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14. ABSTRACT

There is an increasing prevalence of obesity and cardiovascular disease (CVD) risk factors in the military population, which is negatively affecting operational readiness. The ability to prevent heart disease and reduce its overall impact on morbidity would increase the quality of life among military personnel and their dependents, and has the potential to generate enormous cost savings for the DoD. In the Integrative Cardiac Health Program (ICHP), we investigated physiological and molecular responses to risk factor modification interventions in individuals or populations at risk for CVD. We aimed to better understand CVD risk at the molecular level before onset of clinical disease, and develop outcomes-based patient empowering lifestyle solutions to prevent disease. Through this research, our objectives were to (1) identify genetic influences on CVD and integrate information on dietary, behavioral, and lifestyle factors to provide important information on CVD risk reduction and (2) discover new genes in previously associated pathways to reveal new molecular influences on cardiovascular risk reduction.

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The Integrative Cardiac Health Program (ICHP) aims to understand the complex interactions of numerous molecular components that characterize individuals or populations at risk for cardiovascular disease (CVD). In this project, we worked to identify molecular networks that define cardiovascular risk and correspond to lifestyle changes that may influence the trajectory of disease progression. Given the increasing trends of major CVD risk factors in the US military population and potentially adverse consequences on combat readiness, our efforts were directed to (1) better understanding the relationships of war stressors and CVD risk at the molecular level before onset of clinical disease, and (2) outcomes-based patient empowering lifestyle solutions to prevent disease.

WRNMMC ICHP has developed a standardized and personalized lifestyle intervention program that has resulted in significant improvements in cardiovascular risk markers such as C-reactive protein (CRP), glucose, insulin, glycosylated hemoglobin, and lipids. Our previous research was the first to identify significant gene expression changes associated with an ultra-intensive lifestyle change program and to show that genetic variants at genes involved in lipid metabolism influence lipid response. We hypothesized that (1) identifying genetic influences on CVD and integrating information on dietary, behavioral, and lifestyle factors would provide important information on CVD risk reduction and (2) discovering new genes in previously associated pathways would reveal new molecular influences on cardiovascular risk reduction.

This research used state-of-the-art next-generation DNA and RNA sequencing to address the following research questions:

1. Can inherited variants in specific genes that influence cardiovascular disease be identified through whole-genome sequencing of an appropriate population of affected individuals?
2. Does surgically-induced weight loss alter patterns of gene expression in adipose tissue and peripheral blood, and do these molecular changes have prognostic value in predicting weight loss success and improving the effectiveness of treatment programs for obesity?
3. Can changes in whole-transcriptome expression: (a) improve the effectiveness of lifestyle programs by identifying patients who are unlikely to benefit from the conventional program and should follow a customized program tailored to their individual needs; (b) be used to reduce health outcome disparities between men and women and between specific subgroups of patients; and (c) identify previously unknown molecular pathways that influence heart disease, and provide insights into cardiovascular disease development that may have important consequences for cardiac treatment programs?

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1. **INTRODUCTION:** There is an increasing prevalence of obesity and cardiovascular disease (CVD) risk factors in the military population, which is negatively affecting operational readiness. The ability to prevent heart disease and reduce its overall impact on morbidity would increase the quality of life among military personnel and their dependents, and has the potential to generate enormous cost savings for the DoD. In the Integrative Cardiac Health Program (ICHP), we investigated physiological and molecular responses to risk factor modification interventions in individuals or populations at risk for CVD. We aimed to better understand CVD risk at the molecular level before onset of clinical disease, and develop outcomes-based patient empowering lifestyle solutions to prevent disease. Through this research, our objectives were to (1) identify genetic influences on CVD and integrate information on dietary, behavioral, and lifestyle factors to provide important information on CVD risk reduction and (2) discover new genes in previously associated pathways to reveal new molecular influences on cardiovascular risk reduction.

2. **KEYWORDS:** Lifestyle modification, cardiovascular disease, obesity, gene expression, RNA sequencing, gender differences, molecular response, diet, exercise.

3. **OVERALL PROJECT SUMMARY:**
   Throughout this project, our efforts were devoted to generating as much high quality DNA and RNA sequence data as possible. We analyzed DNA methylation changes in response to cardiovascular risk reduction using 132 DNA samples from 66 patients and controls. Significant changes in methylation over one year were detected at 145 chromosomal locations in laparoscopically placed adjustable gastric banding (LAGB) patients compared to controls, at 115 chromosomal locations in intensive lifestyle participants compared to controls, and at 129 chromosomal locations in intensive lifestyle participants compared to LAGB patients.

   Array-based gene expression assays were conducted on 462 RNA samples (time points) from 162 LAGB patients and on 492 peripheral blood samples from 163 moderate lifestyle participants. For the LAGB samples, a comprehensive QC analysis determined that 377 arrays were suitable for subsequent analysis; only 4 arrays did not pass the QC assessment.

   Large-scale RNA sequencing was conducted on 144 RNA samples (time points) from 72 patients and controls. Paired-end sequencing generated ~2,317.26 billion bases of sequence. A preliminary analysis using gene counting and differential expression analysis discovered 1,252 differentially expressed genes among samples using a False Discovery Rate (FDR) $p<0.05$.

   Continuing reviews for all research protocols for this project were submitted to and approved by the Chesapeake IRB throughout the study.

Task #1: Epigenetic changes in DNA (genome-wide patterns of methylation) during CV risk reduction

In this task, we examined patterns of DNA methylation across the entire genome in circulating leukocytes in response to cardiovascular risk reduction (lifestyle and surgically-assisted) using new and current participants in our ICHP programs. Our goal was to identify changes in methylation in specific areas of the genome and relate these changes to known and novel genes influencing heart disease. Results from this research may be useful in further understanding molecular mechanisms associated with changes in cardiovascular risk factors and regulatory processes involved in heart disease development.

During the project, DNA was isolated from 132 whole blood samples using the Quick gDNA Blood Mini kit (Zymo Research) in the following groups: intensive lifestyle baseline (n=28) and one year (n=28), LAGB baseline (n=28) and one year (n=28), and control baseline (n=10) and one year (n=10) as shown in Table 1 below.

Four batches of DNA samples, with aliquots of 1.0 to 1.5 μg, were subjected to methyl-miniSeq analysis. The first two batches had five patients from each group, all age- and gender-matched, in each batch. The next two batches had nine intensive lifestyle participants and nine LAGB patients, age- and gender-matched at baseline and 1 year, in each batch. For all 132 samples included in the analysis, average DNA concentration was 57.70±33.94 ng/μl (range 14.1-269.8 ng/μl), the average 260/280 ratio was 2.01±0.10 (range 1.78-2.60), and the average 260/230 ratio was 1.99±1.78 (range 0.13-15.0).

Methyl-MiniSeq™ libraries were constructed from 200-500 ng of genomic DNA digested sequentially with 60 units of TaqαI and 30 units of MspI (New England Biolabs) and then extracted with the DNA Clean & Concentrator™-5 kit (Zymo Research). Fragments were ligated to pre-annealed adapters containing 5'-methyl-cytosine instead of cytosine according to specified guidelines (Illumina Corporation). Adaptor-ligated fragments of 150–250 bp and 250–350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel (Zymoclean™ Gel DNA Recovery kit, Zymo Research). The recovered fragments were bisulfite-treated using the EZ DNA Methylation-Lightning™ kit (Zymo Research). Following preparative-scale PCR, the resulting products were purified with the DNA Clean & Concentrator™ kit (Zymo Research) for sequencing on an Illumina HiSeq 2000 platform.

