between two sequential periods but only increased at period six when compare with period two ($p=0.0053$). These conflicts might be caused by bypassing pathways like amino acid metabolism.

A set of metabolites including glycine, threonine and methylmalonate reflected a change in protein metabolism. Methylmalonate is a vital intermediate in the metabolism of fat and protein [38], and it had a same trend as urinary free amino acid (glycine, threonine). Viewed from Figure 3f, all these three metabolites had a significant increase in period two ($p<0.05$), and decreased in latter four period till all of them had a same level as initial point in period six. Increasing urinary excretion of these metabolites might be associated with increased consumption of endogenous protein.

O-Acetylcarnitine is a transporter that facilitates movement of acetyl CoA into the matrices of mammalian mitochondria during the oxidation of fatty acids [39]. Isovaleric acid is a fatty acid which is related with uptake of polyunsaturated fatty acids [40]. View from Figure 3g, *O*-acetylcarnitine had a continuous reduction from the first period to the sixth period, and it decreased in a very significant level (6.36×10^{-7}) at the end point comparing with the initial point, suggesting that energy produced from lipid oxidation decreased at the last three periods. Isovaleric acid increased markedly at period two ($p=7.81 \times 10^{-5}$) and also decreased at period four observably ($p=0.0002$). This might be caused by dietary items. Indeed, dietary composition in the first three periods is different from that of the last three periods during MARS-500 project. Foods consumed during the first three periods were provided by German, while foods consumed in the last three periods were self-chosen. Differences in excretion of isovaleric acid might be due to a change in intake of nutrients. Increased urinary excretion of xanthine (Figure 3h), the product of purine metabolism demonstrate an increasing level of intracellular reactive oxygen species (ROS), because ROS can cause increased degradation of adenosine monophosphate into xanthine [41].

N-methylnicotinamide is an intermediate involving in nicotinic acid pathway [42], and it had a significant ($p=0.0107$) elevation comparing the six period with the first period (Figure 3i), suggesting a cognitive dysfunction after the 520 days' isolation. 3-Hydroxymandelate is a metabolite of catecholamine [43] and it has a notable elevation in the last three periods comparing with the second period ($p<0.01$). Taurine can serve as a neurotransmitter in the brain and have a positive effect in promoting human cognitive performance [44]. Urinary taurine had increased at the second period ($p=0.0483$) and reduced during the last three periods ($p<0.01$). Reduction in relative concentrations of 3-hydroxymandelate and taurine during the last three periods might also manifest a weakening in cognitive performance, which was stressed by long-term isolation. Furthermore, it had also been reported that the crews in MARS-500 project had a decrement in their cognitive performance during the 520 days' isolation [45,46]. Moreover, another several studies conducted on isolated environment also have evidence

of weakening in cognitive performance [47,48]. However, exercise has been proven to have some protective effects for cognitive performance [46].

Alterations in gut microbial-host co-metabolites including indole-3-acetate and hippurate might indicate changes in gut microbial activities [49], in which, indole-3-acetate had a persistent elevation from period one to period six, but only showed significant differences at period six in contrast with period one (p=0.0012). However, hippurate showed markedly increasing at period two (p=0.0004) and decreased during later three periods, but increased at period six. These results suggested that 520 days' isolation had an effect on human gut microbial activities. Meanwhile, a metagenomic analysis of the dynamic changes of the composition of the intestinal microbiome of five participants of the MARS-500 experiment had shown that significant changes in the compositions of the microbiomes occurred just 14-30 days after the beginning of the experiment as well as a tendency toward a reversion of the microbiomes to their initial composition was observed two weeks after the end of the experiment, but complete recovery was not achieved [5].

Taken together, isolation environment gives a variety of stress load to human and finally impacts on human psychological and physiological indexes in multiple way (such as emotion, lipid metabolism, and gut microbial activities). Moreover, aside from MARS-500 project, a number of similar expeditions under isolated and confined environments have found evidence of decrement in the variables of cognitive performance [50], mood states [25], locomotor function [47], circadian rhythm and sleep [29], etc. However, results presented here therefore raised another questions: Did crews from MARS-500 project present the same responses for these psychological and physiological indexes?

