Chapter 5: Methodology

Chapter 5

5. Methodology

The present study had two components:

a. The effect of yoga practices on sea voyage
b. The effect of yoga practices during Antarctic stay in summer,
   with non-yoga practicing members of the same expedition exposed to similar stressful conditions.

5.1. Participants / Subjects:

The participants for the study were members who volunteered for the 35th Indian Scientific Expedition to Antarctica (ISEA) between December 2015 and April 2016. The scientific crew following approvals of the project by appropriate agencies applied to National Centre for Antarctic and Oceanic Research, the nodal agency carrying out the Indian Expedition to Antarctica. The non-scientific crew were selected by a committee following receipt of application to participate in the expedition. The current study, to understand the effect of Yoga in extreme climatic conditions is a collaborative research project between Defence Institute of Physiology and Allied Sciences [DIPAS], New Delhi and *Swami Vivekananda Yoga Anusandhana Samsthana* [S-VYASA], Bengaluru.

5.1.1. Screening Procedure

The candidates volunteering and approved by the agencies for participating in the study were subjected to medical examination at All India Institute of Medical Sciences, New Delhi for assessing their health status. Medical assessments included serum biochemistry profile, radiological examination of joints, abdomen and thorax, dental, ophthalmological examination

and consultation with a psychiatrist. Candidates who were free from diseases and were physically and psychologically fit were considered eligible and a final list of members participating in the 35th ISEA was announced by National Centre for Antarctic and Oceanic Research [NCAOR].

Travel to Antarctica from India can be taken by two routes – sea and air. Based on the expeditioners work at Antarctica, the stationing base and mode of stay will be decided by NCAOR.

5.1.2. Inclusion and Exclusion Criteria

Inclusion Criteria:

Subjects matching the following criteria were recruited for the study

- Members undertaking sea voyage
- Willing to participate in the study
- Not diagnosed with any medical illness including non-communicable diseases, psychological illness, nervous system disorders, hypersensitivity disorders, haemorrhoids, arthritis and varicose veins.
- Not under medication at the start of the expedition

Exclusion Criteria:

- Subjects not willing to participate in the study and those who develop any injury during the Antarctic expedition were excluded.

5.1.3. Ethical considerations

The research study was approved by the Institutional Ethics Committee (Appendix 1). The subjects volunteering to participate in the study were detailed regarding the study and a written informed consent was obtained from all the volunteers.

5.1.4. Study Setting

The 35th Indian Scientific Expedition to Antarctica was for a duration of 97 days covering 9700 kilometers by ship in the southern ocean embarking at two Indian stations *Bharati* and *Maitri*. The Yoga practices were performed in the common dining area of the Indian ice-class research vessel *Ivan Papanin* for 1 hour every day, 7 days a week. The candidate was himself a member of the Yoga group. Owing to limited sample size, the candidate himself was also included in the study.

5.1.5. Sample Size Calculations

There are no previous studies conducted in this area of Yoga application. However, owing to the limited availability of Indians taking up sea voyage to Antarctica, all the available volunteers in the 35th Indian Scientific Expedition to Antarctica were considered to be recruited in the study.

5.2. Study Design

Subjects satisfying the inclusion criteria were given the option to be a part of either Yoga group or Control group. Owing to the already stressful nature of Antarctica involving confinement and isolation, it was decided not to randomise the subjects and all the members were free to be a part of either of the two groups. They were also free to withdraw from the study at any of time of the study. The Yoga group was administered with a standardised validated yoga module

specific to the conditions prevailing in Antarctica. The control group followed their usual routine activities.

Customised Yoga modules were developed and validated for sea voyage and Antarctica (*table5.1*). The detailed process of yoga module development and validation are attached as *Appendix-2*. A specific Yogic intervention '*Kunjal kriya* – Voluntarily induced vomiting' to control sea sickness was proposed in the module. A preliminary study to assess its effect on pulmonary function in novices and experienced practitioners were conducted in the mainland on a different set of volunteers before the expedition (*Appendix 3*). But, *'Kunjal kriya'*, was left out the module due to apprehensions from expeditioners that it would aggravate the sea-sickness.

