The heart-brain axis: A proteomics study of meditation on the cardiovascular system of Tibetan Monks

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Summary

Background There have been mixed reports on the beneficial effects of meditation in cardiovascular disease (CVD), which is widely considered the leading cause of death worldwide.

Methods To clarify the role of meditation in modulating the heart-brain axis, we implemented an extreme phenotype strategy, i.e., Tibetan monks (BMI > 30) who practised 19.20 ± 7.82 years of meditation on average and their strictly matched non-meditative Tibetan controls. Hypothesis-free advanced proteomics strategies (Data Independent Acquisition and Targeted Parallel Reaction Monitoring) were jointly applied to systematically investigate and target the plasma proteome underlying meditation. Total cholesterol, low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (Apo B) and lipoprotein (a) [Lp(a)] as the potential cardiovascular risk factors were evaluated. Heart rate variability (HRV) was assessed by electrocardiogram.

Findings Obesity, hypertension, and reduced HRV is offset by long-term meditation. Notably, meditative monks have blood pressure and HRV comparable to their matched Tibetan controls. Meditative monks have a protective plasma proteome, related to decreased atherosclerosis, enhanced glycolysis, and oxygen release, that confers resilience to the development of CVD. In addition, clinical risk factors in plasma were significantly decreased in monks compared with controls, including total cholesterol, LDL-C, Apo B, and Lp(a).

Interpretation To our knowledge, this work is the first well-controlled proteomics investigation of long-term meditation, which opens up a window for individuals characterized by a sedentary lifestyle to improve their cardiovascular health with an accessible method practised for more than two millennia.

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Introduction

World-wide heart disease is the leading cause of death in developed countries including the U.S. Lifestyle changes including meditation offer an attractive complementary strategy to maintain a healthy heart and cardiovascular system in an increasingly greying world. Although a number of studies have reported the benefits to cardiac protection after meditation, two recent

Research in context

Evidence before this study

Cardiovascular disease is the leading cause of death in China and the West, driving the hunt for primary preventions. Several recent reviews posed a number of caveats to the apparent link between meditation and cardiovascular protection, suggesting a need for further research.

Added value of this study

To our knowledge, this work is the first controlled proteomics investigation of long-term meditation practice. We found that although the plasma proteome was distinctly remodelled among three types of meditation, their end-point biological effects converged on cardiovascular protection. We have chosen well-established lipid markers of CVD risk factors based on longitudinal studies across cultures and ethnic groups. Notably, CVD risk factors in plasma were significantly decreased in meditative monks compared with non-meditative Tibetan controls, despite monks having a clinically obese BMI > 30.

Implications of all evidence available

Coupled with an extreme phenotype sampling strategy, this study demonstrates the power of proteome analysis and elucidates how meditation contributes to preventing CVD even in clinically obese subjects. This study suggests that the popular linkage of meditation and a healthier life trajectory may not be too far off track. Meditative practice may thus offer an avenue for reducing the untoward effects of obesity on cardiac health. The current findings underscore the crucial role played by the heart-brain axis in contributing to an extended and healthy life history trajectory.

related proteomics papers. One is based on a 12-week course of Tai Chi[11] and the other is based on a single 20-min yogic breathing exercise.[12] In contrast, the current investigation is designed to minimize the conundrums apparent in previous studies of meditation and CVD by implementing an extreme phenotype sampling strategy, which has been used successfully in both genetic and metabolomic studies.[13,14] Specifically, we recruited 78 Tibetan Buddhist monks who engaged in meditation practice for a remarkable average of 19 years. The monks engaged in at least one hour per day of mediation with a mean time of 3.92 ± 3.21 h (range $1-15$ h). We controlled for age, sex, and dietary habits in the control group of 47 non-meditative Tibetans. To our knowledge, this is the largest sample size so-far studied for long-term Tibetan meditation. We implemented advanced Data Independent Acquisition (DIA)[15,16] and Parallel Reaction Monitoring (PRM)[17] proteomics strategies to systematically interrogate and target the key cardiovascular-related plasma proteome. In addition, total cholesterol, low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (Apo B), and lipoprotein (a) [Lp(a)] as the potential cardiovascular risk factors were evaluated. Heart rate variability (HRV) was assessed by electrocardiogram. Altogether, our overarching goal in this study was to systematically investigate the impact of meditation on the proteome towards a more comprehensive and wide-ranging understanding of its relationship to the health of the cardiovascular system.
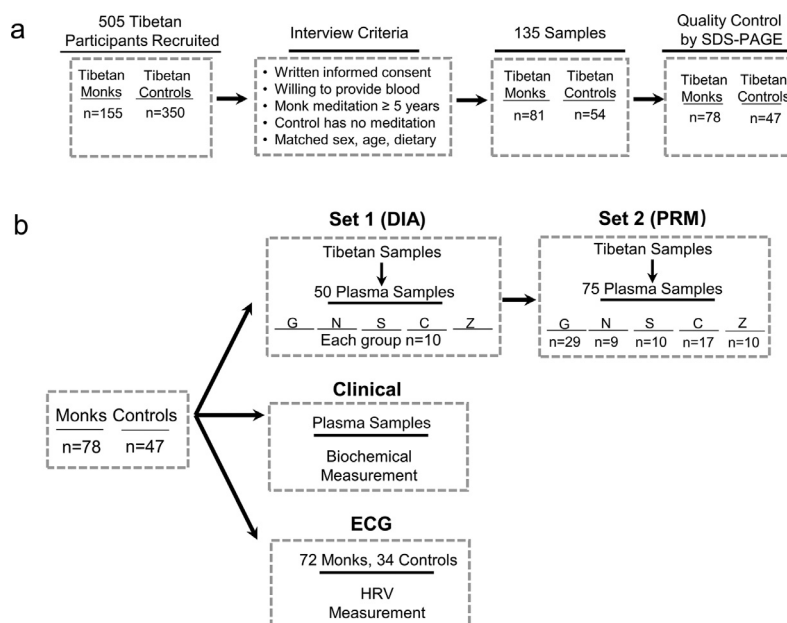
Methods

Ethics

This study was approved by the Institutional Review Board of Shanghai Mental Health Center (SMHC). The reference number of the approval letter is 2016-19. Participants gave their written informed consent.

Tibetan subjects

We interviewed 505 ethnic Tibetan participants including 155 monks and 350 local Tibetans in remote areas of Tibet in 2016. These subjects live at a high altitude of > 3000 m. From this group, we recruited 81 Buddhist monks who were characterized by long-term uninterrupted meditation practice (at least 5 years per monk, 18.54 ± 8.41 years) and 54 local Tibetan controls with no experience in meditation, carefully matched for age (≥ 18), sex (male), and dietary habits (Figure 1a). Exclusion criteria included diagnosis of diabetes or tuberculosis. Monks recruited belong to three types of Tibetan Buddhism: Gelug (G), Nyingma (N), and Sakya (S) sects. It should be noted that meditation styles across sects are not mutually exclusive and differ somewhat in two dimensions. First, the meditation practice of the

reviews[1,2] posed a number of caveats to the apparent link between meditation and cardiovascular protection, suggesting a need for further research. A complementary approach to validate the link between meditation and CVD is to comprehensively examine the plasma proteome, which is a critical mediator of cardiovascular processes, and has been the focus of a number of clinical studies to identify diagnostic biomarkers for CVD.[3-8] Importantly, analogous to genome-wide association studies, examining the proteome is hypothesis-free,[9,10] which is increasingly revealing its power to reliably detect proteins for complex phenotypes. These studies set the stage for an interrogation of the "meditator" proteome towards establishing the role of meditation practice on cardiovascular health.