People differences and its cross-talk with 520-days isolation

Six volunteers each occupy a well-defined region in the broad metabolic space, and they may have their specific alterations in responding to 520 days' isolation environment. Figure 4a and 4b shows the important features identified by two-way ANOVA analysis, where a p value less than 0.05 were considered as significant. As seen by Figure 4b, there were five biomarkers (gentisate, hippurate, *trans*-aconitate, melatonin, and 2-octenoate) that discriminated the six volunteers at a significant level, among of which, 3 of them (hippurate, *trans*-aconitate, melatonin) were also significant in factor time (days that had sustained in the MARS-500 module), but only hippurate (host-gut microbial co-metabolite) is not in the set of factor interactions, suggesting that six subjects each might have a different gut microbial organ, yet this organ showed consistent variation during 520 days' isolation. Another two (*trans*-aconitate, and melatonin) were also significant associated with factor interactions. In contrary with hippurate, urinary excretion of these two metabolites was different among every subject and changing at different directions during the isolation. Namely, each of the six subjects was different in energy expenditure and biological rhythm, and these two physiological status manifested diverse shift for each subject during isolation. Thus might explain the conflicts in variation tendency for succinate, α-ketoglutarate, and citrate which has been mentioned above. On the other hand, 2-octenoic acid only lied in the intersection of people differences and interactions, and this metabolite was significant in discriminating different people but had not notably alteration during isolation. However, differences in urinary excretion of 2-octenoic acid might be magnified by subtle effect of isolation, and this was why 2-octenoic acid just existed in intersection of people differences and interactions. Moreover, 2-octenoic acid is a normal organic acid produced by hepatic microsomal oxidation of aliphatic aldehydes [51] and changes in urinary excretion of it might reflect a different liver response to isolation. In addition, there were 3 biomarkers (biotin, 4-hydroxy-3-methoxymandelate, Nicotinamide *N*-oxide) were significant in the intersection of time and interaction, and among of them, biotin is associated with neurological manifestations [52], and 4-hydroxy-3-methoxymandelate, Nicotinamide *N*-oxide had been shown to be associating with cognitive performance [53,54]. Differences in these three metabolites shed light on that cognitive performance was not markedly deferred between the six subjects but the subjects each presented a different response to 520 days' isolation in cognitive performance. Finally, inosine was only located in the set of interaction without any intersection, and inosine degradation of purines and purine nucleosides to uric acid and in pathways of purine salvage [55], this suggesting that inosine originally had no significant differences among the six subjects and didn't markedly influenced by isolation, but it presented significant different levels among six subjects, which

