NFκB2 gene as a novel candidate that epigenetically responds to interval walking training

Yugen Zhang, Shigenari Hashimoto, Chifumi Fujii, Shigeaki Hida, Kensuke Ito, Tomio Matsumura, Takao Sakaizawa, Mayuko Morikawa, Shizue Masuki, Hiroshi Nose, Keiichi Higuchi, Kouki Nakajima, and Shun`ichiro Taniguchi

Abstract

Physical fitness has been reported to decrease the risk of lifestyle-related diseases. The present study evaluated genome-wide methylation under the hypothesis that interval walking training (IWT) imparted beneficial effects on health, particularly by epigenetically ameliorating susceptibility to inflammation. We screened DNA from peripheral blood samples via genome-wide microarray for genes whose methylation was affected by IWT, paying special attention to promoter regions, and identified over 40 hyper- or hypo-methylated genes following IWT that were not witnessed in controls. We next selected genes in which the degree of methylation change in the promoter region was correlated with energy consumption following IWT. In this way, we found the *NFκB2* gene to have increased methylation in multiple regions of its promoter sequence following participation in an exercise regimen. Next, IWT-induced *NFκB2* hyper-methylation was confirmed by a quantitative PyroSequencing assessment of methylation in samples obtained from independent subjects who also underwent IWT. The increase in *NFκB2* gene promoter methylation by IWT indicates that this regimen may suppress pro-inflammatory cytokines. Thus, these results provide an additional line

of evidence that IWT is advantageous in promoting health from an epigenetic perspective by ameliorating susceptibility to inflammation.

Keywords: interval walking training (IWT), methylation, genome-wide methylation assay, *NFκB*, inflammation, aging

Introduction

Aging brings about numerous changes to the human body, one of which is the increased occurrence of inflammation [6, 10]. Whereas transient inflammation is an inevitable pathophysiological response to pathogenic infection [6], chronic inflammation causes tissue damage and organ dysfunction and underlies other deleterious effects on health. [4, 9, 26, 27]. Aging-related gene regulation in humans may be dependent on modifications that are important for various normal development processes. Genetic characteristics, including single nucleotide polymorphisms [15, 18] and epigenetic modifications such as DNA methylation [19], may alter the duration and degree of chronic inflammation.

To improve the health status of Japan's aging population, Nose et al. developed an exercise program termed "high-intensity interval walking training," or IWT, whereby individuals repeat cycles of 3 minutes of low-intensity walking followed by 3 minutes of high-intensity walking [21]. Conventional physical and biochemical markers, such as

blood pressure and cholesterol level, have been macroscopically examined to evaluate the effects of IWT [17]. However, it is also important to investigate epigenetic alterations at the gene level that are caused by IWT.

There are few reports on epigenetic blood testing that examine the correlation between epigenetic events and improvements of general health via intervention with exercise such as IWT [17], or nutritional supplements. We reported previously that the methylation of the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), which acts as an essential adaptor protein in the production of IL-1β and IL-18, decreased significantly with age but was recovered by IWT training [19]. These results suggest that IWT can induce the modulation of epigenetic signals and gene expression.

In this study, we examined the epigenetic changes associated with IWT using genome-wide DNA methylation and PyroSequencing assays. The purpose of this study was to elucidate the link between DNA methylation and IWT and to uncover genes that responded significantly to IWT exercise based on the inhibitory effect of methylation on inflammation. According to our previous results and in line with the notion that IWT may help epigenetically suppress a pro-inflammatory status, we discuss the significance of methylation changes in several genes, with a particular emphasis on the

hyper-methylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (*NFkB2*), which functions prominently in the induction of inflammation in age-related diseases [11, 24].

Materials and Methods

IWT exercise program and measuring instruments

The IWT regimen consisted of performing several sets of a 3-min low-intensity walking period at 40% of the peak aerobic capacity followed by a 3-min high-intensity walking period at > 70% peak aerobic capacity for as many days as possible over 6 months. Exercise intensity and time were recorded using the Jukudai Mate, which was developed in-house. This device was equipped with two orthogonally attached dual-axis accelerometers that had a detection range of ±10 G, 4 MB flash memory capable of storing minute-by-minute data for 24 h × 60 d, a rechargeable lithium ion battery capable of working for 24 h when fully charged and a USB interface to transfer stored data to an external computer. The Jukudai Mate is a quantity-of-motion measuring instrument that was designed to support the IWT program [28]. The precise IWT conditions were determined for each subject based on individual physical strength, as determined by the data provided by the Jukudai Mate. The intensity and number of steps were monitored using the accelerometer, which was carried on the left or right

midclavicular line of the subjects' waist. During training, a beeping signal alerted subjects when a change of intensity was scheduled, and another beeping signal let them know every minute whether they had accomplished high-intensity walking for the previous minute. Consumed calories and walking intensity were measured and recorded by the Jukudai Mate at Kissei Comtec Co., Ltd., Nagano.