5.3. Trial Profile

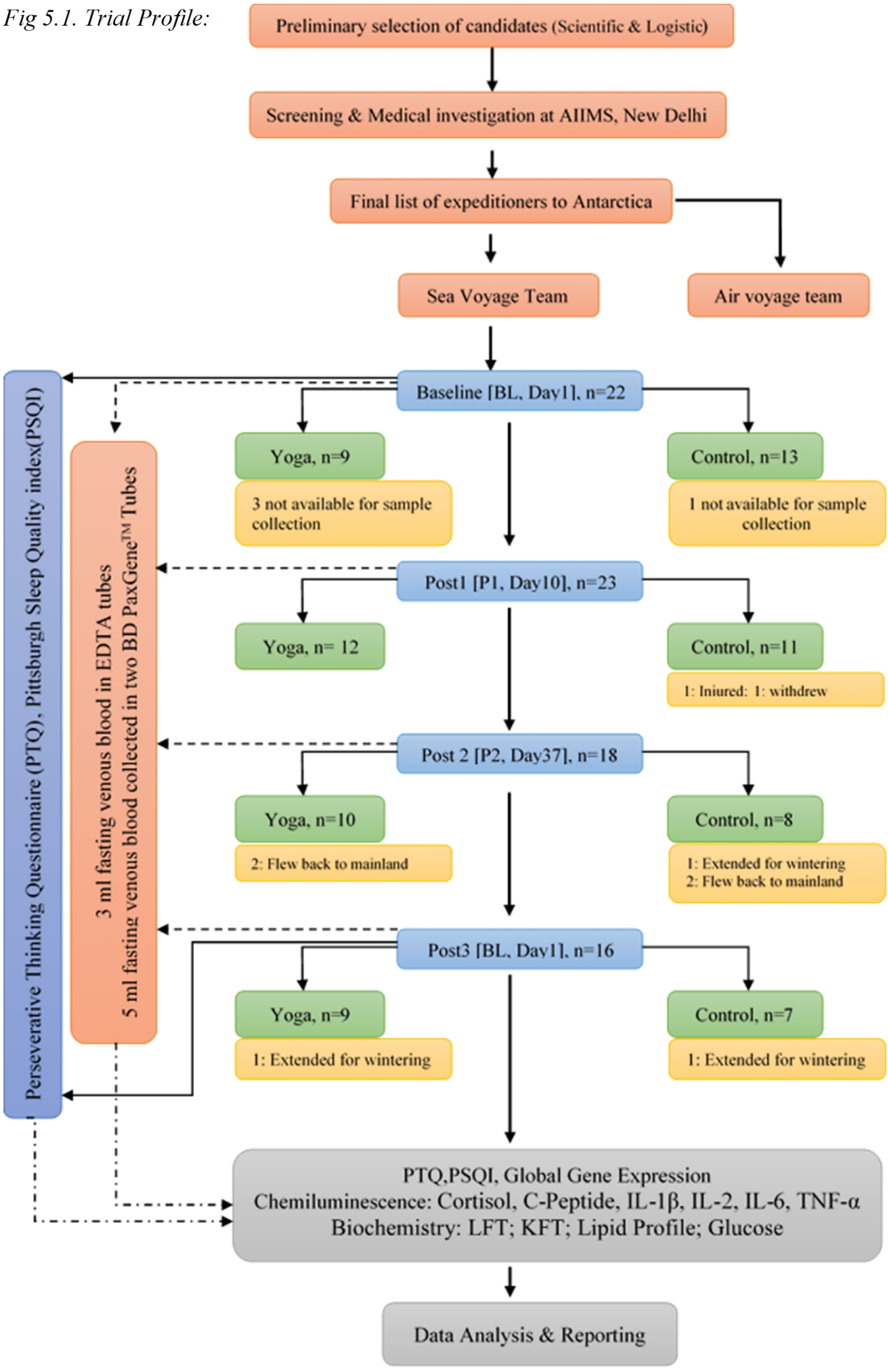
There were 25 members on-board vessel *Ivan Papanin* to Antarctica and all of them consented to be a part of the study. Baseline blood sample collection was not possible in three members due to their non-availability. Two members extended their expedition to wintering over at 1st stop *Bharati* and one member at the 2nd stop *Maitri*. Four members returned back to the mainland by air from the *Maitri*. Summary of members available for data collection is listed in the trial profile *(fig 5.1)*.