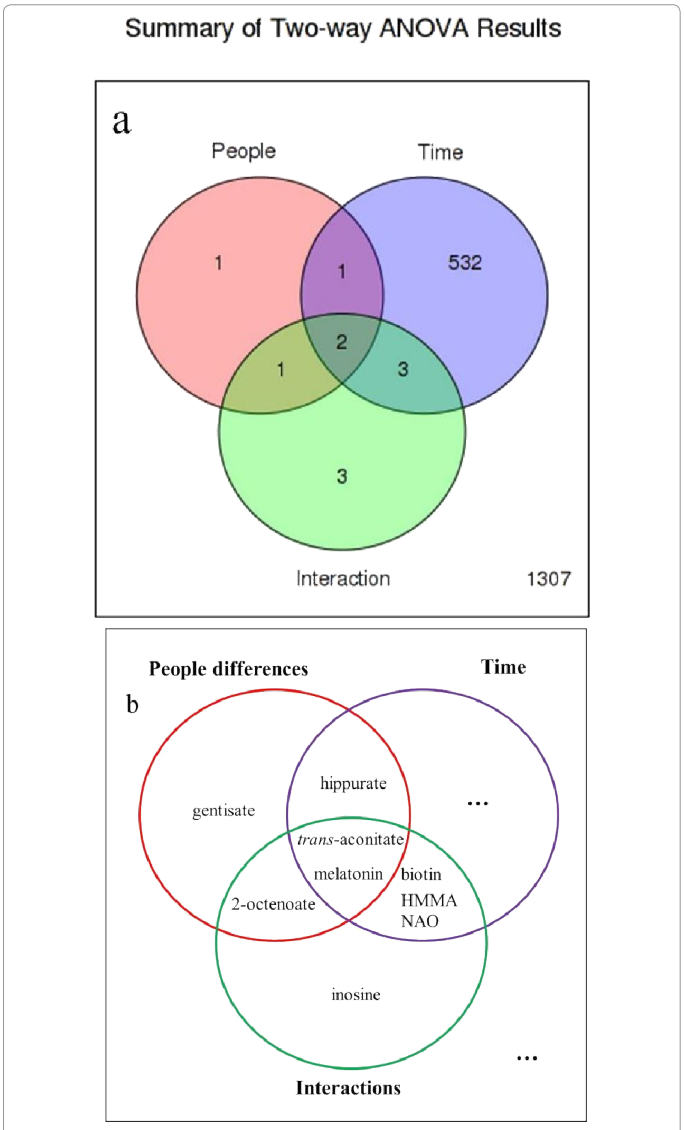


Figure 4: Important Features Selected by two-way ANOVA. Each circle represents a factor (people differences or time since isolation) which have great effects on data variation or the major patterns associated with interaction. a, numbers in this figure refers to the significant chemical shift; b, metabolites assigned according to chemical shift in a.

was affect by long term isolation, suggesting that long-term isolation might have enhanced levels of intracellular reactive oxygen species. All in all, intersections of those significant biomarkers were indicative of individual cross-talk with the long-term isolation.

Correlation structure of biomarkers

The correlation structure of biomarkers that descripted the state of organs in the human body will eventually lead to a functional understanding of the underlining biological changes. So, inter-correlations of endogenous biomarkers can infer multi-organic interactions determined variants in metabolic phenotype that exhibit large effect sizes. As presented in Figure 5, a positive association of serotonin, 4-hydroxyproline, and *L*-glutamine might reflect emotion affecting muscle mass [56]. Accordingly, strong correlation was also observed between glycine and metabolites from lipid metabolism, both of which may be transformed into Acetyl-CoA in latter TCA cycle. In addition, there were a strong positive correlation between taurine and the mentioned two groups of metabolites, suggesting that emotion, degradation of muscle protein, and lipid metabolism had a similar response to 520 days' isolation. Taken together, this correlation shed light on a global physiological regulation of the six crews in coping with isolated environment.

Correlation structures between urinary metabolites can be used to map the most affected pathway by isolation at a global level. As shown in Figure 6, as a chronic stress, 520 days' isolation caused release of neurotransmitter (e.g., serotonin) in brain which further leaded to activation of related enzyme through secretion of hormone. Active enzyme therefore increased degradation of muscle or inherent protein and produced free amino acid (e.g., 4-hydroxyproline, and *L*-glutamine; glycine, alanine, and taurine) for tissue updating. However, imbalance between income and consumption of muscle protein caused decreased extraction of urinary 4-hydroxyproline, and *L*-glutamine, suggesting a decline in muscle mass. Furthermore, the stress also caused decrease in production of energy by β-oxidation of lipid, which can be inferred by decreasing in urinary extraction of *O*-acetylcarnitine which plays an important role in β-oxidation of lipid. Nevertheless, heart activities occupied a main proportional of energy produced by β-oxidation of lipid, and decrement in β-oxidation of lipid may indicate a decrease in heart activities [57]. Moreover, errors in fat utilization can lead to brain deterioration like that of Reye's syndrome, gradually worsening muscle weakness [58]. Under this background, we firstly proposed another pathway which described a connection between human emotion and degradation of muscle protein or β-oxidation of lipid and this proposal need many experimental evidences.

Correlations between metabolic profiles and psychological perturbations

520 days' isolation environment is a chronic stress to the six crews, and a significant drift is observed to the subjects in metabolic space (Figure 2a). Namely, metabolic phenotypes of the subjects drift out from the first period to the farthest at third period, then gradually restored from the third to the six periods. However, there still was gap between the end point and initial point. This suggesting a property of resilience for metabolic phenotypes, which was in accordance with previous findings [10]. However, under the totally isolated and