Training information and energy consumption data are shown in Table 1 for the 13 subjects who underwent the genome-wide microarray experiment.

Blood collection and DNA extraction

Peripheral blood sample collection was performed twice on all subjects before the IWT period and 6 months later, at the end of the study. A MagNa Pure LC DNA isolation kit large volume (Roche, Switzerland) was used to extract genomic DNA from the samples according to the manufacturer's protocol. Briefly, 1 ml of blood was loaded into wells, followed by automated MagNa Pure LC DNA extraction. Extracted DNA was eluted in 100 μl of elution buffer and stored at 4°C.

Subjects for genome-wide microarray experiment

Among the subjects who participated in the Health Promotion Program for Elderly People in Matsumoto (Jukunen Taiiku Daigaku), 7 subjects who followed the IWT regimen for 40 days or more for a 6-month period were randomly chosen for the

exercise group. Of the participants who performed IWT for fewer than 40 days during the same program, 6 were selected for the control group. The characteristics of the subjects enrolled in the genome-wide microarray experiment are presented in Table 2.

This study was approved by the Committee on the Use of Human Research Subjects of Shinshu University School of Medicine and performed in accordance with the ethical standards of the International Journal of Sports Medicine (IJSM) [8]. Informed consent was obtained from each subject after explaining the purpose and significance of the test and the protocols for protecting their personal data.

Genome-wide methylation microarray assay

Genomic DNA samples of 500 ng were sonicated to an average fragment length of 100-600 bp. The DNA was incubated with the MBD2bt protein, which binds to methylated DNA, and collected using nickel-coated magnetic beads. The enriched methylated DNA was then washed 5 times with 1 ml of wash buffer.

A genome-wide methylation microarray assay was performed with Agilent Human CpG Island Array 244K slides containing 237,000 individual oligonucleotide probes (Agilent Technology, Santa Clara, California, USA) according to the manufacturer's instructions. The DNA samples obtained from pre- and post-training subjects were labeled with Cy3 or Cy5 to obtain ratio values for methylation between pre- and post-IWT, respectively,

using Bioprime random primer labeling kits (Invitrogen, USA). An Agilent ChIP-on-chip/aCGH Hybridization kit was adopted for hybridization experiments. Both labeling and hybridization were performed according to the recommended protocols. Arrays were hybridized at 67°C for 40 hours using an Agilent hybridization oven. Hybridization images were analyzed with an Agilent DNA microarray scanner, and data quantification was performed using Agilent Feature Extraction software (Agilent).

Subjects for methylation assay by PyroSequencing analysis

We further assessed DNA methylation to confirm the results obtained by the genome-wide microarray experiment - notably, those for *NFkB2* - using PyroSequencing analysis of 20 separate subjects who had followed the IWT regimen similar to the experimental subjects described above. In this additional cohort, all subjects performed IWT for 40 days or more a period of over 6 months. DNA methylation changes were evaluated by comparing pre- and post-IWT blood samples for each subject.

The characteristics of the subjects are presented in Supplemental Table 1, and the training information and energy consumption data for the 20 subjects who underwent the PyroSequencing assay are shown in Supplemental Table 2.

This study was also approved by the Committee on the Use of Human Research Subjects of Shinshu University School of Medicine and performed in accordance with the ethical standards of the IJSM [8].

DNA methylation analysis by PyroSequencing

PyroSequencing (PyroMark Q24ID, Qiagen, Hilden, Germany) was conducted as an independent method of genome-wide DNA methylation analysis. PCR and sequencing primers were designed using the PyroMark Assay Design 2.0 software (Qiagen), and all procedures were performed according to the recommended protocols. The promoter regions of *NFκB2* (-601 to -348 and -1281 to -1017 upstream of the transcription start site) were amplified by PCR. The primers used are shown in Table 3. Specifically, target regions were 5’-GAGCGTGCAGAGATCTCCCTGTCGCCTGCGCGCCCAGAACCGGTGCG-3’ and 5’-AAAGGGCGCGAGGCGTGACGCACGGAAACGTCA-3’ at -565 to -518 and -1263 to -1203, respectively. Briefly, samples of 200 ng genomic DNA taken from the peripheral blood of 20 individuals before and after the exercise intervention period were used for bisulfite conversion with Qiagen’s EpiTect kit. Bisulfite-converted DNA was amplified by PCR with a reverse primer biotinylated at its 5’ end using a PyroMark PCR Master Mix kit (Qiagen). Biotinylated PCR products were immobilized onto

streptavidin-coated beads (GE Healthcare, Uppsala, Sweden) and the DNA strands were separated using denaturation buffer. After washing and neutralization at a PyroMark Q24 Vacuum Workstation, the sequencing primer was annealed to the immobilized strand. Methylation was analyzed by highly quantitative bisulfite PyroSequencing with the PyroMark Q24 System (Qiagen). Data were analyzed using the PyroMark Q24 software (Qiagen).