To our knowledge, there are no studies that have comprehensively investigated the plasma proteome following long-term meditation. There are two obliquely

Figure 1. Overview of sample collection of proteomics, clinical indexes and HRV measurement. (a) Recruitment of Tibetan monks and matched local Tibetans. 505 Tibetan participants including 155 monks and 350 local controls were reviewed. 81 Tibetan monks and 54 local controls agreed to give written informed consent and provide blood. These monks have meditated for a mean time of 18.54 ± 8.41 years (≥ 5 years for each monk). Local controls were non-meditative and were matched with sex, age and dietary habits. The protein quality of plasma samples was tested through SDS-PAGE. Plasma from 78 Tibetan monks and 47 local controls passed the quality control and were used for further analysis. (b) Workflow of two-stage proteomics, clinical and ECG measurement. Three sects (G: Gelug, N: Nyingma and S: Sakya) were involved. 10 Tibetan monks from each sect and 10 matched local controls (C: controls matched with Gelug monks, Z: controls matched with Nyingma and Sakya monks) were selected for DIA analysis. The remaining 75 Tibetan subjects were used for target validation using the parallel PRM technique. All Tibetan plasma samples were subjected to biochemical measurement for clinical indexes. 72 out of 78 Tibetan monks and 34 out of 47 controls were undertaken ECG detection for HRV measurement.

three sects encompass three stages: practising renunciation, Bodhicitta, and demonstrating the enlightenment of voidness (becoming a Buddha). Overall, the first two stages are essentially similar whereas the three sects diverge when it comes to demonstrating the enlightenment of voidness. Most practitioners of the Gelug and Sakya sects achieve the goal by visualizing and practising energy (Sanskrit: prana), channels (Sanskrit: nadi), and seeds of energy (Sanskrit: bindu). However, Nyingma sect commonly does not depend on such appearances. Second, each sect differs in content and order of visualization albeit each implements both Samatha and Vipasyana practices. In particular, the Gelug sect focuses more on transcendental meditation and focused meditation; the Nyingma sect pays more attention to compassion meditation; the Sakya sect tends to use transcendental meditation. Each meditator practices for one to several hours per day.

Altogether a total of 78 advanced Tibetan Buddhists (19.20 ± 7.82 years) and 47 local Tibetan controls, whose plasma qualified, were used for further proteomics analysis (Figure 1a). We note that in the extreme phenotype sampling strategy, recruitment of unequal sample sizes is practically unavoidable.[14]

Considering their geographic isolation, each meditation type was matched with its respective non-meditative local Tibetan controls. Since Nyingma and Sakya monks lived in the same geographic region, they shared the same controls, briefly named as "Z". Gelug monks live in a separate remote region, hence they have their own controls, named "C". Altogether, we have five subject groups, namely, G, N, S, C, and Z.

DIA-MS and PRM-MS analysis

In this study, DIA discovery proteomics was used to systematically investigate the cardiovascular-related proteome mediated by meditation. PRM targeted proteomics was applied to validate the key cardiovascular-related proteins identified in the DIA stage. The two-stage proteomics procedure was applied on two cohorts of Tibetan samples (Figure 1b). Plasma was collected and abundant proteins were removed using Pierce™Top 12 Abundant Proteins Depletion Spin Columns (85165, Thermo Fisher Scientific, NC, U.S.) prior to enzyme digestion. The Orbitrap Fusion Lumos Tribrid mass spectrometer interfaced with a nano electrospray ion source (Thermo Fisher Scientific, NC, U.S.) was applied

for proteome detection. The detailed mass spectrometry methods are in the Supplementary Method.

ECG recording and HRV analysis

Electrocardiogram (ECG) signals were recorded from 72 out of 78 Tibetan Buddhists and 34 out of 47 local Tibetan controls (Figure 1b). One electrode of the 64-channel Ag/AgCl EasyCapTM (Brain Products GmbH, Munich, Germany) was placed on the chest at a sampling rate of 1000Hz. During the ECG recording, subjects were seated in a cushion and instructed to rest quietly with eye-closed for 10 min. R peaks were detected by an open-source software OSEA-4-Java (Version 1.0.0, https://github.com/MEDEVIT/OSEA-4-Java), and further visually inspected and corrected for false detections. Then based on R-R intervals, HRV indices from the time domain and frequency domain were computed using the HRVAS software package (https//sourceforge. net/projects/hrvas, 2015). The HRV indices analyzed in this study were: standard deviation of R-R interval variability (SDNN); mean heart rate (HR); absolute power of very low frequency (VLF, 0.003-0.04 Hz); absolute power of low frequency (LF, 0.04-0.15 Hz); absolute power of high frequency (HF, 0.15-0.40); ratio of low-frequency to high-frequency power (LF/HF).

Bioinformatics and statistics analysis

This is a very special sample that is extremely hard to collect. There are few if any studies of this special population, so it was not feasible to carry out a sample size estimation. We collected as many samples as practicable, achieving the largest dataset for long-term Tibetan meditation and strictly matched Tibetan controls to date.

To minimize the unwanted effects of sampling and transportation, critical measures were taken. (1) Participants in the same region came to the same site for sample collection, and all samples were collected within one week for each region. (2) Blood was collected by the same professional nurse to ensure consistency. (3) Plasma samples were collected and aliquoted by the same scientific researcher to ensure the consistency of plasma separation. (4) Plasma was immediately stored at -20 °C. When all samples were collected for each region, they were transported to the Shanghai laboratory by airline immediately. Once arrived, samples were transferred to a -80 °C freezer for storage until use. All the above measures ensure the reliability and comparability of our research across the geographically dispersed samples. We conducted a Spearman correlation analysis between the sampling time and protein expression within each group and found there were no significant correlations, suggesting that there were no effects of sampling and transportation for this study (Supplementary Table 1). Demographics

information, blood collection, plasma separation, and data analysis were performed by different staff to ensure the blindness of the study.

For the DIA and PRM proteomics analysis, outliers were removed until the standard deviation of protein intensities of the remaining samples was ≤ 2 in each group. Only proteins quantified in at least 50% of samples in each group were used for subsequent analysis. K-nearest neighbour (KNN) imputation (k =3) was carried out to handle the missing values using the impute package in R (version 4.1.0). Analysis of covariance (ANCOVA) was carried out to investigate the differentially expressed proteins (DEPs) within each compared group, adjusted age and BMI as covariates. Multiple test correction was not performed for the DIA state, in order to minimize the loss of potentially informative data. In the PRM validation stage, FDR correction were applied on the key cardiovascular-related proteins that were found in the DIA stage. Only those that passed the FDR correction and had the same significant regulation as in the DIA stage were reported and used for interpretation of the results. Proteins identified with at least two unique peptides and a fold change of $\geq |1.3|$ and $P < 0.05$ were defined as DEPs

Biological function annotation, enriched pathways, network analysis were conducted using the Ingenuity Pathway Analysis platform (QIAGEN Redwood City). Unsupervised hierarchical clustering was performed with pheatmap package in R. "Complete" was used as the clustering method. "Correlation" was used for distance calculation. Principal Component Analysis (PCA) was conducted with FactoMineR and factoextra packages in R. Pearson correlation were conducted on log-transformed protein intensities using cor () function in R.