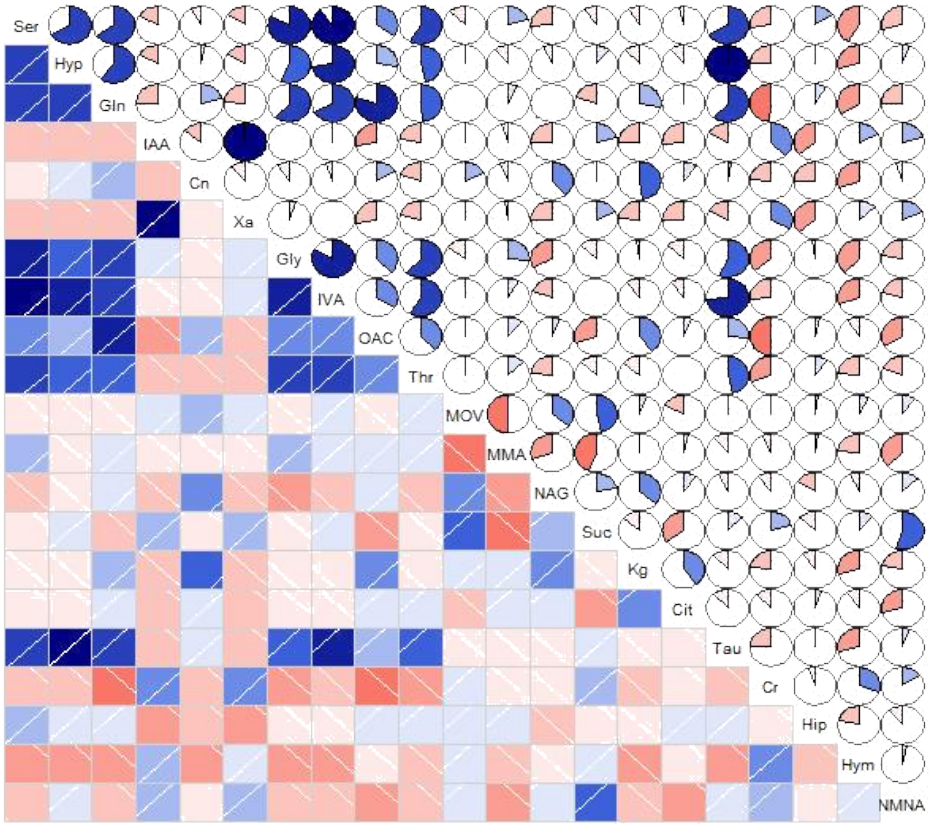


Figure 5: Correlogram of Biomarkers Intercorrelations. Color code: blue is the strongest positive association; red is the strongest negative association. Color gradient from red to blue presents Pearson correlation coefficient from -1 to 1. Area in upper triangular pie shows a positive association if it sweeps in a clockwise. Otherwise, it presents a negative association. The size of filled area presents the value of Pearson correlation coefficient.