Statistical analysis

Student's *t*-test was used to analyze the baseline characteristics of the control and exercise groups before IWT (Table 2), and one-way ANOVA followed by the Tukey post-hoc test was used to examine the methylation ratio values between the pre- and post-IWT samples in the genome-wide methylation microarray assay. The correlations of clinical data with methylation of the *NFκB2* gene were estimated using the Spearman non-parametric method and then analyzed by linear regression. The PyroSequencing data were analyzed using the Wilcoxon test. The association of these data with methylation was evaluated using the SPSS version 11.0J software (SPSS Japan Inc., Japan). A *p*-value less than 0.05 was considered to be statistically significant. Each data point was expressed as the mean ± standard deviation (SD).

Results

Genome-wide methylation assay and gene ontology classification

We performed gene ontology analysis using the Gene Spring analysis software (Agilent Technologies) a major bioinformatics tool that is used to understand the alteration of gene expression and modification, with three different ontologies: biological process, which was used to describe our ontology analysis (Fig. 1A and B); cellular component; and molecular function. We next screened for genes whose ratio value of methylation (post-IWT methylation level/pre-IWT methylation level) differed between the exercise and control groups using one-way ANOVA testing, with special reference to promoter regions, and identified over 40 hyper- or hypo-methylated genes in the exercise group after IWT that were not observed in controls (data not shown). Among the hypermethylated genes, there were 26 genes related to cell growth and maintenance and 15 development-related genes (Fig. 1A). Regarding hypo-methylated genes, the number of cell growth and maintenance-related genes, development-related genes, and genes involved in cell communication was 44, 29, and 11, respectively (Fig. 1B).

Screening of genes with promoter methylation changes correlated with energy consumption in the genome-wide microarray assay

We next examined the correlation between total energy consumption and the methylation status (ratio value calculated as described above) of each gene's promoter region (presumably associated with gene expression) in all of the hyper- and hypo-methylated genes indicated in Figures 1A and B. The genes for which methylation changes showed significantly high correlations with energy consumption are listed in Tables 4 (hyper-methylated genes) and 5 (hypo-methylated genes). As a representative case, the raw ratio values of methylation post-/pre-IWT at the *NFκB2* promoter are shown in Figure 2A, with a comparison between the exercise and control groups (probe locations on the genome-wide microarray are shown in Fig. 2B). Methylation of the *NFκB2* promoter tended to increase following IWT in the regions corresponding to probes 1, 7, 9, 10, 11, 12, 13, 14, and 15. We calculated the correlation between methylation level and energy consumption at these sites. A significant correlation was recognized at three sites corresponding to probes 9 (rho=0.709, p=0.007), 10 (rho=0.747, p=0.003), and 12 (rho=0.555, p=0.049) (Fig. 2C, D, and E).

Quantitative *NFκB2* promoter methylation analysis by PyroSequencing

To confirm the observed hyper-methylation of the *NFκB2* gene following IWT indicated by the microarray assay, we investigated the methylation of the *NFκB2* promoter region using an independent assay and different IWT subjects. We examined the methylation of

CpG sites in the *NFκB2* promoter region by PyroSequencing, which is suitable for quantitative measurement. The region of probe 12 contains binding sites for several transcription factors, including THAP1, NRF1, NHLH1, RUNX2, NFIC, and GATA3, according to the JASPAR database. Because probe 12 demonstrated a methylation increase that was significantly correlated with energy consumption, we chose this region for further examination using the PyroSequencing assay. The region was located -565 to -518 bp upstream of the transcription start site: 5'-GAGCG[1]TGCAGAGATCTCCCTGTCG[2]CCTGCG[3]CG[4]CCCAGAACCG[5]GTGCG[6] -3'. We confirmed a significant increase in the methylation of the post-IWT DNA samples compared with the pre-IWT samples at CpG sites 1, 5, and 6 ($p<0.05$). A tendency toward methylation increase was observed at CpG site 2, whereas there was no remarkable change at CpG sites 3 or 4 (Fig. 3A). We also directed our attention to the probe 5 region because several important transcription factor binding sites, including sites for STAT1, STAT3, NFκB1, REL, RELA, and SP1, have been listed in the JASPAR database. Alterations in methylation were not detected in this region by the genome-wide microarray assay (Fig. 3B). Methylation of the probe 5 region -1236 to -1203 upstream of the transcription start site, 5'-AAAGGGCG[1]CG[2]AGGCG[3]TGACG[4]CACG[5]GAAACG[6]TCA-3', was examined by

the PyroSequencing assay. We observed that the methylation ratios at CpG sites 1, 2, and 5 ($p<0.05$) were significantly increased, whereas those at CpG sites 3, 4, and 6 increased, though without statistical significance (Fig. 3B).