Table 1. Concentrations and purity measures for DNA isolated from whole blood from 28 intensive lifestyle participants, 28 laparoscopically placed adjustable gastric banding patients, and 10 controls.

Patient ID	Time point	DNA Concentration (ng/μl)	Abs 260/280	Abs 260/230

BATCH #1				
047B	Baseline	33.4	1.85	0.93
047Y	1 year	14.1	2.60	3.69
154B	Baseline	65.6	1.97	2.11
154Y	1 year	66.1	1.99	0.92
174B	Baseline	72.3	2.00	2.43
174Y	1 year	76.8	2.02	1.64
185B	Baseline	31.2	2.08	1.31
185Y	1 year	78.3	1.96	2.14
166B	Baseline	63.8	2.12	2.44
166Y	1 year	85.1	2.00	2.00
303B	Baseline	22.1	2.06	10.00
303Y	1 year	35.8	2.09	1.50
589B	Baseline	102.5	2.06	2.35
589Y	1 year	56.4	2.19	4.27
301B	Baseline	49.1	2.07	6.31
301Y	1 year	39.9	2.03	1.42
812B	Baseline	59.8	1.90	3.31
812Y	1 year	61.4	2.15	4.81
919B	Baseline	29.8	2.01	0.45
919Y	1 year	36.7	1.85	4.41
613B-C	Baseline	62.5	1.90	2.70
613Y-C	1 year	35.4	2.04	3.00
377B	Baseline	24.0	2.09	15.00
377Y	1 year	42.1	1.92	4.60
709B	Baseline	38.8	1.93	1.62
709Y	1 year	35.3	1.83	0.55
743B	Baseline	43.1	2.00	0.18
743Y	1 year	35.5	1.88	0.46
226B	Baseline	39.3	1.89	0.88
226Y	1 year	35.3	1.78	10.26
BATCH #2				
304B	Baseline	29.2	1.98	1.42
304Y	1 year	47.4	1.90	1.93
342B	Baseline	104.8	2.02	1.55
342Y	1 year	19.2	2.04	0.92
825B	Baseline	41.6	1.87	0.24
825Y	1 year	33.00	2.11	1.58
751B	Baseline	67.1	1.97	2.31
751Y	1 year	37.8	1.95	0.78

414B	Baseline	80.3	2.00	2.07
414Y	1 year	78.8	1.98	2.29
030B	Baseline	41.4	1.95	2.06
030Y	1 year	74.9	1.92	1.13
046B	Baseline	72.6	2.02	2.19
046Y	1 year	23.4	1.99	1.99
062B	Baseline	96.2	1.99	1.45
062Y	1 year	32.8	2.03	1.52
136B	Baseline	26.2	1.91	0.95
136Y	1 year	32.4	1.97	1.41
142B	Baseline	37.5	1.93	0.37
142Y	1 year	34.8	2.04	0.83
505B	Baseline	33.5	1.88	1.25
505Y	1 year	54.00	1.91	1.21
705B	Baseline	28.5	2.17	1.92
705Y	1 year	44.8	2.02	2.34
708B	Baseline	50.1	1.96	1.46
708Y	1 year	70.2	1.95	1.73
400B	Baseline	29.8	2.28	2.59
400Y	1 year	63.2	2.01	2.00
956B	Baseline	55.00	1.88	1.98
956Y	1 year	46.3	2.04	2.23
BATCH #3				
003B	Baseline	67.7	2.03	1.04
003Y	1 year	64.1	2.10	2.15
148B	Baseline	125.4	1.99	2.16
148Y	1 year	23.3	2.10	1.47
163B	Baseline	31.2	1.98	2.26
163Y	1 year	80.6	2.14	2.08
177B	Baseline	65.0	1.99	0.80
177Y	1 year	62.5	2.11	1.63
179B	Baseline	134.8	1.99	2.26
179Y	1 year	113.7	1.99	2.14
193B	Baseline	40.4	2.01	0.24
193Y	1 year	79.2	2.04	2.01
194B	Baseline	75.6	2.06	1.80
194Y	1 year	77.9	2.05	1.85
198B	Baseline	150.3	1.96	2.09
198Y	1 year	51.6	2.14	2.29
287B	Baseline	80.8	2.05	2.45
287Y	1 year	88.5	2.06	1.25
288B	Baseline	41.0	2.12	2.19

288Y	1 year	60.0	2.06	1.57
308B	Baseline	127.2	1.94	2.24
308Y	1 year	269.8	1.98	1.93
350B	Baseline	53.6	2.07	2.06
350Y	1 year	29.1	2.13	1.94
613intB	Baseline	38.6	1.98	1.00
613intY	1 year	47.9	1.98	1.21
749B	Baseline	36.6	2.11	1.15
749Y	1 year	32.1	2.16	1.83
823B	Baseline	70.7	2.01	2.11
823Y	1 year	24.7	2.23	1.42
884B	Baseline	145.9	2.05	2.00
884Y	1 year	60.5	2.05	1.88
157B	Baseline	57.1	2.01	1.90
157Y	1 year	73.5	1.97	2.17
579B	Baseline	76.3	2.01	1.69
579Y	1 year	49.7	1.96	1.70
BATCH #4				
004B	Baseline	156.4	1.98	1.40
004Y	1 year	71.0	1.91	1.23
035B	Baseline	49.2	1.91	0.70
035Y	1 year	19.1	1.87	1.63
162B	Baseline	22.4	2.15	0.96
162Y	1 year	86.7	1.98	1.02
176B	Baseline	97.3	2.00	2.00
176Y	1 year	110.6	2.03	2.03
178B	Baseline	105.3	2.04	2.21
178Y	1 year	83.4	2.04	2.25
186B	Baseline	64.2	2.02	2.51
186Y	1 year	52.7	2.04	3.07
211B	Baseline	44.7	1.88	0.80
211Y	1 year	46.2	1.99	1.75
225B	Baseline	78.5	1.96	1.97
225Y	1 year	72.3	1.96	1.71
427B	Baseline	39.4	1.98	2.90
427Y	1 year	50.9	1.98	1.15
428B	Baseline	41.8	2.02	1.55
428Y	1 year	34.1	1.92	1.20
432B	Baseline	33.2	1.88	1.61
432Y	1 year	35.1	1.84	0.91
669B	Baseline	43.6	1.99	1.03
669Y	1 year	49.9	2.00	1.39

687B	Baseline	32.3	2.11	2.34
687Y	1 year	52.0	1.93	1.44
882B	Baseline	36.3	1.97	0.13
882Y	1 year	32.3	1.93	0.52
918B	Baseline	35.4	1.95	1.47
918Y	1 year	27.7	1.96	2.10
928B	Baseline	22.1	2.04	1.28
928Y	1 year	27.4	2.03	1.72
181B	Baseline	31.2	1.98	0.82
181Y	1 year	55.0	1.93	0.93
434B	Baseline	42.7	2.02	0.21
434Y	1 year	69.4	2.00	2.01

Library construction was completed on all 132 samples and all samples passed QC requirements. One microgram of DNA from each library was sequenced using Methyl-Mini Sequencing, which is a Reduced Repression Bisulfite Sequencing method that allows for detection of 3-4 million CpG sites throughout the genome.