For clinical and HRV measurements, qualitative data were analyzed by chi-square test. For quantitative data, normality was tested using the Shapiro-Wilk test. Levene test was used to examine the homogeneity of variance. If data were normally distributed and showed equal variances, Student's t-test or one-way ANOVA was applied. If data were normally distributed whereas with unequal variances, Welch's test or Welch ANOVA was used. If data were not normally distributed, the Mann-Whitney U test was conducted. For correlation analysis we used, Pearson, Pearson partial, and semi-Pearson partial correlation tests; Spearman, partial Spearman, and semi-Spearman partial correlation tests were conducted as appropriate. Linear mixed-effects analysis was conducted using lem4 package in R. P-values were obtained by likelihood ratio tests. During analysis, age and BMI were adjusted as covariates as needed. Multiple test correction was applied for P-values using the Benjamini-Hochberg method.

Additional information is provided in the Supplementary Materials & Method's section.

Roles of funding source

The funding sources supported the study design, sample collection and preparation, mass spectrometry detection, data collection and analysis, writing of the manuscript and fees for publication.

Results

Meditation remodeled plasma protein profiles (DIA-MS)

In this study, DIA-MS discovery and PRM-MS targeted proteomics were jointly applied to investigate and validate the key proteins related to the cardiovascular system, mediated by long-term meditation. Two cohorts of Tibetan samples were involved, respectively (Figure 1b).

First, in the DIA-MS discovery phase, we chose 10 samples from each of the five subject groups (G, N, S, C, Z), matched for age, sex, and dietary habits (Sample Set 1) (Figure 1b, Table 1). The DIA-MS technique identified a total of 963 proteins (Supplementary Table 2). The median coefficients of variation (CVs) of protein intensities in each group ranged from 30% to 35% (Supplementary Figure 1a), consistent with previous reports of biological variation.[18] The quality of the data was further strengthened by the high reproducibility of results by the repeated analysis of randomly selected samples (Supplementary Figure 1b, R = 0.95 on average).

Unsupervised hierarchical clustering (HC) was used to characterize the protein expression profiles. Results showed that the controls for each geographical group are close to their corresponding meditative group, providing the face validity that the controls and monks were well matched. In addition, the proteins of subjects within each group were closer to each other compared to subjects from other groups, indicating that plasma proteome differed between the meditative groups and their control groups (Figure 2a). Principal Component Analysis (PCA) supported the HC findings that meditation likely remodels plasma proteome, viz. the two principal components (PC1 and PC2) that accounted for the largest variations were distinctly segregated between the monks and the control groups in each geographical region (Figure 2b, c).

Differentially regulated plasma proteome across three sects (DIA-MS)

To avoid the possible conundrum of geographic isolation, DEPs were analyzed within each sect and compared to its matched control (viz. G vs C, N vs Z, and S vs Z). Since BMI has been shown associated with widespread changes in the plasma proteome,[19] we adjusted BMI as the covariate during DEPs analysis. Consequently, we quantified 83, 93, and 73 DEPs ($|FC| \geq 1.3$ and $P < 0.05$, ANCOVA) in each comparison group (Figure 2d, Supplementary Table 3). We found that Nyingma and Sakya shared the greatest number of DEPs (26 in Figure 2d), whereas Gelug and Nyingma shared the least (9 in Figure 2d). Notably, five proteins were significantly differentially regulated across all three comparison groups (Figure 2d). One protein in particular, CD5L was consistently down-regulated, regardless of meditation types (Figure 2e). Altogether, our results revealed that the plasma proteome was significantly regulated under meditation. Interestingly, such regulation was practice-type dependent.

In addition, in the Nyingma sect, some monks have experienced a three-year retreat practice (at least 8 h/day) in addition to their daily practice. Here, we compared the impact between daily plus retreat (n =6) vs daily practice (n = 6). These two groups have comparable demographics and dietary habits (Supplementary Table 4). We identified a total of 24 DEPs ($|FC| \geq 1.3$ and $P < 0.05$, ANOVA), which were mainly involved in immune, transcription, vesicle transportation, and lipid process regulation (Supplementary Table 4, Supplementary Figure 2). This exploratory result indicates that retreat experience could to some extent further alter the plasma proteomics compared to single daily practice.

Enrichment analysis of cardiovascular-related functions underlying meditation (DIA-MS)

For DEPs identified in each comparison group (G vs C, N vs Z, and S vs Z), we found they were mainly enriched in functions related to CVD, metabolism, and immune response (Supplementary Figure 3a−c), regardless of meditation types. In this study, we further analyzed DEPs related to CVD and metabolism. We found all three sects were significantly involved in atherosclerosis-related processes (including vessel blocking and lipid accumulation) and angiogenesis associated processes, which were involved in heart development, such as the development of endothelial cells and vasculature. Specifically, DEPs of meditators from the Nyingma sect were additionally enriched in oxidative metabolism. In addition, DEPs of meditators from the Sakya sect were specifically enriched in glycolysis (Figure 3). Moreover, these DEPs displayed considerable interactions with one another (Supplementary Figure 4a−d).

Next, we applied the PRM-MS technique to validate CVD and metabolism related DEPs found in the DIA-MS stage. Validation was performed on Sample Set 2, comprising the remaining samples of 75 Tibetan subjects (Figure 1b, Table 2). DEPs within each compared group were analyzed using ANCOVA, adjusted with age and BMI. To ensure accuracy, proteins deemed successfully validated were required to show consistent differential-regulation patterns with DIA samples ($|FC| \geq 1.3$ and $P_{BH\ adjusted} < 0.05$, Supplementary Table 5). The following results were validated from the PRM-MS study.