Discussion

In the present study, we identified 7 genes whose methylation changes were significantly correlated with energy consumption following an IWT regimen using a genome-wide microarray method. Among these genes, *NFκB2*, which is a well-known transcriptional regulator of inflammation, was hyper-methylated at multiple CpG islands in the promoter region. We therefore focused on *NFκB2* and further examined this gene to confirm its methylation changes using the PyroSequencing assay system in additional subjects who had completed the IWT program. Although an increase in methylation had not been predicted in the probe 5 region by the genome-wide microarray assay (Fig. 2A), we observed by PyroSequencing that the methylation ratio at this site was significantly higher (Fig. 3B). We believe that the different results were due to the more quantitative and direct nature of PyroSequencing versus the genome-wide microarray assay. The fraction of lymphocytes, monocytes, eosinophils, and basophils in white blood cells did not change following IWT (unpublished observation), suggesting that the alteration of methylation was not due to a shift in blood constituents.

In the ontology study, we observed the number of hyper- and hypomethylated genes involved in cell growth and maintenance and development was 41 and 73, respectively. These data provide a basis for future research into the epigenetics of exercise.

NFκB is composed of homodimers and heterodimers of 5 distinct subunits. Activated *NFκB* has been reported to regulate the transcription of over 400 genes that are involved in inflammation and are associated with age-related disease [11, 24]. It has also been reported that the NFκB signaling pathway regulates skeletal myogenesis [1]. Because NFκB2 is a component of NFκB, hyper-methylation of the *NFκB2* gene might reflect a decrease in NFκB2 expression, leading to a suppression of NFκB activity.

We reported that *ASC* [25], a major inflammasome regulator that promotes the activation of the inflammatory IL-1β cytokine, became significantly more methylated in subjects performing IWT (6.29 ± 0.26%) compared with controls (5.33 ± 0.14%) among a cohort of more than 400 subjects [19]. A subtle increase in *ASC* methylation was also observed in the genome-wide microarray assay in this study, but this findingwas not statistically significant, likely due to the limited number of subjects.

Apart from *NFκB2*, *HELT* and *PRRC2A/BAT2* [7] displayed a significantly positive correlation between methylation ratio and energy consumption following IWT (Table 4). In contrast, *EGR2* [14], *SLIT2*, *NKX2-5* [16], and *TBX18* showed a significant inverse

correlation between methylation ratio and energy consumption after training (Table 5). According to the reported biological functions of these genes, the observed alterations in methylation levels following IWT are intriguing and noteworthy. Because Helt determines GABAergic over glutamatergic fate [20], the hyper-methylation of *HELT* by IWT might indicate an enhancement in neural activity. Meanwhile, the transcription factor Egr2 has been reported to be essential for the control of inflammation and antigen-induced proliferation of B and T cells together with Egr3 [14]. Thus, the hypo-methylation of *EGR2* by IWT may be advantageous to suppress chronic inflammation in a manner similar to *NFkB2*. The cell motility modulator Slit2 is a potent inhibitor of platelet function [23]. The hypo-methylation of *SLIT2* may attenuate thrombosis, which could ameliorate blood viscosity. Finally, because *Tbx18* induces the direct conversion of quiescent cardiomyocytes to pacemaker cells [12], the hypo-methylation of this gene may lead to an improvement in cardiac function.

Acute bouts of exercise have been shown to activate a number of cellular pathways. For instance, several reports have described the effects of exercise on the activation of *PGC-1α*, *PDK4*, *PPAR-δ*, *PPKAB1*, *MAPK1*, and *NDUFC2* genes by assessing methylation in promoter regions and mRNA expression in skeletal muscle [2, 22]. Conversely, chronic exercise in rats has been shown to produce systemic

anti-inflammatory effects [13], which is consistent with our results, although the mechanism for this phenomenon remains unclear.

Several studies have described the DNA methylation programming of rat offspring by maternal behavior in the early period after birth [5]. Thus, methylation status is thought to be highly sensitive to environmental factors [3]. The epigenetic changes in some genes are also believed to reflect the process of aging [5], for which methylation status, in addition to the conventional physical and biochemical markers of blood pressure and cholesterol level, may be a predictor of physical age or health condition. A general improvement in health condition was noted in the clinical data of subjects after a 6-month IWT regimen, as indicated by decreases in BMI, although the difference between IWT and non-IWT participants was not statistically significant (data not shown). This might have been due to the small number of samples in the test groups. However, significant methylation changes brought about by IWT in *NFκB* and several other genes were observed, indicating that methylation change can be a sensitive and accurate biomarker with which to judge whether a particular training regimen is improving the health of a subject.

We conclude that the augmentation of methylation in the *NFkB2* promoter by IWT is advantageous in promoting a healthy state from an epigenetic perspective by

ameliorating the susceptibility to inflammation. The methylation changes in several other interesting genes are also suspected to be beneficial in promoting well-being, which is critical in aging populations. Based on our findings, the significance and mechanism of the gene methylation changes induced by IWT merit further investigation in larger human studies and in molecular and cellular analyses using animal models.

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Figure legends

Figure 1. Gene ontology classification for genes hyper- and hypo-methylated by IWT

Genes hyper-(A) and hypo-methylated (B) by IWT were assigned to categories reflecting putative roles in growth and maintenance, development, cell communication, and others.