Methyl-MiniSeq™ sequence alignments and data analysis: Sequence reads were derived from bisulfite-treated EpiQuest libraries using standard Illumina base-calling software. Sequences were analyzed using a proprietary analysis pipeline written in Python, which used Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) to perform sequence alignments. Index files were constructed using the "bismark_genome_preparation" command and the entire reference genome. The "non_directional" parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed when conducting methylation calling. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C residue divided by the total number of reads reporting a C or T. Fisher's exact test or a t-test was performed for each CpG site that had at least five reads of coverage. Promoter, gene body, and CpG island annotations were added for each CpG included in the comparison.

The average number of total reads per sample was 38,258,843±6,375,753. Mapping efficiency was 52.1%±4.2%. All samples had a bisulfite conversion rate of >98.0% (Table 2). The number of CpG (methylated) sites per sample was 8,410,571±374,803 with minimum coverage of 5X per sample.

Table 2. Results of Reduced Repression Bisulfite Sequencing on 28 intensive lifestyle participants, 28 laparoscopically placed adjustable gastric banding patients, and 10 controls.

Sample	No. Total Reads	Mapping Efficiency	Unique CpGs	Average CpG Coverage	BS Conv. Rate
BATCH #1					
047B	36,705,287	59%	8,380,942	8X	99%
047Y	39,480,981	56%	8,449,711	8X	99%
154B	39,344,051	57%	8,427,361	9X	99%
154Y	46,518,809	49%	8,034,750	10X	98%
174B	37,535,399	58%	8,380,190	8X	99%
174Y	35,271,999	55%	8,107,389	8X	99%
185B	43,264,229	54%	8,589,618	9X	98%
185Y	29,840,305	55%	8,009,735	7X	99%
166B	35,539,525	56%	8,284,017	7X	99%
166Y	38,190,627	56%	8,356,105	8X	99%
303B	35,952,416	57%	8,418,795	8X	99%
303Y	40,202,579	59%	8,916,549	8X	99%
589B	35,525,846	61%	8,765,446	7X	99%
589Y	39,022,506	61%	9,056,597	8X	99%
301B	31,184,095	59%	8,391,100	7X	99%
301Y	32,292,061	56%	8,089,337	7X	99%
812B	34,115,895	57%	8,133,044	8X	99%
812Y	38,180,314	56%	8,235,083	8X	99%
919B	41,441,318	57%	8,451,510	9X	99%
919Y	35,784,427	60%	8,387,346	8X	99%
613B-C	42,637,181	55%	8,391,188	9X	99%
613Y-C	39,364,561	55%	8,267,203	9X	99%
377B	39,764,053	57%	8,343,424	9X	99%
377Y	39,329,753	57%	8,349,635	9X	99%
709B	32,495,359	55%	7,872,673	7X	99%
709Y	37,557,280	57%	8,098,635	9X	98%
743B	40,901,341	57%	8,448,724	9X	99%
743Y	39,784,766	58%	8,366,518	9X	99%
226B	34,963,505	58%	8,254,083	8X	99%
226Y	37,830,893	58%	8,367,000	8X	99%
BATCH #2					
304B	38,199,408	57%	8,794,890	9X	99%
304Y	35,345,851	60%	8,757,535	8X	99%
342B	42,523,679	57%	9,028,714	9X	99%
342Y	37,077,680	58%	8,699,549	9X	99%
825B	40,134,188	56%	8,728,369	9X	99%
825Y	39,005,801	54%	8,817,675	8X	99%

751B	41,909,407	50%	8,701,899	8X	98%
751Y	38,697,833	52%	8,646,752	8X	99%
414B	45,736,781	48%	8,919,426	8X	98%
414Y	39,939,001	50%	8,797,138	8X	99%
030B	36,310,000	54%	8,721,595	8X	99%
030Y	36,406,225	55%	8,764,649	8X	99%
046B	41,278,186	56%	8,856,851	9X	99%
046Y	40,229,529	53%	8,590,640	9X	99%
062B	33,271,228	57%	8,566,252	8X	99%
062Y	40,383,241	54%	8,808,181	9X	99%
136B	29,267,480	52%	7,796,970	7X	98%
136Y	40,686,881	53%	8,795,929	9X	99%
142B	47,748,368	56%	8,842,608	11X	99%
142Y	32,195,094	53%	8,532,504	7X	99%
505B	34,840,496	57%	8,453,948	8X	99%
505Y	39,787,631	56%	8,742,775	8X	99%
705B	36,032,892	54%	8,667,754	8X	98%
705Y	32,772,795	56%	8,686,925	7X	99%
708B	37,642,450	52%	8,717,964	8X	99%
708Y	39,318,228	51%	8,990,244	7X	99%
400B	43,509,397	49%	8,759,136	8X	98%
400Y	40,113,401	49%	8,830,726	8X	99%
956B	48,029,519	47%	9,060,199	9X	99%
956Y	28,842,827	50%	8,271,275	6X	99%

BATCH #3

003B	41,333,425	52%	8,675,027	9X	99%
003Y	34,879,594	51%	8,167,391	8X	99%
148B	44,803,977	53%	8,606,916	9X	99%
148Y	37,091,352	52%	8,281,401	8X	99%
163B	40,540,138	50%	8,372,643	8X	99%
163Y	43,601,517	50%	8,631,038	8X	99%
177B	43,952,958	48%	8,631,479	9X	99%
177Y	39,789,225	46%	8,458,777	8X	99%
179B	48,849,550	46%	8,630,762	9X	99%
179Y	40,181,802	45%	8,208,028	9X	99%
193B	44,020,201	46%	8,514,858	9X	99%
193Y	47,605,225	47%	8,534,253	9X	99%
194B	47,252,731	45%	8,462,727	10X	99%
194Y	41,354,429	45%	8,291,267	8X	99%
198B	51,392,609	40%	8,463,087	10X	99%
198Y	42,124,468	43%	8,171,994	7X	99%
287B	45,067,689	44%	8,417,606	9X	99%