Members participating in the 35th ISEA, gathered at National Centre for Antarctic and Oceanic Research, Goa and travelled to Cape Town as four different batches based on the availability of flights. After two days of rest, the members embarked the expedition vessel *Ivan Papanin* on December 23rd 2015. The baseline assessments were performed on the 24th December 2015 (Day1), while the expeditioners were in empty stomach.

On the 2nd January 2016 (Day 10), the 2nd time-point assessments were made when the vessel reached ~200 km from the *Bharati* research base. As the objective of this time-point will be to answer the impact of adverse sea conditions, the data was collected before any member onboard research vessel would touch land.

Following 37 days of stay at *Bharati*, the third time-point data was collected on the 9th February 2016. And the final data point was collected before departing from *Maitri* (India Bay, ~90Km from *Maitri* research base) on the 19th March 2016 (Day 87). This time point was collected to understand the effect of stay in Antarctica on the expeditioners. The limitation in the study procedure is that, data was not collected on arriving at mainland from Antarctica.

Fig 5.1. Trial Profile:



S. No	Practices for Antarctica		Practices for Sea Voyage
	Sūkṣma vyāyāma [7 min]		***Sūkṣma vyāyāma***
1.	Grīvā śakti vikāsaka	1.	Grīvā śakti vikāsaka
2.	Aṅguli śakti vikāsaka	2.	Aṅguli śakti vikāsaka
3.	Maṇibandha śakti vikāsaka	3.	Maṇibandha śakti vikāsaka
4.	Kāraprastha śakti vikāsaka	4.	Kāraprastha śakti vikāsaka
5.	Kati śakti vikāsaka i & ii	5.	Kati śakti vikāsaka i & ii
6.	Jānu śakti vikāsaka	6.	Jānu śakti vikāsaka
7.	Piṇḍali śakti vikāsaka	7.	Quick relaxation technique
8.	Gulpha – pāda – prastha – pāda – tala – śakti – vikāsaka		***Yogāsanā***
9.	Instant relaxation technique	8.	Supta Udarakarshanāsanā
10.	Sūryanamaskāra		***Prāṇāyāma***
11.	Quick relaxation technique	9.	Vibhāgīya śvasana
	Yogāsanā [15 min]	10.	Kapālabhāti kriyā
12.	Ardhakati cakrāsana	11.	Nāḍī śuddhi prāṇāyāma
13.	Trikoṇāsana	12.	Bhrāmarī
14.	Parivṛtta trikoṇāsana		***Meditation***
15.	Pārśvakoṇāsana	13.	Nādānusandhāna / AUM chanting
16.	Vajrāsana		
17.	Uṣṭrāsana		***Modified Practices included***
18.	Paścimottānāsana	14.	Modified Ardhakati cakrāsana
19.	Vakrāsana	15.	Supported Lumbar Twist
20.	Arddha matsyendrāsana		
21.	Bhūnamanāsana		
22.	Cakki cālanā		
23.	Bhūjaṅgāsana		
24.	Setubandhāsana		
25.	Viparītakaraṇī		
	Prāṇāyāma [14 min]		
26.	Vibhāgīya śvasana		
27.	Kapālabhāti kriyā [60 strokes]		
28.	Nāḍī śuddhi prāṇāyāma [6 rounds]		
29.	Bhrāmarī [9 rounds]		
	Meditation [9 min]		
30.	Nādānusandhāna / AUM chanting		

Table 5.1: Validated Yoga module administered during sea voyage and at Antarctica

5.4. Data Collection:

5.4.1. Blood sample Collection

In this study 8 ml of fasting blood samples were collected from the antecubital vein at four time-points [5 ml in two BD PaxGene tubes + 3 ml in EDTA tubes]

a. Baseline, onboard research vessel *Ivan Papanin*, Capetown [Baseline, Day1]
b. On reaching Antarctic continent (Post 1, ~200 Kms away from *Bharati*, Larsemann Hills) [Post1, Day10]
c. On the day of departing from *Bharati* Station (Post 2, At Quilty Bay, *Bharati*, Antarctica) [Post 2, Day37]
d. Before departing from Antarctica (Post 3, At India Bay, ~90 Kms from *Maitri*, Antarctica) [Post3, Day87]