Figure 2. Hyper-methylation of *NFκB*2

(A) The genome-wide methylation microarray assay was performed with microarray slides containing individual oligonucleotide probes (1-17). The ratio of methylation levels post-/pre-IWT is shown at sites corresponding to each probe. Open and closed bars indicate control and exercise groups, respectively. (B) The location of 17 probes used in a genome-wide microarray assay to evaluate the methylation level of the *NFκB2* promoter. (C, D, and E) Regression analysis of methylation status of the *NFκB2* gene promoter region versus total energy consumption divided by body weight. The vertical axis shows the ratio value of the post-IWT methylation/pre-IWT methylation in the

promoter region for probes 9 (C), 10 (D), and 12 (E). The correlation between methylation and energy consumption was analyzed using the Spearman non- parametric test. TSS: Transcription Start Site; ○: Non-exercise group; Δ: Exercise group.

Figure 3. Methylation of the *NFκB2* promoter region following IWT according to the PyroSequencing method for bisulfite-treated genomic DNA

(A) The *NFκB2* target region -565 to -518 upstream of the transcription start site. This region corresponds to probe 12, as shown in Figs 2A, B, and E. From left to right, CpG sites 1, 2, and 3 at top and CpG sites 4, 5, and 6 at bottom. (B) The *NFκB2* target region -1236 to -1203 upstream of the transcription start site corresponding to probe 5 is shown in Fig 2A and B. From left to right, CpG sites 1, 2, and 3 at top and CpG sites 4, 5, and 6 at bottom. The methylation differences between pre- and post-IWT among 20 subjects were compared using the Wilcoxon test. *: $p<0.05$, **: $p<0.01$

Table 1. IWT data and energy consumption of subjects undergoing the genome-wide microarray experiment

	Non-exercise						Exercise						
Subject	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Weight (kg)	**81.6**	**65.3**	**56**	**51**	**73.5**	**72**	**68.3**	**61.7**	**60.8**	**46.1**	**72.5**	**45.3**	**55.1**
Training days	**38**	**23**	**19**	**19**	**8**	**13**	**122**	**125**	**84**	**69**	**75**	**103**	**129**
EC (kcal)/day	**165**	**201**	**193**	**111.01**	**321**	**290**	**146**	**112**	**121**	**85**	**159**	**87.27**	**223.78**
Total EC (kcal)	**6270**	**4623**	**3667**	**2109.19**	**2568**	**3770**	**17812**	**14000**	**10164**	**5685**	**11925**	**8988.81**	**28867.62**
Total EC/weight (kcal/kg)	**76.84**	**70.80**	**65.48**	**41.36**	**34.94**	**52.36**	**260.79**	**226.90**	**167.17**	**127.22**	**164.48**	**198.43**	**523.91**

EC: Energy Consumption. Seven subjects who performed IWT for 40 days or more during a 6-month program were randomly chosen for the exercise group. Six subjects who performed IWT for less than 40 days during the same program were selected for the control group.

Table 2. Baseline characteristics of the control and exercise groups before IWT

	Non-exercise (n=6)	Exercise (n=7)	*p*-value
Age (years)	65.7±5.1	68.4±6.7	0.213
Height (cm)	157.3±7.3	157.6±8.3	0.477
Weight (kg)	66.6±11.5	58.5±11.2	0.213
BMI (kg/m^2)	26.8±3.2	25.0±3.3	0.104
Body fat (%)	31.7±7.1	25.5±4.5	0.082
Systolic BP	142.7±19.6	137.7±15.4	0.620
Diastolic BP	83.0±12.7	80.9±8.3	0.722
HDL cholesterol (mg/dl)	62.3±15.7	72.4±29.3	0.467
LDL cholesterol (mg/dl)	142.7±38.8	144.7±54.8	0.941
Triglycerides (mg/dl)	103.7±42.5	119.6±83.9	0.683
VO_{2max} (ml/kg/min)	20.2±3.5	21.4±3.7	0.588
Glucose (mg/dl)	110.0±22.1	105.7±11.3	0.661

Data are expressed as mean ± SD. *p*-values were determined using the Student's *t*-test. BMI, Body Mass Index; BP, Blood Pressure; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein

Table 3. Primers used for the PyroSequencing assay

Amplification site	Forward primer (5' to 3')	Reverse primer (5' to 3')	Sequencing primer (5' to 3')
-601 to -348	TGTGATGGAGATATATTTTTGTATTAGG	Biotin-ACCTCCCCTTCCAACAACCTATAA	-569-GGGGGGGGGGGGGGA
-1281 to -1017	GGGTTGGTTGAGTTAGTTTAGAGTTAAAT	Biotin-CCTCCTCCCTCTTTTCTCTTATCC	-1263-AGAGTTAAATTTTTAGTTAATGAA

Table 4. Genes with hyper-methylated status correlating to energy consumption following IWT