	Gelug Monks (G, n=10)		Controls (C, n=10)				Nyingma Monks (N, n=10)		Controls [a] (Z, n=10)				Sakya Monks (S, n=10)		Controls [a] (Z, n=10)			
Sex	**N**	**%**	**N**	**%**	**χ2**	**P**	**N**	**%**	**N**	**%**	**χ2**	**P**	**N**	**%**	**N**	**%**	**χ2**	**P**
Male	**10**	**100**	**10**	**100**	**0.00**	**1.00**	**10**	**100**	**10**	**100**	**0.00**	**1.00**	**10**	**100**	**10**	**100**	**0.00**	**1.00**
	Mean	SD	Mean	SD	W/T	P	Mean	SD	Mean	SD	T	P	Mean	SD	Mean	SD	T	P
Age(y)	42.70	6.63	39.30	8.31	T=-1.01	0.33	40.90	7.43	44.00	10.79	-0.75	0.46	38.20	8.77	44.00	10.79	-1.32	0.20
Meditation (y)	16.80	5.96					20.00	7.03					19.90	8.14				
BMI	34.17	4.92	28.83	4.57	T=-2.47	0.03(*)	29.79	5.26	24.42	3.93	2.42	0.03(*)	26.97	5.26	24.42	3.93	1.23	0.23
SBP (mmHg)	131.40	10.54	136.75	10.9	W=50.00	0.40	125.56	14.54	128.70	14.48	-0.47	0.64	139.30	20.25	128.70	14.48	1.35	0.19
DBP (mmHg)	83.60	8.59	84.25	8.35	T=0.16	0.87	73.11	8.81	83.50	12.53	-2.07	0.054	89.10	12.84	83.50	12.53	0.99	0.34
	N	%	N	%	χ2	P	N	%	N	%	χ2	P	N	%	N	%	χ2	P
Smoking	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00
Alcohol	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00
Dietary	N	%	N	%	χ2	P	N	%	N	%	χ2	P	N	%	N	%	χ2	P
Highland barley	9	90	10	100	1.05	0.31	8	80	8	80	0.00	1.00	9	90	8	80	0.39	0.53
Rice	8	80	7	70	0.27	0.61	8	80	8	80	0.00	1.00	7	70	8	80	0.27	0.61
Noodles	4	40	5	50	0.20	0.65	7	70	8	80	0.27	0.61	7	70	8	80	0.27	0.61
Vegetables	9	90	8	80	0.39	0.53	7	70	7	70	0.00	1.00	8	80	7	70	0.27	0.61
Tibetan tea	2	20	2	20	0.00	1.00	0	0	1	10	1.05	0.31	2	20	1	10	0.39	0.53
Yoghourt	5	50	4	40	0.20	0.65	5	50	7	70	0.83	0.36	7	70	7	70	0.00	1.00
Pork/beef	6	60	8	80	0.95	0.33	8	80	7	70	0.27	0.61	6	60	7	70	0.22	0.64

Table 1: **The demographic characteristics for the monks and controls involved in the DIA proteomics analysis.**
Life Occupation: Monks sit for a long period of time every day to chant and meditate, without physical activity.
Tibetan controls work, often in some highly active and energy demanding occupations such as shepherds.
Note:
[a]Since Nyingma and Sakya monks lived in the same geographic region, they shared the same controls (Z).
χ2: Chi-square test; W: Mann-Whitney U test; T: Welch's test or students' t-test. *P < 0.05.
BMI: Body mass index; SBP: Systolic Pressure; DBP: Diastolic Pressure.
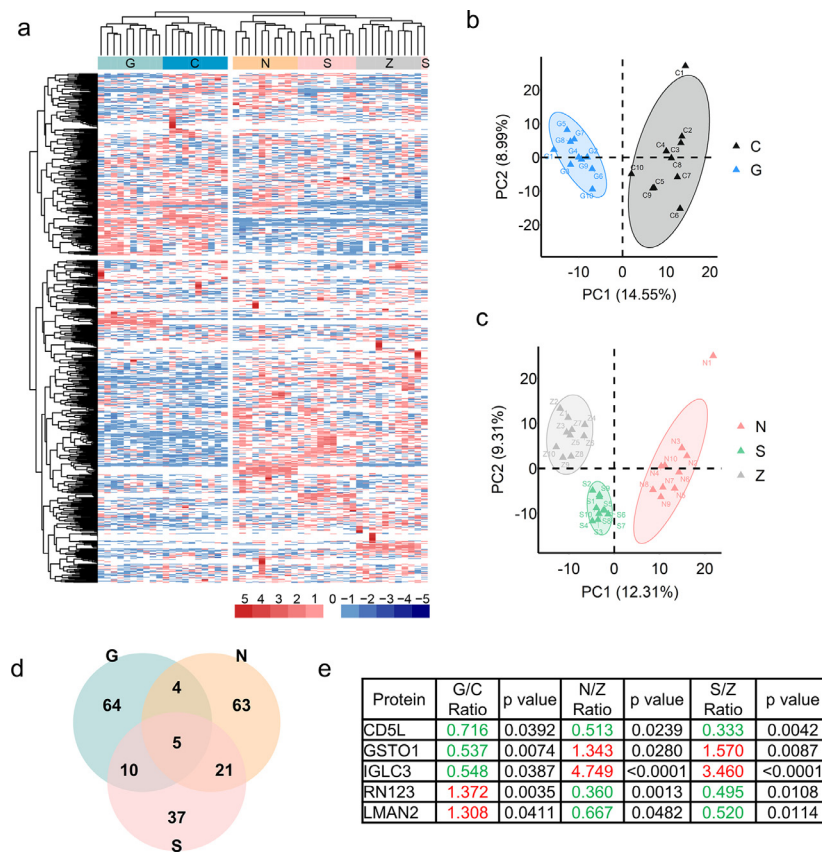
Figure 2. Plasma proteome signatures in three types of Tibetan meditations. (a) The proteins quantified across all groups were used for unsupervised hierarchical clustering analysis. Rows were scaled. "Complete" was used as the clustering method. "Correlation" was used for distance calculation. Legend represents z score of protein expression. (b, c) The quantitative proteins in each group were used for principal component analysis to group samples based on different regions. (d) The overlap of differentially expressed proteins (DEPs) of DIA proteomics among three comparison groups. Fold change ≥ |1.3|, P-value < 0.05. (e) The five overlapped DEPs among three comparison groups. Red: up-regulated. Green: down-regulated. Analysis of covariance (ANCOVA) was carried out to investigate the DEPs within each comparison group, adjusted BMI as a covariate. Multiple test correction was not performed for this stage. n = 10 for each group.

Reduced risk of atherosclerosis after meditation (PRM-MS)

Inflammation is an active driver of atherosclerotic plaque development and a risk factor for atherosclerotic related illness.[20] Consistent with the DIA finding, the PRM-MS based validation also showed a consistent down-regulation of CD5L across all three types of meditation (Figure 4a, b, d). Additionally, CD163 was validated down-regulated in Gelug monks (Figure 4a). Both CD5L and CD163 are well recognized inflammatory factors that play key roles in driving atherosclerotic development and its progression.[21,22] In Nyingma monks, up-regulation of TSP[23] was validated (Figure 4b). TSP is secreted by and adheres to inflammatory cells such as neutrophil macrophages and has been implicated in various inflammatory processes. Studies have shown that TSP1 deficiency enhanced inflammation by macrophages in animal models of atherosclerosis, thereby accelerating atherosclerosis plaque maturation.[24]

Another feature of atherosclerosis is an accumulation of fat deposits within the arterial wall. We also validated that FINC,[25] APOC2,[26] and SAP[27] were significantly down-regulated in Nyingma monks (Figure 4b). These proteins mediate lipid metabolism and were significantly increased in atherosclerotic plaques. Together with CD5L and TSP1, APOC2 and FINC participated in a protein-protein interaction network associated with atherosclerosis (Figure 4c). Taken together, these validated DEPs underscore that meditation leads to the reduction of risk factors for atherosclerosis, such as inflammation and hyperlipidemia.