The blood samples were collected at four time-points on empty stomach. The day prior to the blood collection day was adhered as a dry day i.e., the subjects were not permitted to consume alcohol. The blood samples were collected from Left Ante-cubital vein using BD Vacutainer Safety Lock blood collection set *(product ID: 367292, BD Biosciences, USA)*. The blood samples were first collected in 3 ml BD K2 EDTA vacutainer *(product ID:367856, BD Biosciences, USA)* and then in two BD Paxgene blood RNA tubes *(product ID: 762165, BD Biosciences, USA).*

The collected blood samples were centrifuged within 1 hour from the time of blood collection at 1500 RPM for 10 minutes and the supernatant serum was separated using a pipette and stored in 1.5ml microcentrifuge tubes as 2 aliquots. The processed samples were stored at -20^{o}C throughout the expedition and were transported to the lab on dry ice. Following receipt, the samples were stored in -80^{O}C until they were analysed in biochemistry analyser.

The collected 2.5 ml of whole blood in each BD Paxgene blood RNA tube was mixed with prefilled 6.9 ml of preservative by gently rotating the tube for 8-10 times. The Paxgene tube was allowed to stand for 2 hours in room temperature and then shifted to -20^{0}C freezer and transported back to the laboratory. Following receipt of the tubes, they were stored in -80^{0}C until further experiments were performed.

5.4.2. Psychological assessments

Psychological assessments for Perseverative Cognition and Sleep quality were performed at time-point Baseline and Post 3

5.5. Assessments

5.5.1. Psychological Assessments:

Psychological assessments were made twice: once at the baseline (BL), onboard vessel *Ivan Papanin,* CapeTown and the post assessments were made before departing from Antarctica (P3).

Perseverative Thinking Questionnaire

The Perseverative Thinking Questionnaire (PTQ) is a 15-item scale to measure repetitive thinking with three subscales representing (1) the core characteristics of RNT (repetitiveness, intrusiveness, and difficulties with disengagement), (2) perceived unproductiveness of RNT and (3) RNT capturing mental capacity. The scale has an internal consistency with Cronbach alpha = 0.95 for total score and Core Characteristics of RNT: α = 0.92–0.94; Unproductiveness of RNT:α = 0.77–0.87; RNT Capturing Mental Capacity: α=0.82–0.90 for subscales (Ehring et al., 2011). The greater the global score, more is the perseverative cognition.

Pittsburgh Sleep Quality Inventory

The Pittsburgh Sleep Quality Index (PSQI) (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989) is a self-rated questionnaire which assesses sleep quality and disturbances over a 1-month time interval. Nineteen individual items provide seven "component" scores: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. The sum of scores for these seven components yields global score. A global PSQI score greater than 5 yielded a diagnostic sensitivity of 89.6% and specificity of 86.5% (kappa = 0.75, p less than 0.001) in distinguishing good and poor sleepers.

The script used for analysing PTQ and PSQI is mentioned as A*ppendix 4*

5.5.2. Biochemical Tests

Biochemical tests were performed for Serum Glucose, Lipid Profile (Total Cholesterol, Low Density Lipoprotein, High Density Lipoprotein, and Triglycerides), Liver Function Tests (serum glutamic-pyruvic transaminase (SGPT/ALT), serum glutamic oxaloacetic transaminase (SGOT/AST), Gamma-Glutamyl Transferase (GGT), Bilirubin, Total Protein, Albumin) and Renal Function Tests (Urea, Uric Acid, Creatinine). Detailed protocols of the assessments are mentioned as A*ppendix 5*.

5.5.3. Serum Biomarker Assessments

Serum levels of Biomarkers: Cortisol, C-Peptide, IL-1β, IL-2, IL-6, TNF-α

The serum biomarker levels using custom multiplex Chemiluminescence assays from Q-Plex Array *(Quansys Biosciences, USA)*. Q-Plex chemiluminescence kits are built by printing nano spots in defined arrays of multiple distinct capture antibodies in each well of a 96 well plate.