287Y	48,338,833	51%	8,558,662	10X	99%
288B	43,494,249	52%	8,673,095	10X	99%
288Y	41,486,890	48%	8,464,926	9X	99%
308B	37,512,424	46%	8,242,475	8X	99%
308Y	42,170,237	43%	8,112,620	8X	99%
350B	38,076,502	51%	8,447,075	8X	99%
350Y	49,388,567	46%	8,613,276	9X	99%
613intB	44,628,358	47%	8,366,027	8X	99%
613intY	31,938,077	51%	8,098,581	7X	99%
749B	30,043,658	52%	8,136,628	6X	99%
749Y	46,928,508	51%	8,796,963	10X	99%
823B	48,197,637	48%	8,594,779	10X	99%
823Y	43,759,996	48%	8,432,129	9X	99%
884B	43,968,414	51%	8,580,772	9X	99%
884Y	41,284,737	46%	8,441,629	8X	99%
157B	46,849,718	49%	8,602,170	9X	99%
157Y	37,100,301	50%	8,280,525	8X	99%
579B	50,347,474	49%	8,661,293	9X	99%
579Y	42,288,448	50%	8,492,666	9X	99%
BATCH #4					
004B	35,867,921	52%	8,257,447	8X	99%
004Y	37,170,944	50%	8,160,428	9X	99%
035B	43,023,305	51%	8,680,924	9X	99%
035Y	41,551,938	51%	8,612,844	9X	99%
162B	45,679,166	51%	8,666,481	10X	98%
162Y	42,246,419	51%	8,520,678	10X	99%
176B	42,981,984	51%	8,703,974	9X	99%
176Y	42,531,890	49%	8,506,566	10X	99%
178B	39,250,172	53%	8,368,582	8X	99%
178Y	32,862,952	52%	8,106,986	8X	99%
186B	35,618,917	55%	8,496,807	8X	99%
186Y	22,850,758	50%	7,136,798	7X	99%
211B	36,583,762	53%	8,455,360	8X	99%
211Y	19,669,957	55%	7,484,190	5X	99%
225B	21,432,042	52%	7,460,294	5X	99%
225Y	19,407,868	50%	7,187,842	5X	99%
427B	17,894,904	51%	7,108,588	5X	99%
427Y	18,028,715	47%	6,685,900	5X	99%
428B	29,572,443	49%	8,032,282	7X	99%
428Y	32,764,380	50%	8,262,974	8X	99%
432B	34,617,424	52%	8,430,039	8X	99%
432Y	32,380,240	50%	8,239,536	7X	99%

669B	29,599,875	53%	8,420,485	7X	99%
669Y	33,501,227	53%	8,565,568	8X	99%
687B	32,158,299	49%	7,966,504	8X	99%
687Y	33,204,275	49%	8,004,952	8X	99%
882B	32,923,516	51%	8,283,218	8X	99%
882Y	34,420,830	51%	8,302,455	8X	99%
918B	35,493,478	54%	8,275,355	8X	99%
918Y	31,326,253	51%	8,016,992	8X	99%
928B	41,383,298	50%	8,496,786	8X	99%
928Y	40,230,489	48%	8,415,111	8X	99%
181B	36,624,767	54%	8,703,498	8X	99%
181Y	35,515,323	53%	8,422,506	8X	99%
434B	39,160,124	49%	8,283,130	9X	99%
434Y	41,736,587	49%	8,384,979	9X	99%

Comparative analyses showed that significant changes in methylation over one year occurred at 145 chromosomal locations in LAGB patients compared to controls (Table 3). Similarly, significant changes in methylation over one year occurred at 115 chromosomal locations in intensive lifestyle participants compared to controls (Table 4), and significant changes in methylation over one year occurred at 129 chromosomal locations in intensive lifestyle participants compared to LAGB patients (Table 5). Only three sites (Start BP 179675453, 100301187, 76171144) showed changes in methylation in multiple comparisons.

Table 3. Changes in genome-wide methylation at CpG sites from Baseline to One Year in 28 laparoscopically placed adjustable gastric banding patients compared to 10 non-intervention controls.

Chromo	Start BP	End BP	Strand	Meth Diff 1YR vs Base LAGB	Meth Diff 1YR vs Base Control	Diff of Meth Diff
chr15	80310167	80310168	-	-0.15	-0.15	0
chr10	38883558	38883559	+	0.12	0.12	0
chr20	3725620	3725621	+	-0.12	-0.12	0
chr15	79033171	79033172	-	-0.12	-0.12	0
chr3	137481936	137481937	-	0.11	0.11	0
chr19	584979	584980	+	-0.15	-0.15	0
chr3	73005896	73005897	+	-0.13	-0.13	0
chr17	29852692	29852693	-	-0.14	-0.14	0
chr7	69047606	69047607	+	-0.14	-0.15	0.01

chr6	40317694	40317695	+	-0.15	-0.14	0.01
chr22	48788912	48788913	-	-0.15	-0.14	0.01
chr7	31136269	31136270	+	-0.15	-0.14	0.01
chr3	75827848	75827849	-	-0.18	-0.17	0.01
chr8	21288258	21288259	-	-0.11	-0.10	0.01
chr16	54611262	54611263	+	-0.11	-0.12	0.01
chr7	24229695	24229696	+	0.10	0.11	0.01
chr22	38453843	38453844	-	-0.12	-0.11	0.01
chr17	77328149	77328150	-	-0.11	-0.10	0.01
chr21	44001123	44001124	+	-0.11	-0.12	0.01
chr19	51433059	51433060	+	-0.11	-0.12	0.01
chr17	7339885	7339886	+	-0.13	-0.12	0.01
chr6	6969472	6969473	+	-0.13	-0.12	0.01
chr20	3140891	3140892	-	-0.14	-0.13	0.01
chr4	1492475	1492476	-	-0.16	-0.15	0.01
chr1	179675453	179675454	+	0.17	0.16	0.01
chr2	152958888	152958889	+	-0.13	-0.14	0.01
chr12	57997901	57997902	+	-0.12	-0.13	0.01
chr5	140264045	140264046	-	-0.13	-0.15	0.02
chr10	100301187	100301188	-	-0.10	-0.12	0.02
chr19	4635428	4635429	-	-0.16	-0.14	0.02
chr20	57179919	57179920	-	-0.12	-0.10	0.02
chr7	128050909	128050910	-	-0.14	-0.16	0.02
chr16	19906397	19906398	+	-0.13	-0.11	0.02
chr3	21854148	21854149	+	0.13	0.11	0.02
chr5	16626987	16626988	+	-0.14	-0.12	0.02
chr5	132159035	132159036	+	-0.12	-0.14	0.02
chr5	140530473	140530474	+	0.15	0.17	0.02
chr19	31050422	31050423	+	-0.15	-0.12	0.03
chr10	23004088	23004089	+	-0.17	-0.14	0.03
chr10	73111789	73111790	+	-0.17	-0.14	0.03
chr8	27436499	27436500	-	-0.12	-0.15	0.03
chr1	11863037	11863038	-	-0.16	-0.13	0.03
chr22	49438930	49438931	+	-0.16	-0.13	0.03
chr1	147190288	147190289	+	0.18	0.15	0.03
chr3	10487994	10487995	-	0.14	0.11	0.03
chr12	131488480	131488481	+	-0.14	-0.11	0.03
chr1	182922366	182922367	+	-0.11	-0.14	0.03
chr10	36498238	36498239	+	-0.11	-0.14	0.03
chr2	9562532	9562533	+	0.18	0.14	0.04
chr14	101923926	101923927	+	0.15	0.11	0.04
chr6	139583814	139583815	-	-0.13	-0.17	0.04
chr11	67200965	67200966	+	-0.19	-0.15	0.04