Elevated glycolysis and oxygen release from hemoglobin after meditation (PRM-MS)

We also validated the DEPs involved in glycolysis found at the DIA-MS stage in Sakya monks. We found a consistent up-regulation of G3P, PGK1, PGAM1, and
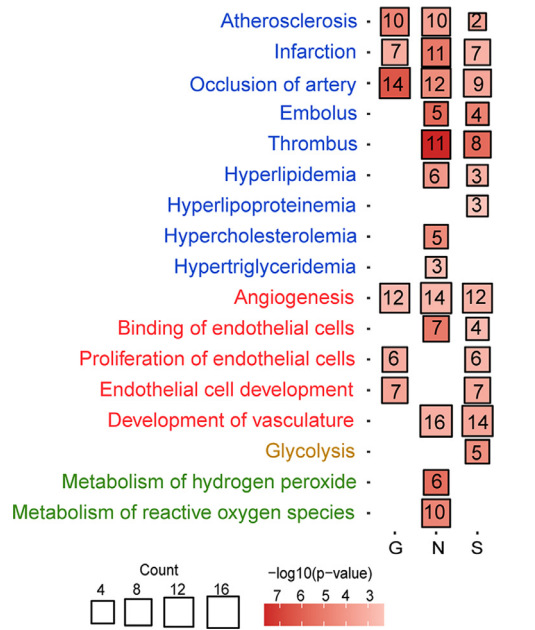
Figure 3. The details of enriched biological items associated with the cardiovascular system for each type of meditation. Square size and inside numbers represent the number of DEPs involved in this enriched function. The red colour of squares represents the -log10 (P-value) of enrichment significance. Blue: functions associated with atherosclerosis and related processes. Red: functions associated with angiogenesis and related processes. Yellow: functions associated with glycolysis. Green: functions associated with oxidative metabolism. G: Gelug vs Control; N: Nyingma vs Control; S: Sakya vs Control. n = 10 for each group.

ENOA (Figure 4d). These proteins/enzymes are involved in the latter steps of anaerobic glycolysis (Figure 4e). In addition, we validated the increase of HSP7C (Figure 4d), which induces increased activity of glycolytic enzymes and elevated glucose consumption.[28] These observations point to enhanced glycolysis ability in Sakya monks compared to controls. In addition, the level of enzyme PMGE (biphosphoglycerate mutase) was also validated up-regulated in Sakya monks (Figure 4d). PMGE regulates the level of 2,3-bisphosphoglycerate (2,3-BPG) in human blood cells by catalytic synthesis of 2,3-BPG from 1,3 bisphosphoglycerate (Figure 4e). In red blood cells, 2,3-BPG is the main allosteric effector of hemoglobin. It shifts the equilibrium between the oxy and deoxy conformations, and thus promotes oxygen release.[29] The up-regulation of PMGE suggests an elevated oxygen release in Sakya monks, thereby reducing the workload on the heart.

Association between the length of meditation and key cardiovascular-related proteins

The above joint proteomics analysis (DIA & PRM) provided us with a group of key cardiovascular-related proteins modulated by meditation. Notably, each sect has its own key regulating proteins. We next analyzed the relationship between the length of time the monks engaged in meditation and the expression of these key proteins within each sect (Supplementary Table 6). BMI was adjusted as a covariate if needed. We found for Gelug monks, there was no significant correlation between the length of practice and their key cardiovascular-related proteins (CD5L and CD163, all P > 0.05, Spearman correlation analysis or Semi-spearmen partial correlation analysis) (Supplementary Figure 5a, b). In Nyingma monks, only the expression of TSP1 had a positive correlation with the length of practice (R = 0.48, P = 0.045, Semi-spearman partial correlation analysis) (Supplementary Figure 5c−g). For Sakya monks, only CD5L showed a negative correlation with the length of practice (R = -0.51, P = 0.030) (Supplementary Figure 5h−n). In interpreting these results, we note that our study was cross sectional, not longitudinal, and it is therefore unlikely that we would be able to observe the effect of years of meditation in monks who at the time of sampling have already engaged in significant periods of meditation.

Clinical profiles

The proteomics data suggest that long-term Tibetan meditation supports a healthy cardiovascular system through reduced risk of atherosclerosis, improved glycolysis, and enhanced oxygen release from hemoglobin. We next asked whether there were other biological consequences besides these proteomic results. To address this question, we examined an additional set of cardiovascular risk factors including age, BMI, blood pressure, lipid metabolism, and HRV in each comparison group (G vs C; N vs Z; S vs Z). In this part, we included all available subjects for Gelug, Nyingma, Sakya sects and their matched controls. We found no significant difference in age for each comparison group (all P > 0.05, Student's t-test or Welch's t-test, Figure 5a, Supplementary Table 7). However, the monks were obese, whose BMI were significantly higher than that of matched controls (30.66 ± 5.26 vs 27.27 ± 4.92, Welch's t-test, P < 0.001, Figure 5b, Supplementary Table 7).

Significantly, in addition to a healthy heart proteome, the monks also showed clinical indicators indicative of reduced vulnerability to CVD. Apo B and LDL-C were lower in both Nyingma and Sakya monks compared to their matched Tibetan controls (all $P_{BH\ adjusted}$ < 0.05, ANCOVA, adjusted BMI; Figure 5d, e, Supplementary Table 7). In addition, plasma total cholesterol and lipoprotein (a) were specifically lower in Nyingma monks (all $P_{BH\ adjusted}$ < 0.05, ANCOVA, adjusted BMI, Figure 5c, f, Supplementary Table 7). The level of high-density lipoprotein-cholesterol (HDL-C) was not changed in Gelug and Sakya monks ($P_{BH\ adjusted}$ > 0.05) or a bit lower in Nyingma monks (N vs Z: 1.13 ±

	Gelug Monks (G, n=29)		Controls (C, n=17)				Nyingma Monks (N, n=9)		Controls[a] (Z, n=10)				Sakya Monks (S, n=10)		Controls[a] (Z, n=10)			
Sex	N	%	N	%	χ2	P	N	%	N	%	χ2	P	N	%	N	%	χ2	P
Male	29	100	17	100	0.00	1.00	9	100	10	100	0.00	1.00	10	100	10	100	0.00	1.00
	Mean	SD	Mean	SD	T	P	Mean	SD	Mean	SD	T	P	Mean	SD	Mean	SD	T	P
Age(y)	39.59	9.41	49.06	15.24	2.32	0.03	39.67	6.48	40.20	8.20	-0.16	0.88	40.70	7.48	40.20	8.20	0.14	0.89
Meditation (y)	18.28	9.18					19.33	5.48					22.80	7.45				
BMI	31.89	5.28	29.07	5.46	-1.70	0.10	28.87	3.76	25.86	4.07	1.67	0.11	29.52	4.71	25.86	4.07	1.87	0.079
SBP (mmHg)	141.41	17.98	139.06	30.83	-0.33	0.74	129.00	10.42	129.40	14.16	-0.07	0.95	134.60	11.90	129.40	14.16	0.89	0.39
DBP (mmHg)	86.03	9.81	84.59	16.02	-0.38	0.71	81.78	9.05	83.50	10.35	-0.38	0.71	84.80	11.90	83.50	10.35	0.27	0.79
	N	%	N	%	χ2	P	N	%	N	%	χ2	P	N	%	N	%	χ2	P
Smoking	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00
Alcohol	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00	0	0	0	0	0.00	1.00
Dietary	N	%	N	%	χ2	P	N	%	N	%	χ2	P	N	%	N	%	χ2	P
Highland barley	29	100	17	100	0.00	1.00	9	100	9	90	0.95	0.33	9	90	9	90	0.00	1.00
Rice	21	72.41	13	76.47	0.091	0.76	6	66.67	7	70	0.02	0.88	7	70	7	70	0.00	1.00
Noodles	20	68.97	12	70.59	0.01	0.91	5	55.56	4	40	0.46	0.50	7	70	4	40	1.82	0.18
Vegetables	24	82.76	13	76.47	0.27	0.60	8	88.89	6	60	2.04	0.15	8	80	6	60	0.95	0.33
Tibetan tea	2	6.90	3	17.65	1.28	0.26	0	0	0	0	0.00	1.00	1	10	0	0	1.05	0.31
Yoghurt	19	65.52	12	70.59	0.12	0.72	5	55.56	5	50	0.06	0.81	6	60	5	50	0.20	0.65
Pork/beef	19	65.52	13	76.47	0.61	0.44	7	77.78	8	80	0.01	0.91	9	90	8	80	0.39	0.53