Gene symbol	Chr	Probe region (TSS)	Spearman's rho	*P*-value	Function
HELT	4	-148	0.736	0.004	Determines GABAergic over glutamatergic fate [20].
PRRC2A/BAT2	6	-595	0.769	0.002	Involved in the inflammatory response [7].
NFκB2	10	-565	0.709	0.007	"Master gene" of inflammation. NFκB plays a key role in inflammatory disease [24].
		-709	0.747	0.003	
		-787	0.555	0.049	

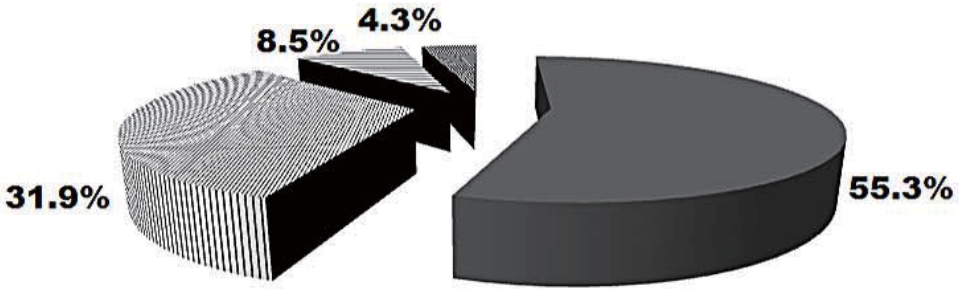
Chr: Chromosome; TSS: Transcription Start Site

Table 5. Genes with hypo-methylated state correlating to energy consumption following IWT

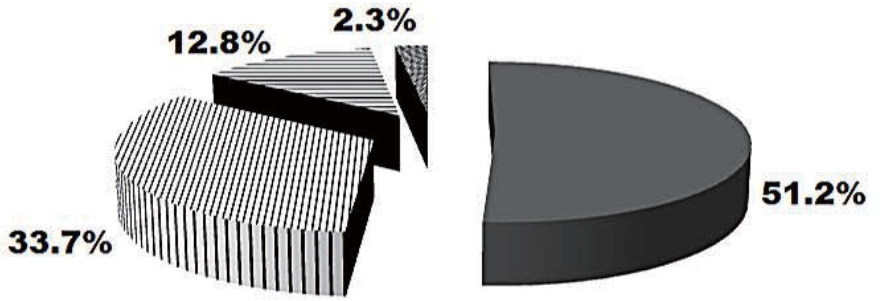
Gene symbol	Chr	Probe region (TSS)	Spearman's rho	*P*-value	Function
SLIT2	4	-598	-0.786	0.001	Potent inhibitor of platelet function, maybe advantageous against thrombosis [23].
NKX2-5	5	-3266	-0.753	0.003	Central regulator of heart development that is affected in congenital heart disease [16].
TBX18	6	-9439	-0.841	0.0003	Induces direct conversion of quiescent cardiomyocytes into pacemaker cells, indicating the improvement of cardiac function [12].
EGR2	10	-1900	-0.714	0.006	Essential for the control of inflammation and antigen-induced proliferation of B and T cells together with *Egr3*, maybe advantageous in suppressing chronic inflammation [14].
		-1983	-0.769	0.002	