chr22	46262571	46262572	+	-0.12	-0.16	0.04
chr12	122079087	122079088	-	-0.16	-0.12	0.04
chr1	153916734	153916735	+	-0.16	-0.12	0.04
chr17	75363197	75363198	+	-0.12	-0.16	0.04
chr17	79535220	79535221	+	-0.12	-0.16	0.04
chr20	22144025	22144026	-	0.14	0.10	0.04
chr20	3140906	3140907	-	-0.18	-0.13	0.05
chr18	77918253	77918254	-	-0.15	-0.10	0.05
chr20	58325835	58325836	+	-0.14	-0.19	0.05
chr12	109916472	109916473	+	-0.16	-0.11	0.05
chr13	66425319	66425320	-	-0.16	-0.11	0.05
chr5	153896906	153896907	-	0.16	0.11	0.05
chr19	22990024	22990025	-	0.17	0.12	0.05
chr5	149978524	149978525	+	-0.10	-0.16	0.06
chr19	588097	588098	+	0.16	0.10	0.06
chr12	49760013	49760014	+	-0.20	-0.14	0.06
chr8	127889337	127889338	+	-0.18	-0.11	0.07
chr6	94570539	94570540	-	-0.18	-0.11	0.07
chr7	2164711	2164712	+	0.18	0.11	0.07
chr13	30330068	30330069	+	0.13	0.20	0.07
chr16	67969366	67969367	+	-0.20	-0.12	0.08
chr12	50432868	50432869	+	-0.23	-0.12	0.11
chr20	56247624	56247625	-	-0.26	-0.15	0.11
chr14	63623934	63623935	+	-0.10	0.10	0.20
chr7	23720805	23720806	+	0.11	-0.10	0.21
chr6	41337060	41337061	+	-0.11	0.10	0.21
chr9	133781625	133781626	-	0.10	-0.11	0.21
chr5	65917990	65917991	+	-0.12	0.10	0.22
chr15	77861673	77861674	-	0.10	-0.12	0.22
chr8	145939423	145939424	+	0.12	-0.10	0.22
chr3	128525592	128525593	-	0.10	-0.12	0.22
chr4	1401611	1401612	+	0.12	-0.10	0.22
chr17	29852636	29852637	-	-0.11	0.12	0.23
chr2	1506856	1506857	-	0.11	-0.12	0.23
chr16	11517862	11517863	-	-0.11	0.12	0.23
chr17	38024471	38024472	-	0.10	-0.13	0.23
chr12	65515861	65515862	+	0.13	-0.11	0.24
chr20	44001832	44001833	-	0.12	-0.12	0.24
chr16	88814192	88814193	-	0.12	-0.12	0.24
chr6	41337039	41337040	+	-0.15	0.10	0.25
chr19	719693	719694	-	-0.15	0.10	0.25
chr17	48661402	48661403	+	-0.14	0.11	0.25
chr1	226163502	226163503	+	-0.14	0.11	0.25

chr12	67198030	67198031	-	0.13	-0.12	0.25
chr9	139379125	139379126	+	0.11	-0.15	0.26
chr12	125628536	125628537	-	-0.14	0.12	0.26
chr19	51220083	51220084	-	0.15	-0.11	0.26
chr19	45669588	45669589	+	0.16	-0.10	0.26
chr16	15083470	15083471	-	-0.14	0.12	0.26
chr16	86592606	86592607	-	0.11	-0.16	0.27
chr18	52369320	52369321	-	0.16	-0.11	0.27
chr19	3112852	3112853	+	0.12	-0.15	0.27
chr4	48987802	48987803	-	-0.15	0.12	0.27
chr7	146137071	146137072	+	-0.11	0.16	0.27
chr9	127015821	127015822	+	0.13	-0.14	0.27
chr12	34483695	34483696	+	0.15	-0.13	0.28
chr7	4338562	4338563	-	-0.17	0.11	0.28
chr16	87769491	87769492	-	-0.16	0.12	0.28
chr9	132631702	132631703	-	0.14	-0.14	0.28
chr13	78957734	78957735	-	0.12	-0.16	0.28
chr11	69559545	69559546	+	0.10	-0.18	0.28
chr4	170213983	170213984	+	0.13	-0.16	0.29
chr14	56471843	56471844	+	-0.16	0.13	0.29
chr14	91740445	91740446	+	0.16	-0.13	0.29
chr7	68950148	68950149	-	-0.15	0.14	0.29
chr9	133567800	133567801	-	0.13	-0.16	0.29
chr2	63323386	63323387	+	-0.19	0.11	0.30
chr3	56938979	56938980	-	0.12	-0.18	0.30
chr1	27865546	27865547	+	-0.15	0.15	0.30
chr4	4341354	4341355	+	0.13	-0.17	0.30
chr7	43153974	43153975	-	-0.14	0.16	0.30
chr3	56818508	56818509	+	-0.13	0.17	0.30
chr4	67576944	67576945	+	-0.19	0.12	0.31
chr13	27979107	27979108	+	-0.18	0.13	0.31
chr16	46438923	46438924	-	0.14	-0.17	0.31
chr6	74164575	74164576	+	0.14	-0.17	0.31
chr16	11666473	11666474	-	0.14	-0.17	0.31
chr2	149296612	149296613	+	0.20	-0.12	0.32
chr11	2154156	2154157	+	0.17	-0.15	0.32
chr2	220072674	220072675	+	0.20	-0.12	0.32
chr19	50732263	50732264	-	0.18	-0.14	0.32
chr14	103564277	103564278	+	-0.15	0.17	0.32
chr15	41803454	41803455	+	0.17	-0.15	0.32
chr18	24673384	24673385	-	-0.18	0.15	0.33
chr11	1965447	1965448	-	0.11	-0.22	0.33
chr17	76171144	76171145	+	0.14	-0.19	0.33

chr4	785503	785504	+	-0.17	0.16	0.33
chr3	9988439	9988440	-	0.15	-0.19	0.34
chr19	45608026	45608027	-	0.14	-0.2	0.34
chr15	44068755	44068756	-	0.18	-0.17	0.35
chr13	103424761	103424762	+	-0.25	0.10	0.35
chr5	132166565	132166566	+	0.21	-0.19	0.40
chr14	105633736	105633737	-	0.18	-0.22	0.40

Table 4. Changes in genome-wide methylation at CpG sites from Baseline to One Year in 28 intensive lifestyle modification participants compared to 10 non-intervention controls.