Table 2: **The demographic characteristics for the monks and controls involved in PRM proteomics analysis.**

Life Occupation: Monks sit for a long period of time every day to chant and meditate, without physical activity.
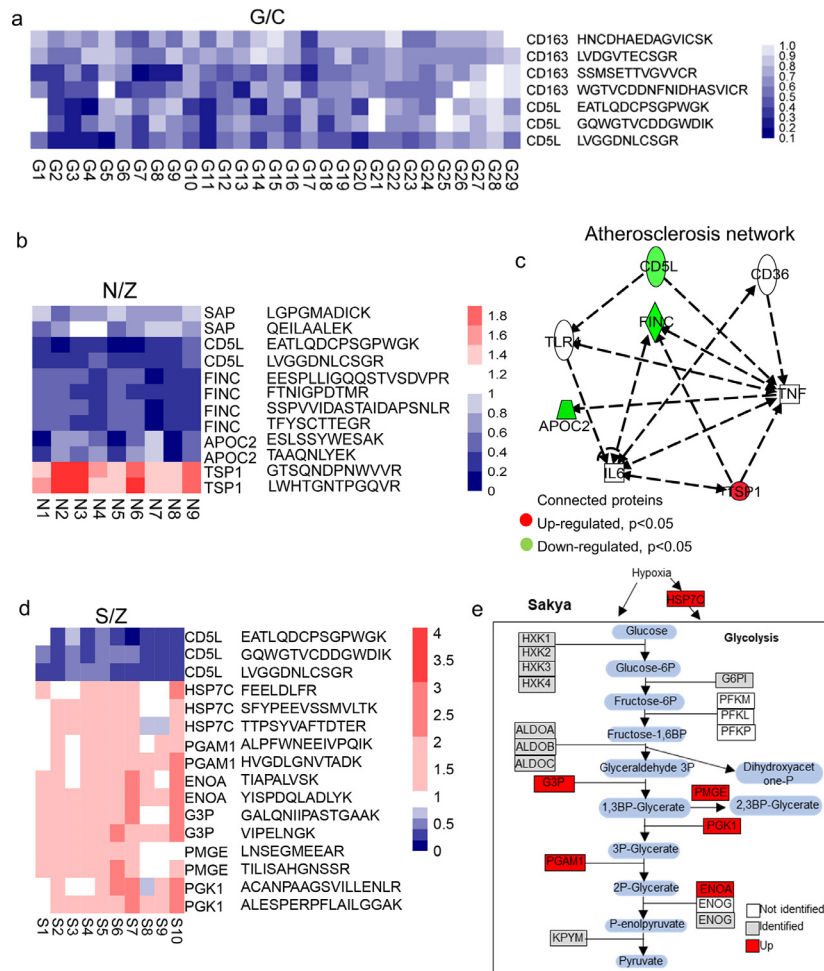
Tibetan controls work, often in some highly active and energy demanding occupations such as shepherds.

Note:

[a]Since Nyingma and Sakya monks lived in the same geographic region, they shared the same controls (Z).

χ2: Chi-square test; T: Welch's test or students' t-test. * P < 0.05.

BMI: Body mass index; SBP: Systolic Pressure; DBP: Diastolic Pressure.

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Figure 4. PRM validation on the effect of meditation on cardiovascular protection. (a, b) Heatmap of validated DEPs indexing atherosclerosis in Gelug and Nyingma sects. Each square represents the expression ratio of each targeted peptide of this protein for each individual (monks vs controls). Red: up-regulated. Blue: down-regulated. Fold changes≥|1.3|, $P_{BH\ adjusted} < 0.05$. Legend represents expression ratio. (c) DEPs in the atherosclerosis network in the Nyingma sect. Coloured: DEPs. (d) Heatmap of validated DEPs involved in atherosclerosis, glycolysis and O_2 releasing in Sakya sects. Each square represents the expression ratio of each targeted peptide of this protein for each individual (monks vs controls). Red: up-regulated. Blue: down-regulated. Fold changes≥|1.3|, $P_{BH\ adjusted} < 0.05$. (e) Pathways of glycolysis and O_2 release (hemoglobin). Proteins marked in grey were those identified and / or validated in DIA and PRM analysis with no significant expression. Proteins in red means up-regulated, and green is down-regulated in both DIA identification and PRM validation. Those not identified in either DIA or PRM were marked in white. Analysis of covariance (ANCOVA) was carried out to investigate the targeted DEPs within each comparison group, adjusted age (if needed) and BMI as covariates. 5% FDR was applied for multiple test correction. n = 29 for G; n = 9 for N; n = 10 for S; n = 17 for C; n = 10 for Z.

0.19 vs 1.32 ± 0.18, $P_{BH\ adjusted} = 0.046$, ANCOVA, adjusted BMI, Supplementary Table 7). Despite being overweight or even obese, which is frequently comorbid with hypertension[30] and reduced HRV,[31] all three sects of monks have both blood pressure (all $P_{BH\ adjusted} > 0.05$, Student's t-test or Mann-Whitney U test, Supplementary Table 7) and HRV measures (all $P_{BH\ adjusted} > 0.05$, Mann-Whitney U test, Supplementary Figure 6) comparable to their matched Tibetan controls. In addition, we determined the relationship between the length of meditation and these clinical indicators. We found there was no significant correlation for either monks

across all sects or within each sect (Supplementary Table 8, Supplementary Figure 7).

Altogether, our findings indicate that meditation is protective for the cardiovascular system, and strikingly even in clinically overweight and obese Tibetan monks.