The standards were prepared in duplicates. The serum samples from P1, P2 and P3 were diluted as prescribed and added to the micro-well plate. The plate was incubated, added detection mix and washed as prescribed by the manufacturer. Finally, after addition of streptavidin beads and stop solution, the microwell plate was imaged using Syngene GBox Chemidoc system *(Syngene, USA).* The standard curve was calculated using Q-Plex software and the serum concentration of the analytes were determined. Detailed Chemiluminescence protocol is mentioned as A*ppendix 6*.

5.5.4. Global Gene Expression

Blood sample Collection

The blood samples were collected in BD Paxgene Blood RNA tubes and stored in -20^{O}C during the expedition and shifted to -80^{O}C during transport and further storage at the laboratory.

Total RNA Isolation & Testing RNA Integrity

The samples were thawed and RNA was isolated using BD Paxgene RNA kit following the manufacturer's instruction. The quality and yield of RNA was initially measured using a spectrophotometer *(Thermo Scientific NanoDrop 6000C, USA)* to determine the RNA concentration and the A260/A280 ratio. The integrity of RNA was tested using capillary electrophoresis method by Agilent 2100 Bioanalyzer *(G2939A Bioanalyser, USA)* and electropherogram profiles were generated. Samples with RNA Integrity Number ≥6.5 and presence of two distinct peaks for 18S and 28S ribosomal RNA were only used for gene expression experiments. If the quality was lesser, the RNA extraction procedure was repeated from the 2nd aliquot of the Paxgene blood RNA tube. Detailed protocol for RNA extraction and Capillary Electrophoresis is mentioned as A*ppendix 7 & 8.*

Gene Expression Analysis was performed on twelve subjects, 6 from each group from three time-points:

a. Baseline, on departing from Capetown
b. On reaching Antarctica, at *Bharati*
c. On departing from Antarctica, at *Maitri*

Global gene expression was performed using Agilent Sureprint v3 8X60k *(Product Number: G4851C; Design ID: 072363)*. The slides that we had used for our experiments consisted of 26,083 enterez genes and 30,606 lnc RNA. There were 3000 replicates of biological features with 96 ERCC & 10 E1A control probes in the slide. The protocol for gene expression was performed as per the manufacturer's guidelines (*Appendix 9*). The hybridised slides were scanned using Agilent D Scanner (Agilent Technologies, USA).

Five slides containing forty arrays were used (n=36) for the microarray study. There were 6 samples from yoga and control groups each and three timepoints for each samples were present. The samples were allocated to random arrays to avoid any bias in the comparison of treatments. The allocated position of each of the samples are mentioned *Appendix 10*

The glass slides containing the RNA samples were scanned using Agilent D scanner as per the standard settings for the slide, suggested by the manufacturer.

Microarray Feature extraction and Normalisation

The scanned microarray slide was processed using the Feature Extraction software (version 12.0.3.2) supplied by Agilent Technologies. The 'gProcessedSignal' from the output file was used for further downstream analysis. The gProcessedSignal was calculated by following the standard settings recommended by the manual for the Agilent feature extraction software for one colour.

Quality control check were performed on the microarray data output generated from Feature Extraction software. The quality control parameters followed were as recommended by the manual for the Agilent feature extraction software *(version 12.0.3.2, Agilent Technologies, USA)* for one colour. The standard QC metrics for one colour arrays included spot finding at four corners of each array, spatial distribution of the outliers and histogram of the signals across all non-control probes. The output profiles from the feature extraction indicated that the microarray data generated were reliable and of good quality. In this regard, the quality control reports from the feature extraction showed that there were no spatial abnormalities and the arrays were performing normally. The chip spike-in controls also behaved similarly and were no obvious outliers.

Identification of Differentially regulated genes

Two analysis strategies were used to identify the Differentially regulated genes in the present study.

i. Conventional analysis
ii. Un-conventional exploratory analysis

Conventional analysis (Stringent Protocol)

The genes that were flagged as 'Detected' across all the 36 samples across two groups (6 samples in each group across three time-points) were filtered and the signals were log-transformed using Gene Spring software (Version 14.8). This strategy was adopted to ensure that an appropriate comparison is made while estimating the fold change values.