Chromo	Start BP	End BP	Strand	Meth Diff 1YR vs Base Lifestyle	Meth Diff 1YR vs Base Control	Diff of Meth Diff
chr12	2017276	2017277	+	-0.14	-0.14	0
chr15	20487877	20487878	+	-0.12	-0.12	0
chr17	12493310	12493311	-	-0.13	-0.13	0
chr9	133535082	133535083	-	-0.11	-0.11	0
chr18	44285482	44285483	+	-0.11	-0.11	0
chr4	4379916	4379917	+	-0.12	-0.12	0
chr2	130345186	130345187	+	-0.12	-0.12	0
chr17	56350224	56350225	+	0.14	0.14	0
chr20	51218989	51218990	+	-0.18	-0.17	0.01
chr12	53108569	53108570	+	-0.15	-0.14	0.01
chr19	42503269	42503270	-	-0.11	-0.12	0.01
chr5	1706816	1706817	-	-0.11	-0.12	0.01
chr20	34893806	34893807	-	-0.11	-0.10	0.01
chr22	27518706	27518707	-	-0.12	-0.11	0.01
chr4	37044420	37044421	-	-0.11	-0.10	0.01
chr2	105401305	105401306	-	-0.11	-0.10	0.01
chr17	10031557	10031558	-	-0.11	-0.10	0.01
chr2	242788671	242788672	+	0.13	0.12	0.01
chr8	42623717	42623718	+	-0.16	-0.15	0.01
chr1	4015040	4015041	+	0.15	0.13	0.02
chr22	29875591	29875592	+	0.11	0.13	0.02
chr9	139542990	139542991	+	0.11	0.13	0.02
chr16	34659939	34659940	-	0.11	0.13	0.02
chr18	34906080	34906081	+	-0.14	-0.12	0.02

chr22	36960356	36960357	+	0.14	0.12	0.02
chr17	20799270	20799271	-	-0.12	-0.14	0.02
chr19	50659902	50659903	+	-0.15	-0.17	0.02
chr2	49826975	49826976	-	-0.12	-0.14	0.02
chr3	48448489	48448490	+	-0.17	-0.14	0.03
chr11	121107635	121107636	-	-0.13	-0.10	0.03
chr13	52175653	52175654	-	-0.12	-0.15	0.03
chr11	2467032	2467033	+	-0.13	-0.16	0.03
chr15	56242876	56242877	+	0.10	0.13	0.03
chr15	23455141	23455142	-	-0.11	-0.14	0.03
chr1	4697768	4697769	-	0.14	0.11	0.03
chr17	7894043	7894044	-	-0.21	-0.17	0.04
chr16	20303449	20303450	-	-0.15	-0.11	0.04
chr16	50637680	50637681	+	-0.12	-0.16	0.04
chr1	3281166	3281167	+	-0.14	-0.10	0.04
chr15	41113256	41113257	+	-0.13	-0.17	0.04
chr9	133006260	133006261	-	-0.14	-0.10	0.04
chr16	1227474	1227475	+	-0.10	-0.14	0.04
chr8	141229444	141229445	-	-0.17	-0.13	0.04
chr17	15530816	15530817	+	-0.18	-0.13	0.05
chr12	133100332	133100333	+	-0.10	-0.15	0.05
chr3	101406057	101406058	-	-0.13	-0.18	0.05
chr2	232348565	232348566	+	-0.17	-0.12	0.05
chr9	131761978	131761979	+	-0.10	-0.16	0.06
chr2	121279971	121279972	-	-0.11	-0.17	0.06
chr8	142592849	142592850	+	0.17	0.11	0.06
chr19	3548617	3548618	-	0.14	0.21	0.07
chr1	210410305	210410306	+	-0.13	-0.20	0.07
chr20	43945607	43945608	-	-0.21	-0.13	0.08
chr3	195616738	195616739	+	-0.21	-0.29	0.08
chr20	36543997	36543998	-	-0.20	-0.12	0.08
chr9	138065432	138065433	+	0.22	0.12	0.10
chr15	34191962	34191963	-	0.10	-0.11	0.21
chr1	17028048	17028049	+	-0.10	0.11	0.21
chr19	3636639	3636640	+	-0.12	0.10	0.22
chr11	67283712	67283713	-	-0.11	0.11	0.22
chr8	76316466	76316467	-	0.12	-0.10	0.22
chr7	152160229	152160230	-	0.11	-0.11	0.22
chr1	228774166	228774167	+	0.12	-0.11	0.23
chr2	199587847	199587848	+	-0.12	0.11	0.23
chr10	100301187	100301188	-	0.11	-0.12	0.23
chr16	34740679	34740680	+	0.11	-0.12	0.23
chr10	87928783	87928784	+	0.11	-0.12	0.23

chr10	99709447	99709448	-	0.13	-0.10	0.23
chr6	163755600	163755601	-	0.10	-0.13	0.23
chr16	68742006	68742007	-	0.13	-0.11	0.24
chr17	7921167	7921168	-	-0.12	0.12	0.24
chr17	80126207	80126208	+	-0.11	0.13	0.24
chr17	79922011	79922012	-	0.12	-0.12	0.24
chr1	2899789	2899790	+	0.12	-0.12	0.24
chr16	34442282	34442283	+	-0.10	0.15	0.25
chr6	106440967	106440968	-	0.13	-0.12	0.25
chr16	89489021	89489022	-	-0.13	0.12	0.25
chr22	33157193	33157194	-	0.14	-0.11	0.25
chr16	1079220	1079221	-	-0.14	0.11	0.25
chr5	140557227	140557228	-	0.12	-0.13	0.25
chr14	106938274	106938275	+	0.11	-0.15	0.26
chr19	485654	485655	+	0.16	-0.10	0.26
chr6	9025639	9025640	-	-0.13	0.13	0.26
chr22	24890529	24890530	-	-0.14	0.12	0.26
chr1	214406447	214406448	-	0.14	-0.12	0.26
chr17	70379406	70379407	+	0.15	-0.11	0.26
chr9	138439476	138439477	+	0.11	-0.16	0.27
chr2	236044667	236044668	+	0.12	-0.15	0.27
chr19	55428607	55428608	-	0.12	-0.15	0.27
chr1	179675453	179675454	+	-0.11	0.16	0.27
chr14	100771141	100771142	+	0.15	-0.12	0.27
chr17	20799676	20799677	-	0.15	-0.13	0.28
chr8	41521439	41521440	+	-0.12	0.16	0.28
chr1	228112760	228112761	+	0.12	-0.16	0.28
chr2	225862040	225862041	+	-0.15	0.13	0.28
chr16	63464092	63464093	+	0.13	-0.15	0.28
chr12	130648818	130648819	+	0.17	-0.11	0.28
chr9	91150908	91150909	+	-0.16	0.12	0.28
chr22	49412405	49412406	+	0.14	-0.14	0.28
chr17	48545943	48545944	-	0.14	-0.15	0.29
chr17	71541581	71541582	+	0.14	-0.15	0.29
chr12	123450264	123450265	-	0.12	-0.17	0.29
chr16	596481	596482	-	-0.18	0.12	0.30
chr11	128647106	128647107	+	0.14	-0.16	0.30
chr12	28063997	28063998	+	-0.16	0.14	0.30
chr17	7702855	7702856	+	0.14	-0.16	0.30
chr5	131593473	131593474	-	0.17	-0.15	0.32
chr5	177577190	177577191	-	0.17	-0.15	0.32
chr6	39196525	39196526	+	0.17	-0.15	0.32
chr10	134232289	134232290	-	-0.12	0.21	0.33

chr1	11517803	11517804	-	-0.21	0.12	0.33
chr3	46759102	46759103	-	0.14	-0.19	0.33
chr10	120207130	120207131	+	0.19	-0.14	0.33
chr4	174454506	174454507	-	0.24	-0.11	0.35
chr17	76171144	76171145	+	0.21	-0.19	0.40

Table 5. Changes in genome-wide methylation at CpG sites from Baseline to One Year in 28 intensive lifestyle modification participants compared to 28 laparoscopically placed adjustable gastric banding patients.