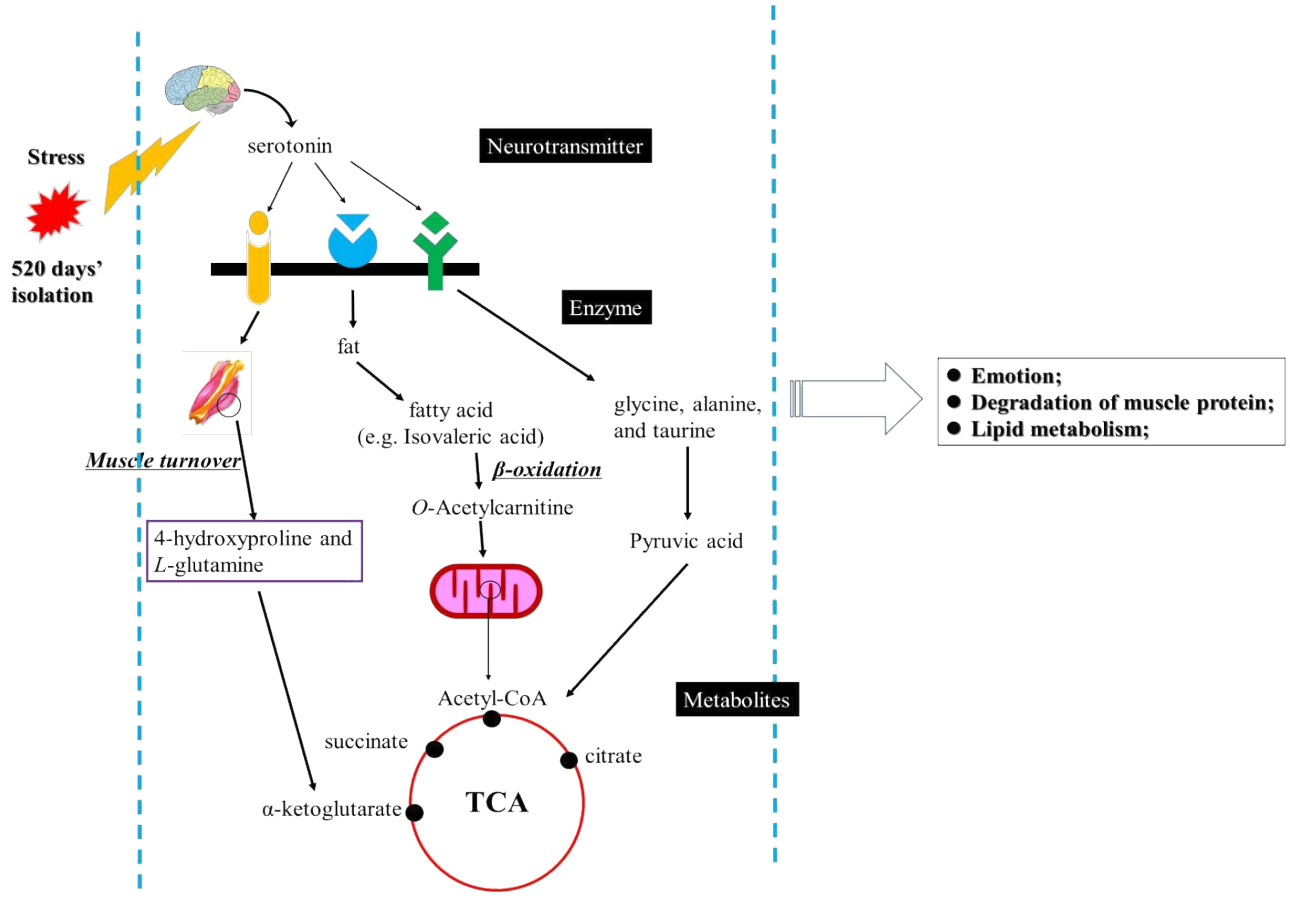


Figure 6: Mapping of urinary metabolite excretion pattern involved in emotion, degradation of muscle protein, lipid metabolism, and TCA cycle.

confined conditions in the MARS-500 habitat over 520 days, organisms adapted themselves to these perturbations by metabolic control [59] to meet those changing demands, resulting in a shift in metabotypes to the preferred metabolic state that an organism aimed to attain, which exhibited altered concentrations or populations in metabolites (Table 2). Strikingly, emotion was the most susceptible factor during the isolation. Feichtinger et al. [4] had shown in their publication that private communication was very important to the crews' psychological state during 520 days' isolation. However, it yet lacks of underlying physiological proofs. In contrary, we have found that serotonin might be served as the biomarker of psychological state, and metabolic control may be a new strategy in psychological state monitoring. Furthermore, other groups of biomarkers which were indicative of muscle turnover, circadian rhythms, energy expenditure, lipid metabolism, oxidative activity, cognitive performance, and gut-microbial activities may provide a more comprehensive insight into human state [1-5].

In addition, people differences played an important role in responding to isolated environment, which has been well descripted by the results of two-way ANOVA (Figure 4b). In this study, we have presented those six crews each had their own gut-microbes, but their microbes exhibited a consistent response to the isolated environment. In contrast, different crews have been proven to be different in response of cognitive performance and circadian rhythms to the stress of isolation. What's more, alterations of some metabolites (like inosine) that did not contribute to people differences had been magnified by isolation, although these metabolites did not significantly discriminate the days for isolation.

Eventually, correlation structure of metabolic biomarkers from different pathway may reveal an interaction between different organs. In present study, biomarker which is indicative of emotion has a strong correlation with that of degradation of muscle protein and β-oxidation of lipid. There are some publications proving that emotion can impact on human cognitive performance [48] and gut-microbial activities [60]. Therefore, it is reasonable to know the correlation in emotion, cognitive performance, and gut-microbial activities. However, impactions of emotion on degradation of muscle protein and β-oxidation of lipid may pick up much interest in future experiment, especially under isolated conditions.

Conclusion

NMR spectroscopy-based metabonomics [61] is undoubtedly able to provide a comprehensive and untargeted insight into the metabolic homeostasis of the human body, then it therefore subscribes the risk for environmental challenges. Based on metabolic profiles, this study demonstrated that when human faced with multiple challenges living under totally isolated and confined conditions, the final metabolic phonotypes had changed although they exerted some degree of resilience. In addition, people difference might play an important role in response to the isolation as *trans*-aconitate and melatonin discriminated both people differences and time after 520 days' isolation starting (Figure 4). So, our strategies in promoting human healthy state during future space mission should aim at practice of precision medicine [62,63]. Namely, reinforcing the human performance to

revert to his or her specific metabotype through personalized health care. In short, metabolic strategies provide us a new window into healthy promotion during future space mission.

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