Association between the cardiovascular-related proteome and clinical indicators

To investigate the relationship between the cardiovascular-related proteome and clinical indicators, we performed a linear mixed-effects analysis with BMI as a
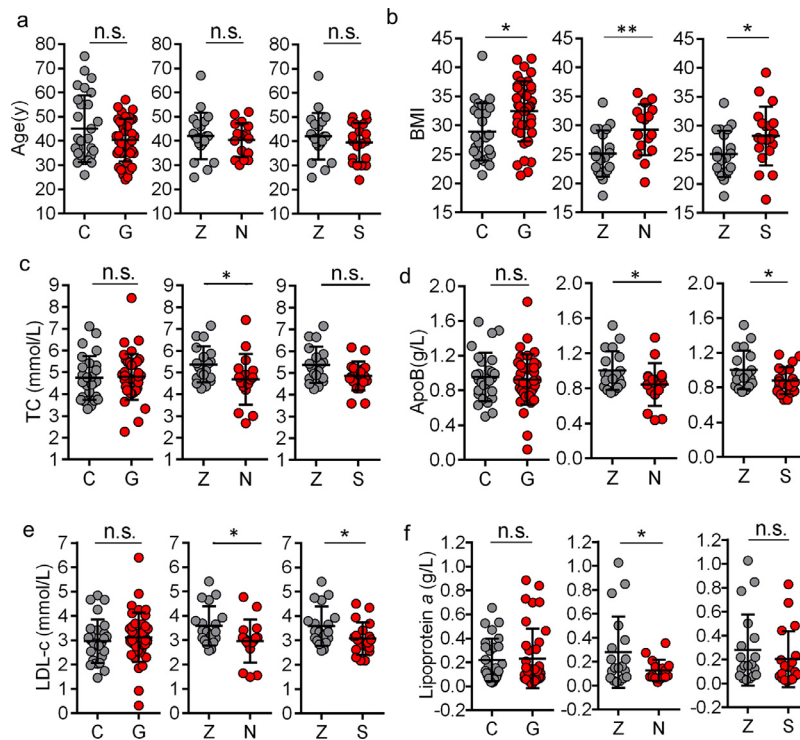
Figure 5. Clinical measurement of cardiovascular risks in Tibetan monks and matched local controls. (a) The age distribution of monks and controls. G vs C: T = 1.68, P = 0.10. G: M = 40.38, SD = 8.81. C: M = 45.44, SD = 13.78. N vs Z: T = 0.67, P = 0.51. N: M = 40.32, SD = 6.83. Z: M = 42.10, SD = 9.53. S vs Z: T = 0.95, P = 0.35. S: M = 39.45, SD = 8.04. Z: 42.10, SD = 9.53. T: Students' t-test or Welch's test. (b) The BMI distribution of monks and controls. G vs C: T = -2.65, P = 0.01. G: M = 32.47, SD = 5.22. C: M = 28.98, SD = 5.02. N vs Z: W = 76, P = 0.0078. N: M = 29.27, SD = 4.34. Z: M = 25.14, SD = 3.96. S vs Z: W=119.5, P = 0.030. S: M = 28.25, SD = 5.03. Z: M = 25.14, SD = 3.96. W: Mann-Whitney U test; T: Students' t-test or Welch's test. (c) Plasma total cholesterol in monks and controls. G vs C: F = 0.79, $P_{BH\ adjust}$ = 0.78. G: M = 4.79, SD = 1.05. C: M = 4.69, SD = 0.99. N vs Z: F = 9.12, $P_{BH\ adjust}$ = 0.024. N: M = 4.66, SD = 1.21. Z: M = 5.37, SD = 0.83. S vs Z: F = 4.15, $P_{BH\ adjust}$ = 0.09. S: M = 4.97, SD = 0.86. Z: 5.37, SD = 0.83. (d) Clinical measures of Apo B. G vs C: F = 2.82, $P_{BH\ adjust=}$ 0.69. G: M = 0.93, SD = 0.29. C: M = 0.94, SD = 0.27. N vs Z: F = 10.19, $P_{BH\ adjust}$ = 0.024. N: M = 0.84, SD = 0.25. Z: M = 1.00, SD = 0.22. S vs Z: F = 5.59, $P_{BH\ adjust}$ = 0.049. S: M = 0.89, SD = 0.18. Z: M = 1.00, SD = 0.22. (e) Clinical measures of LDL-C. G vs C: F = 0.08, $P_{BH\ adjust}$ = 0.78. G: M = 3.12, SD = 1.02. C: M = 2.89, SD = 0.86. N vs Z: F = 9.12, $P_{BH\ adjust}$ = 0.024. N: M = 2.95, SD = 0.92. Z: M = 3.59, SD = 0.81. S vs Z: F = 6.20, $P_{BH\ adjust}$ = 0.049. S: M = 3.08, SD = 0.66. Z: M = 3.59, SD = 0.81. (f) Clinical measures of lipoprotein (a). G vs C: F = 0.15, $P_{BH\ adjust}$ = 0.78. G: M = 0.23, SD = 0.25. C: M = 0.22, SD = 0.18. N vs Z: F = 4.41, $P_{BH\ adjust}$ = 0.045. N: M = 0.11, SD = 0.065. Z: M = 0.27, SD = 0.30. S vs Z: F = 1.31, $P_{BH\ adjust}$ = 0.30. S: M = 0.19, SD = 0.23. Z: 0.27, SD = 0.30. (c-f) Analysis of Covariance (ANCOVA). Multiple test corrections have been applied for P-values using Benjamini-Hochberg method. BMI was adjusted as a covariant. Data were represented as mean ± SD. **P < 0.01, *P < 0.05. n = 39 for G; n = 19 for N; n = 20 for S; n = 27 for C; n = 20 for Z.

covariate (Supplementary Table 9). Since each type of meditation has its own cardiovascular-related proteome, we analyzed them separately. We found for Nyingma monks and their matched controls, that there was a significant relationship between key cardiovascular-related proteins (SAP, FINC, and CD5L) and clinical measures (SBP, DBP, total cholesterol, HDL-C, LDL-C, Apo B, and lipoprotein (a)). There were no significant correlations for other monks and their matched controls (Supplementary Table 9).

Discussions

The overarching goal of the current investigation is to examine the link between meditation and protection against CVD. This paper has implemented an extreme phenotype strategy coupled with a parallel intervention study to investigate the role of meditation in CVD. To evaluate cardiovascular health, we used a two-stage hypothesis-free interrogation of the proteomics (DIA (discovery) and PRM (validation)), and searched for protein biomarkers and their interactions underpinning cardiovascular health. Despite being generally obese, Buddhist Tibetan monks were characterized by a cardiovascular protective profile of plasma biomarkers including total cholesterol, LDL-C, Apo B, and lipoprotein (a), compared to non-meditative Tibetan controls. Additionally, monks had blood pressure and HRV measurements also comparable to the Tibetan controls. The obesity of monks is likely attributable to their relatively sedentary lifestyle.

They sit for a long period of time every day to chant and meditate, without physical activity.

Notably, since life style and level of stress may be confounding factors when investigating the effects of meditation, and to minimize these effect, our study strictly matched age, sex, smoking, alcohol, and dietary habit between monks and Tibetan controls. For monks, one main activity in life is meditation practising. In contrast, Tibetan controls work, often in some highly active and energy demanding occupations such as shepherds, albeit not psychologically stressful. Although they have different activities in daily life, both monks and Tibetan controls are generally characterized by low levels of mental stress. Hence, the changes we observed in the proteome are very likely to be specifically associated to the practice of meditation and not to generically stress-less life style.