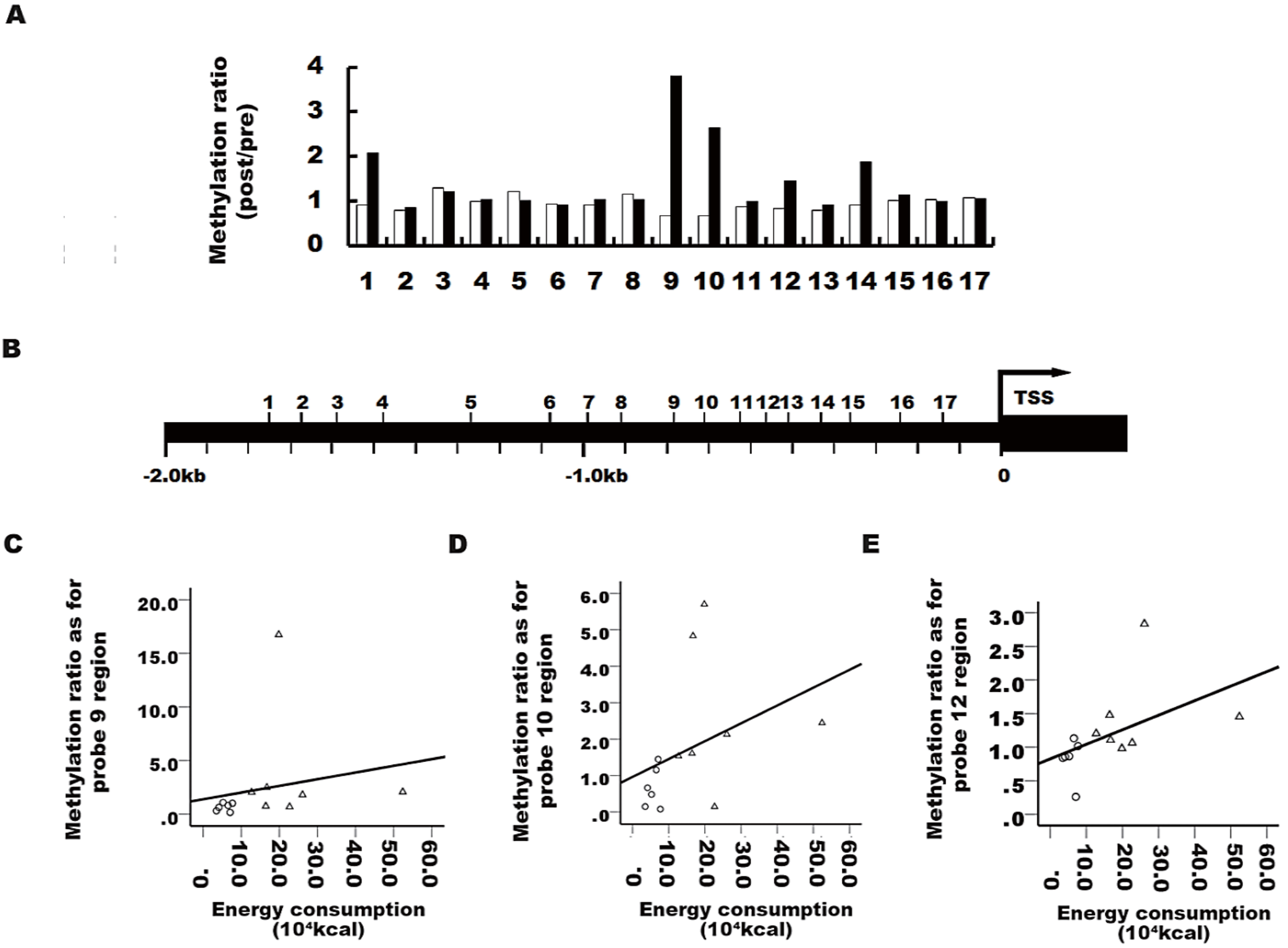
Chr: Chromosome; TSS: Transcription Start Site