Genes which are more than 2-fold regulated in each of the groups were tabulated [BL-P1; P1-P3; BL-P3 of Yoga and Control groups]. Only the genes that had EnterezID annotation were

used for further over-representation analysis, enrichment analysis and profiling of transcription factors.

Unconventional Exploratory Analysis

In this analysis, each sample was treated as an individual experiment. The genes that were flagged 'Detected' across three time-points of the sample were alone filtered and the intensity values were log-transformed. Genes which were more than 2-fold regulated were tabulated for each sample. The DEGs from all the samples of yoga and control groups were pooled and the fold change values were averaged for the genes that were more than 2-fold regulated in multiple samples of the same group. However, not all the genes in the slide were having EntrezID. So, only the genes annotated with EnterezID were used for further analysis.

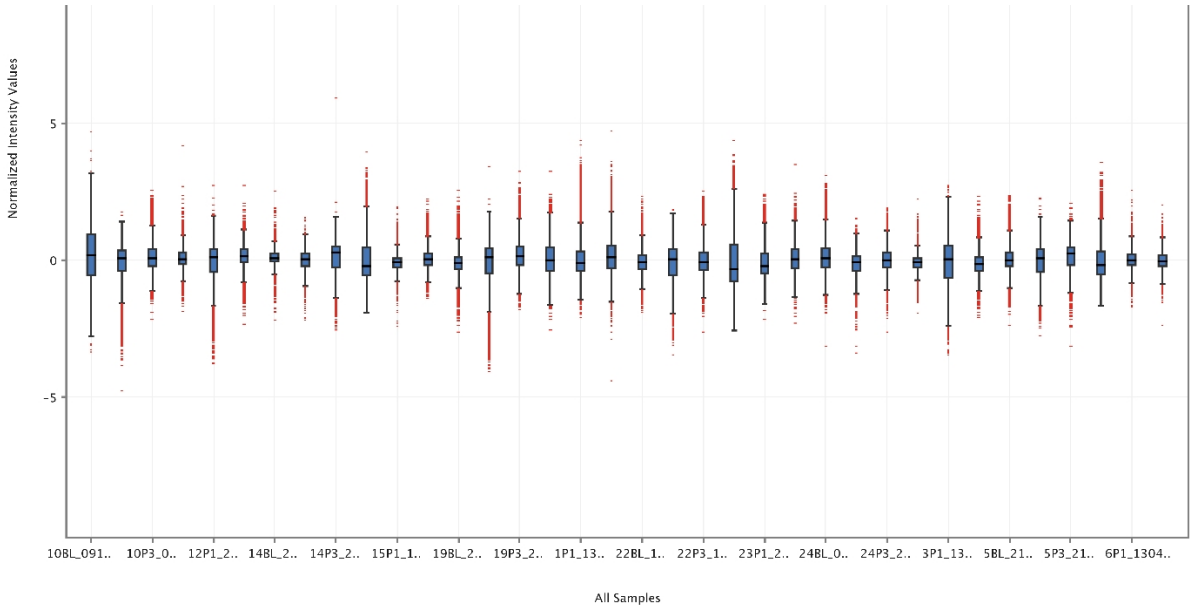


Figure 5.5.4: Box plot of Normalised intensity values of all samples included for microarray analysis

5.6. Statistical Analysis

5.6.1. Biochemical and Psychological parameters:

Test for Normality

For both biochemical and psychological parameters the change observed for each variable was calculated by subtracting the post values from the baseline values. Kolmogorov Smirnov's test was performed on the difference values to check for normal distribution of data. All the data except Pittsburgh Sleep Quality Index were normally distributed.

Statistical tests

Following the normal distribution of Biochemical test data, paired t-test was employed for ascertaining within-group differences. Paired *t*-test was employed for different time points instead of Repeated Measures Analysis of Variance [RMANOVA] because of the dropouts at every time-point.