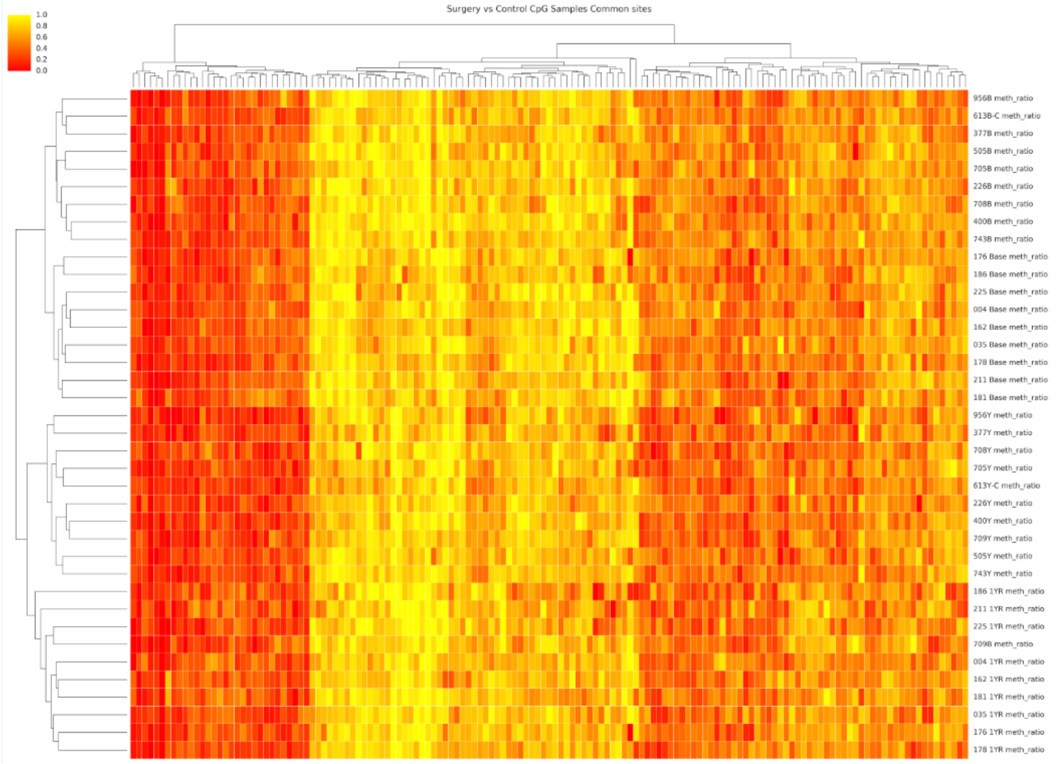
Chromo	Start BP	End BP	Strand	Meth Diff 1YR vs Base Lifestyle	Meth Diff 1YR vs Base Control	Diff of Meth Diff
chr11	70646912	70646913	-	-0.13	-0.13	0
chr20	49252126	49252127	+	-0.15	-0.15	0
chr16	2892558	2892559	-	-0.11	-0.11	0
chr16	790212	790213	-	0.11	0.11	0
chr15	81294575	81294576	-	0.12	0.12	0
chr12	81101900	81101901	+	0.11	0.11	0
chr10	21788828	21788829	-	-0.12	-0.12	0
chr7	1576955	1576956	+	-0.16	-0.16	0
chrX	107681396	107681397	+	0.13	0.13	0
chr1	22155369	22155370	+	0.10	0.10	0
chr2	47268748	47268749	-	0.17	0.18	0.01
chr20	56247617	56247618	-	-0.18	-0.17	0.01
chr10	124909272	124909273	-	-0.11	-0.10	0.01
chr10	37626772	37626773	-	-0.10	-0.11	0.01
chr12	132662669	132662670	+	0.12	0.11	0.01
chr5	132159033	132159034	+	-0.12	-0.11	0.01
chr10	681835	681836	-	-0.19	-0.18	0.01
chr1	201814439	201814440	-	0.13	0.14	0.01
chr6	22435995	22435996	+	-0.12	-0.13	0.01
chr1	37517302	37517303	+	0.12	0.13	0.01
chr1	42196366	42196367	+	-0.13	-0.14	0.01
chr12	54763995	54763996	+	-0.17	-0.16	0.01
chr3	49219282	49219283	-	0.13	0.12	0.01
chr20	57429781	57429782	-	0.12	0.13	0.01
chr19	38877196	38877197	-	0.15	0.13	0.02
chr8	140015721	140015722	-	-0.14	-0.16	0.02
chr20	2704120	2704121	+	-0.15	-0.13	0.02