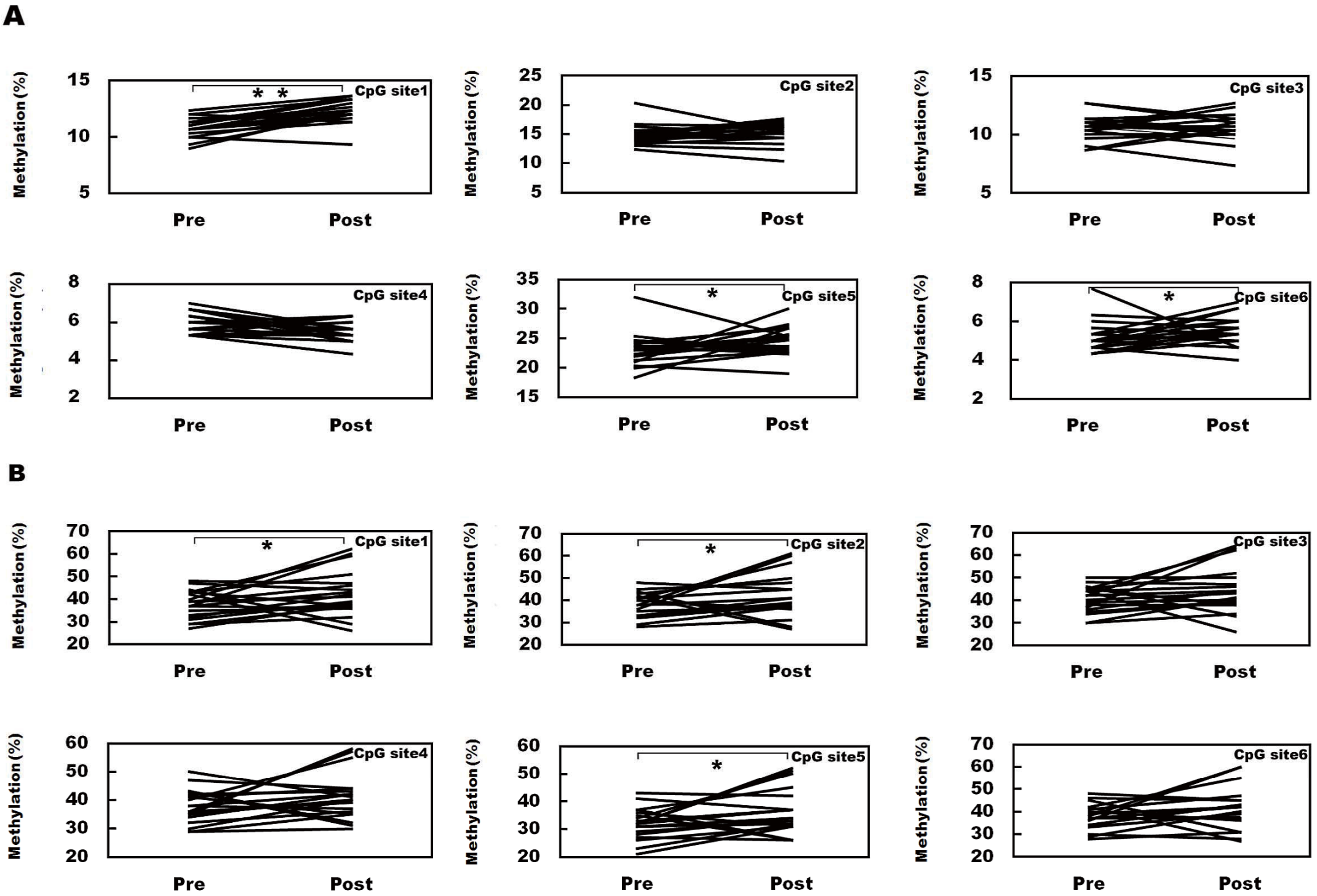
A


8.5%
4.3%
31.9%
55.3%
Biological process
Cell growth and maintenance
Cell communication
Development
Others

B


12.8%
2.3%
33.7%
51.2%
Biological process
Cell growth and maintenance
Cell communication
Development
Others


A
Methylation ratio (post/pre)
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TSS
-2.0kb
-1.0kb
0
C
Methylation ratio as for probe 9 region
20.0
15.0
10.0
5.0
.0
.0 10.0 20.0 30.0 40.0 50.0 60.0
Energy consumption (10⁴kcal)
D
Methylation ratio as for probe 10 region
6.0
5.0
4.0
3.0
2.0
1.0
.0
.0 10.0 20.0 30.0 40.0 50.0 60.0
Energy consumption (10⁴kcal)
E
Methylation ratio as for probe 12 region
3.0
2.5
2.0
1.5
1.0
.5
.0
.0 10.0 20.0 30.0 40.0 50.0 60.0
Energy consumption (10⁴kcal)


A
Methylation (%)
15
10
5
* *
CpG site1
Pre
Post
Methylation (%)
25
20
15
10
5
CpG site2
Pre
Post
Methylation (%)
15
10
5
CpG site3
Pre
Post
Methylation (%)
8
6
4
2
CpG site4
Pre
Post
Methylation (%)
35
30
25
20
15
*
CpG site5
Pre
Post
Methylation (%)
8
6
4
2
*
CpG site6
Pre
Post
B
Methylation (%)
70
60
50
40
30
20
*
CpG site1
Pre
Post
Methylation (%)
70
60
50
40
30
20
*
CpG site2
Pre
Post
Methylation (%)
70
60
50
40
30
20
CpG site3
Pre
Post
Methylation (%)
60
50
40
30
20
CpG site4
Pre
Post
Methylation (%)
60
50
40
30
20
*
CpG site5
Pre
Post
Methylation (%)
70
60
50
40
30
20
CpG site6
Pre
Post

Supplement table 1. Baseline characteristics of the subjects in PyroSequencing analysis

	Subjects (n=20)
Age (years)	61.6±9.2
Height (cm)	157.6±8.1
Weight (kg)	58.1±9.1
BMI (kg/m^2)	23.3±2.7
Body fat (%)	30.0±7.3
Systolic BP	134.0±19.9
Diastolic BP	78.6±9.3
HDL cholesterol (mg/dl)	68.1±18.0
LDL cholesterol (mg/dl)	150.3±35.4
Total cholesterol(mg/dl)	228.6±33.6
Glucose (mg/dl)	96.3±8.2

Data are expressed as mean ± SD. BMI, Body Mass Index; BP, Blood Pressure; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein

Supplement table 2. IWT data and energy consumption of subjects undergoing the PyroSequencing experiment

Subject	Weight (kg)	Training days	EC (kcal)/day	Total EC (kcal)	Total EC/weight (kcal/kg)
1	51.3	50	163.50	8174.83	159.35
2	65.4	55	173.92	9565.63	146.26
3	48	77	124.53	9588.88	199.77
4	49.2	98	180.94	17732.21	360.41
5	59.4	74	220.33	16304.10	274.48
6	51	73	116.91	8534.27	167.34
7	75.2	77	178.44	13740.15	182.71
8	65.5	49	159.92	7835.85	119.63
9	57.9	55	183.48	10091.36	174.29
10	48.5	71	116.51	8272.43	170.57
11	61.5	53	171.19	9073.19	147.53
12	46.6	66	132.53	8746.67	187.70
13	57.7	103	411.25	42358.80	734.12
14	56.2	59	240.42	14184.72	252.40
15	51.1	69	152.71	10537.04	206.20
16	56.4	36	155.27	5589.60	99.11
17	55.1	43	142.71	6136.57	111.37
18	55.4	88	190.33	16749.35	302.33
19	77.5	86	464.01	39904.77	514.90
20	72.7	68	255.10	17346.80	238.61

EC: Energy Consumption.