For both psychological & biochemical parameters between-group differences were assessed using Analysis of Co-variance [ANCOVA] with respective baseline values as covariates to check for between-group differences.

The statistical tests adopted for Gene expressions are mentioned below

5.6.2. Gene expressions

The scanned image obtained was processed with Agilent Feature Extraction software. Standard settings recommended by Agilent were used for data normalisation and feature extraction. The features were labelled as Detected, Not Detected and Compromised based on several algorithms to quantify the signals. Even though, the manufacturer recommends using

both detected and not-detected flags for downstream analysis, only features with the flag 'detected' was used and the other probes were not included in the analysis.

The features that were flagged 'Detected' in all the three time-points of each sample alone were filtered, log-transformed and fold change was calculated. Shapiro-Wilk's test for normality was performed with the level of significance fixed at $p \geq 0.1$. Two-way ANOVA was performed. P-value was computed Asymptotically and Benjamini-Hochberg test for multiple testing correction was performed. Genes that were ≥2-fold change values for each time point comparison [BL-P1; P1-P3 and BL-P3] for Yoga group and control group were tabulated for further analysis.

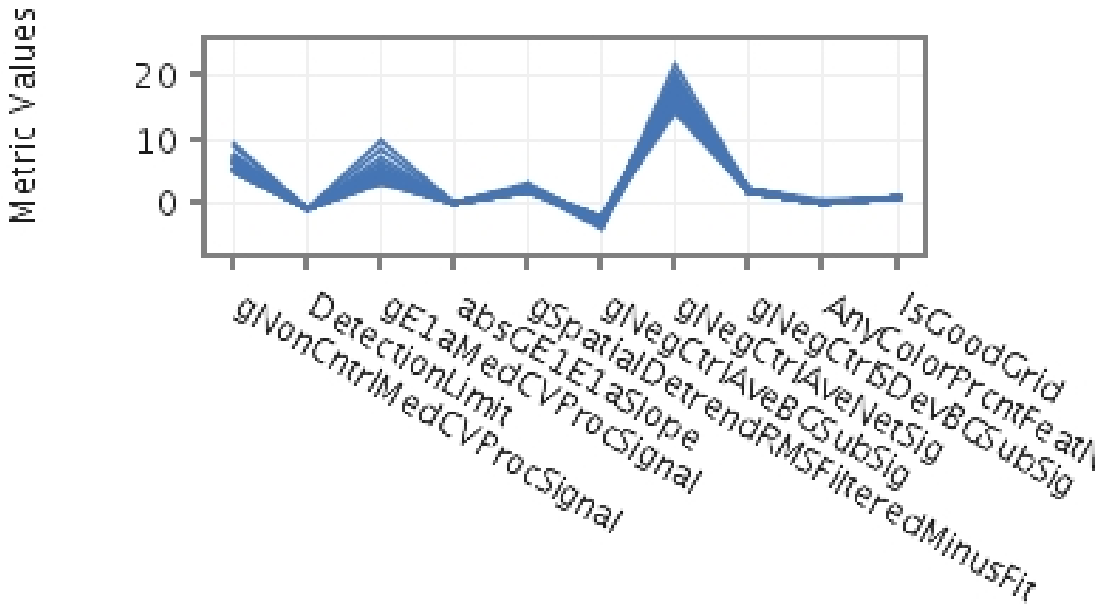


Image 5.6.2: PCA plot for control probes in all the 36 samples across two groups and three timepoints

Following analysis were performed on ≥ 2 fold differentially regulated genes obtained from two analysis methods.

a. Gene Ontology Analysis
b. Over Representation Analysis

c. Gene Set Enrichment Analysis
d. Genes involved in common pathological conditions
e. Pathway Analysis

Gene Ontology Analysis

The genes are classified across a universally agreed classification system into three sub-categories representing gene-product categories, covering three domains:

a. Cellular Component, the parts of the cell or its extracellular environment
b. Molecular Function, the elemental activities of a gene product at the molecular level, such as binding or catalysis
c. Biological Processes, operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms

For Conventional Stringent Analysis:

Gene Ontology analysis was performed on ≥ 2 fold regulated genes in Yoga and Control groups obtained between two time-points [BL-P1, P1-P3, BL-P3]. Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8) was used for computing gene ontology classes for the differentially regulated genes for each time point comparisons. Fischer Exact's test for multiple testing correction was used to identify the significantly regulated gene ontologies with level of significance fixed at $p \leq 0.05$. GOTERM_BP_Direct; GOTERM_CC_Direct and GOTERM_MF_Direct were considered for reporting the direct ontology classes.