It has been widely observed that a stressful life style is associated with higher risk of cardiovascular disease.[32,33] Our study revealed the beneficial effect of long-term meditation on cardiovascular protection even in obese and overall physically inactive individuals. Consistent with our findings, several studies have suggested an improvement of cardiovascular risks (e.g., stress, hypertension, smoking, and obesity), following a daily or twice a day 13−30 min meditation.[34−38] Although these results require further validation, and especially by implementing more robust methodologies, they nevertheless provide us with clues that daily meditation even for a short time, could nevertheless be beneficial for individuals particularly those engaging in stressful occupations (e.g. CEO's, military personnel and fire-fighters). Future studieswould gainfully carry out a similar investigation of the cardiovascular-related proteome as currently undertaken, towards examining the potential effect of short-term meditation on such individuals with stressful life style.

Although some prior work has demonstrated reduced blood pressure in randomized controlled meditation trials, our study did not observe such difference between monks and their controls. It is possible that our subjects come from high altitudes, and they have developed compensatory higher blood pressure to cope with the high altitude,[39,40] suggesting there might be no further capacity for meditation to significantly reduce their blood pressure. Similar to our findings, no significant blood pressure changes were observed in Sri Lanka long-term meditative Buddhist monks compared with non-meditative monks.[41] In addition, although it is considered that transcendental meditation may modulate blood pressure, other forms of meditation do not show consistent changes.[2] Since Tibetan meditation in this study is a combination of various complex forms of training undergone in a unique geographical setting, it is ipso facto difficult to predict how meditation will, if at all, modulate blood pressure.

We have chosen well-established lipid markers for our study of CVD risk factors and meditation. The decrease in lipid profile seen in this study is in agreement with the earlier studies.[42,43] Repeated stress is known to lead to persistent elevation of cortisol, which promotes mobilization of fatty acids from adipose tissue and is linked to dyslipidemia.[44] Meditation could stabilize the hypothalamic−pituitary−adrenal axis and thus reduce cortisol secretion induced by stress.[45] The decreased cortisol may be one mechanism by which mediation improves the lipid profile observed in our study. In addition, meditation has been reported to increase the plasma level of melatonin.[46,47] Melatonin has been shown to improve lipid metabolism and gut microbiota communities in animals and humans.[48,49] Also, the improvement in the lipid profile after meditation may be due to increased hepatic lipase and lipoprotein lipase at the cellular level, which affects the metabolism of lipoprotein.[50]

The advantageous cardiovascular proteome in these monks is likely mediated by the reduction of the inflammatory burden and/or reduced hyperlipidemia. It is noteworthy that CD5L, a key immune protein and important in inflammatory disease,[22] is down-regulated across all Tibetan monks. Macrophages are the main source of CD5L, and its expression is up-regulated under inflammatory conditions of infectious origins, as well as in the course of cardiovascular and metabolic pathologies, such as in atherosclerotic lesions[51] and in the adipose tissue of obese mice.[52] Hence, the down-regulation of CD5L observed in the Buddhist monk meditators in this study likely conferred marked protection against CVD.

High altitude living and hypoxia are risk factors for cardiovascular dysfunction.[53] High altitude dwellers have been reported to show right ventricular hypertrophy, caused by cardiac pressure overload and often associated with heart failure.[54,55] Results from mouse models have demonstrated that elevated glycolysis is an adaptive response to cardiac pressure overload.[56,57] In addition, hypoxia is challenging for people in terms of both energy depletion and oxidative stress.[53] By promoting glycolysis to generate ATP under aerobic conditions, vascular endothelial cells can save oxygen for the adjacent cardiomyocytes and protect themselves from mitochondrial electron leakage and the generation of reactive oxygen species.[58] Indeed, enhanced glycolysis in human red blood cells has been observed at high altitudes.[59−61] Studies have also reported genetic variants in *EPAS1*, associated with increased glycolysis, in Tibetans.[62,63] Altogether, the current findings of over-expression of proteins involved in glycolysis in meditator Tibetan monks suggest better physiological adaptation to living at > 3000.

Several investigations have identified an altered gene expression profile and DNA methylation indicating a

reduced inflammatory activity and enhanced innate immune responses following meditation.[64–67] Additionally, significant changes in gene expressions are known to be involved in cellular metabolism and oxidative stress response.[68] Notably, mRNA levels are not always directly correlated with protein expression[69] due to substantial post-transcriptional regulation. Consequently, such measurements may not adequately reflect the true biological process compared to the direct measure of proteins. In this study, the mass spectrometry-based proteomic analysis of plasma is our applied methodology. It well performs in small sample sizes and a limited number of targeted proteins. For large scale populations, multiplexed affinity-based proteomics approaches using antibodies or specifically designed aptamers[70,71] or recently emerging protein sequencing methods,[72] have emerged as an attractive alternative to mass spectrometry by allowing quantification of proteins at a higher throughput and sensitivity. Recently, several studies have used the affinity-based plasma proteomics to investigate cardiovascular-related biomarkers.[73,74] In addition, larger scale genome-wide association studies coupled with affinity-based plasma proteomics have recently gained increased interest in human disease study, including the cardiovascular system.[73–77] This advanced method is powerful to explore the genetic architecture of the plasma proteome, reveal disease cause, and point to drug targets.

There are several limitations of the current study. Although our dataset is the largest to date, nevertheless the difficulty to collect data from this special population precluded the collection of a larger sample size in this exploratory study. Hence, we did not perform the FDR correction during DIA proteomics analysis to minimize the loss of any potentially informative data. However, our findings are reliable to the extent that the key findings in the DIA stage have been verified in the PRM stage in another cohort. We had three measures of cardiac health, viz. plasma proteome, heart rate variability using electrocardiogram and blood pressure. Additional measures in future studies would include echocardiogram, MRI and stress tests. However, the geographical isolation of the monasteries, the relative difficulty in travel arrangements, and non-interference to the monks' intensive schedule of meditation constrained us in this first study to carry out all such measures. In addition, in this cross-sectional study, we perforce cannot understand the dynamic effects of meditation on the heart-brain axis over time. Lastly, since our study was carried out in Tibetan populations, we cannot generalize our results to other populations. We await studies on other groups of long-term meditators to confirm the findings from the current investigation.

Despite the only partially overlapping proteome profiles of the three monk sects, the differentially expressed proteome biomarker patterns in three sects converge to a recognizable signature of cardiovascular protection. This paper suggests that the myriad of parallel pathways of the complex cardiovascular system can accommodate diverse heart-brain interactions to module the proteome signatures while benefiting heart health. The current findings strengthen the scientific basis for this significant path to stress reduction and cardiovascular health as well as underscore how important the heart-brain axis is in contributing to an extended and healthy life history trajectory.

Contributors

DHC: supervision and conceptualization; RPE: conceptualization, writing, review and editing data analysis, statistical methodology and analysis tools; CSH: review and editing, formal analysis, visualization, formulation, organization; TX: sample collection, mass spectrometry sample preparation, data analysis and manuscript writing; BC: review and editing, formal analysis, analysis of data and statistical methodology; TJX, HL, YS, KS and YC: Tibetan sample collection. XLG, SBT, and MLG: EEG data collection and analysis. RPE, TX, and BC verified the underlying data. All authors read and approved the final version of the manuscript.

Data sharing statement

We declare that all data supporting the findings of this study are available within the paper and its supplementary information files or upon request. All mass spectrum raw data and search results have been deposited to iProx and can be accessed with the iProx accession: IPX0001342000.

Declaration of interests

The authors declare no competing interests.

Acknowledgements

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