For Exploratory Analysis

Gene Ontology analysis was performed on ≥ 2 fold regulated genes in Yoga and Control groups obtained between two time-points [BL-P1, P1-P3, BL-P3]. PANTHER (Protein ANalysis THrough Evolutionary Relationships) database [Version 12, released 10-07-2017] was used to classify the differentially expressed genes [DEG] based on the gene ontology. This strategy was used to understand the ontology class of the DEGs(Mi, Muruganujan, Casagrande, & Thomas, 2013).

Over-Representation Analysis

Over-representation analysis is a technique to determine if a set of categories in the list of differentially expressed genes are present more than expected. ClueGo application in Cytoscape was used to visualise the over-represented ontologies (Bindea et al., 2009).

Overrepresentation analysis was performed on ≥2 DE genes from Yoga and Control groups for each time point comparison of the exploratory analysis individually using binomial distribution test and Bonferroni correction for multiple testing was performed (PANTHER, Version 12, released 10-07-2017). For the conventional analysis, the same was performed using Fischer's exact test with FDR correction for multiple testing (PANTHER, version 13.1, released 03-02-2017) [The difference in statistical algorithms used are due to the change in the version of database used].

Statistical Enrichment Analysis

Enrichment Analysis is a computational method that determines whether an *a priori* set of genes that shows statistically significant, concordant differences between two biological states [between group analysis]. This algorithm involves categorisation of genes (from the input list for a specific timepoint between yoga and control groups) and ascertains if the

regulation observed is by chance using the Wilcoxon signed-rank test and a P-value for the significantly regulated pathways is given. The p-value are corrected for multiple testing using FDR and q-values are estimated.

The significantly regulated pathways between the Yoga and Control groups at any given timepoint [BL-P1, P1-P3, BL-P3] were determined using the Consensus Path Signalling Database. The enriched pathways were obtained from 11 databases: Wikipathways, Netpath, Kegg, Humancyc, Ehmn, Inoh, Smpdb, Biocarta, Reactome, Signalink, and Pid (Kamburov et al., 2011).

Pathway Analysis

The participating differentially regulated genes from were visualised using Paintomics database (Garcia-Alcalde, Garcia-Lopez, Dopazo, & Conesa, 2011). Paintomics is a web tool for the integrative visualisation of multiple omic-datasets onto KEGG pathways.

Genetic Association of Disease

The expression of genes associated with common pathologies associated with Antarctica was of our interest. To understand the expression of disease associated genes, DisGeneT database was used. DisGeneT is presently the largest publicly available discovery platform on genes and variants associated to human diseases (Janet Piñero et al., 2017; J Piñero, Queralt-Rosinach, Bravo, & Deu-Pons, 2015). A database of DisGeneT was downloaded and the genes associated with the diseases of our interest were tabulated.

To understand the genes that were expressed in common and unique to both yoga and control groups, InteractiVenn was used. InteractiVenn is an online Java based application to generate venn diagrams and also provide tabulated data on genes that are common and unique to multiple groups (Heberle, Meirelles, da Silva, Telles, & Minghim, 2015).

Transcription Factor Profiling

The differentially regulated gene list was used identifying the transcription factors that were differentially regulated in either of the groups. The differentially regulated genes at each time-points in Yoga group and control group were given as input to the PANTHER database [Version 12, released 10-07-2017] and the transcription factors were tabulated. The identified transcription factors were explored in ClueGo to map them based on their ontology.

Stringent Analysis

The genes that are found to be detected in all the 36 conditions (12 samples across 3 timepoints) alone were filtered out and processed for obtaining differentially regulated genes making a stringent condition. Over-representation analysis was performed using PANTHER database [Version 12, released 10-07-2017] to understand the processes regulated in the Yoga group and Control group.