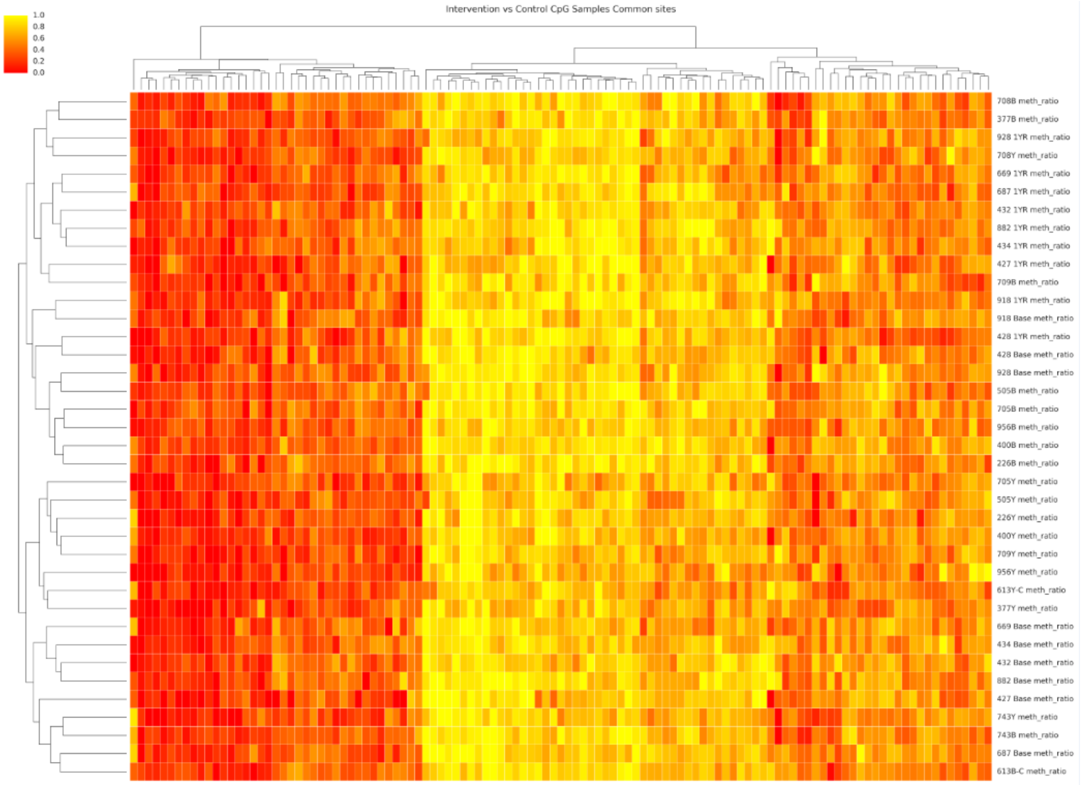
chr1	150194638	150194639	-	0.19	0.21	0.02
chr8	42888651	42888652	-	-0.12	-0.10	0.02
chr14	105451683	105451684	-	0.16	0.18	0.02
chr13	22447169	22447170	-	-0.16	-0.14	0.02
chr18	6742419	6742420	+	-0.13	-0.11	0.02
chr2	90484692	90484693	-	-0.11	-0.13	0.02
chr20	57429792	57429793	-	0.12	0.14	0.02
chr5	14358152	14358153	+	0.20	0.23	0.03
chr14	92651007	92651008	+	0.15	0.18	0.03
chr16	1361829	1361830	-	-0.12	-0.15	0.03
chr3	129295095	129295096	-	-0.15	-0.12	0.03
chr15	45402716	45402717	-	0.16	0.13	0.03
chr19	17393401	17393402	-	0.11	0.14	0.03
chr12	111650974	111650975	+	0.18	0.14	0.04
chr3	50314172	50314173	+	0.11	0.15	0.04
chr11	46381418	46381419	+	-0.15	-0.11	0.04
chr8	144403358	144403359	-	0.10	0.14	0.04
chr1	211688041	211688042	-	-0.15	-0.19	0.04
chr12	5020978	5020979	+	0.12	0.16	0.04
chr7	188970	188971	-	0.10	0.14	0.04
chr12	53746265	53746266	+	0.20	0.16	0.04
chr20	22567723	22567724	+	0.13	0.18	0.05
chr17	7246933	7246934	-	0.10	0.15	0.05
chr3	183987357	183987358	+	0.11	0.16	0.05
chr20	47131884	47131885	+	-0.17	-0.12	0.05
chr13	48415467	48415468	-	0.16	0.10	0.06
chr14	104645707	104645708	+	-0.14	-0.20	0.06
chr5	135266866	135266867	+	-0.19	-0.13	0.06
chr7	138847855	138847856	+	-0.23	-0.17	0.06
chr3	15677781	15677782	+	-0.12	-0.18	0.06
chr1	21901191	21901192	+	-0.12	-0.18	0.06
chr4	49053812	49053813	+	-0.18	-0.12	0.06
chr1	229283100	229283101	-	0.11	0.17	0.06
chr17	76171144	76171145	+	0.21	0.14	0.07
chr16	57563447	57563448	-	-0.15	-0.22	0.07
chr17	73010381	73010382	-	0.20	0.13	0.07
chr19	18016303	18016304	-	-0.15	-0.22	0.07
chr17	45607982	45607983	-	-0.11	-0.19	0.08
chr11	75139523	75139524	-	-0.12	-0.20	0.08
chr2	1800756	1800757	+	-0.23	-0.14	0.09
chr7	155592256	155592257	-	0.10	0.19	0.09
chr4	182985627	182985628	+	0.13	0.23	0.10
chr17	78169988	78169989	-	-0.22	-0.12	0.10

chr14	71288197	71288198	+	0.12	0.23	0.11
chr11	134347520	134347521	+	-0.13	-0.26	0.13
chr2	233387878	233387879	+	0.15	0.28	0.13
chr10	100301187	100301188	-	0.11	-0.10	0.21
chr6	26571058	26571059	-	0.10	-0.11	0.21
chr16	86794240	86794241	-	-0.11	0.10	0.21
chr12	117500505	117500506	-	-0.10	0.12	0.22
chr8	99985283	99985284	-	0.12	-0.10	0.22
chr10	2544080	2544081	-	0.11	-0.11	0.22
chr10	111003219	111003220	-	0.12	-0.11	0.23
chr10	82167473	82167474	+	0.11	-0.12	0.23
chr20	29611809	29611810	+	0.12	-0.11	0.23
chr19	38909085	38909086	+	-0.12	0.12	0.24
chr19	35389122	35389123	+	0.11	-0.13	0.24
chr16	31005528	31005529	+	0.12	-0.12	0.24
chr17	40823473	40823474	+	-0.12	0.12	0.24
chr11	126162696	126162697	+	-0.13	0.11	0.24
chr7	1714085	1714086	-	0.11	-0.13	0.24
chr3	11331390	11331391	-	0.11	-0.13	0.24
chr12	104697948	104697949	+	0.11	-0.14	0.25
chr12	52258618	52258619	+	0.13	-0.12	0.25
chr3	73683837	73683838	+	-0.12	0.13	0.25
chr11	2801229	2801230	-	-0.14	0.12	0.26
chr11	109760460	109760461	-	-0.14	0.12	0.26
chr19	35389134	35389135	+	0.12	-0.14	0.26
chr22	46262549	46262550	+	0.11	-0.15	0.26
chr4	96695163	96695164	-	-0.11	0.15	0.26
chr5	145711411	145711412	-	-0.15	0.12	0.27
chr19	57440032	57440033	-	-0.13	0.15	0.28
chr17	7643951	7643952	-	0.15	-0.13	0.28
chr10	134019555	134019556	+	-0.16	0.12	0.28
chr1	179675453	179675454	+	-0.11	0.17	0.28
chr22	41846368	41846369	+	-0.15	0.13	0.28
chr14	21191419	21191420	+	-0.11	0.18	0.29
chr7	30202902	30202903	+	-0.17	0.12	0.29
chr1	6368382	6368383	+	-0.12	0.17	0.29
chr11	35966346	35966347	-	0.17	-0.12	0.29
chr3	193587436	193587437	+	0.12	-0.17	0.29
chr11	69552826	69552827	+	-0.14	0.15	0.29
chr16	85560853	85560854	+	-0.17	0.12	0.29
chr11	74035019	74035020	+	0.16	-0.13	0.29
chr10	135259568	135259569	-	0.16	-0.13	0.29
chr6	47638175	47638176	-	0.17	-0.12	0.29

chr5	176154060	176154061	-	0.15	-0.15	0.30
chr16	5092855	5092856	+	0.18	-0.12	0.30
chr19	48768956	48768957	+	0.15	-0.15	0.30
chr1	59090259	59090260	+	-0.16	0.15	0.31
chr10	94531260	94531261	+	0.17	-0.14	0.31
chr21	46712204	46712205	-	0.19	-0.13	0.32
chr17	4835640	4835641	-	0.14	-0.18	0.32
chr19	18543887	18543888	-	0.16	-0.17	0.33
chr10	132203214	132203215	+	0.22	-0.12	0.34
chr11	3804077	3804078	-	0.16	-0.18	0.34
chr17	7255801	7255802	-	0.17	-0.18	0.35
chr8	11323534	11323535	-	0.21	-0.15	0.36
chr2	162284555	162284556	-	0.16	-0.20	0.36
chr11	62618703	62618704	-	-0.21	0.18	0.39
chr12	111982854	111982855	+	0.22	-0.17	0.39
chr22	37858963	37858964	-	0.29	-0.16	0.45

Heat maps showing the clustering of sample time points and specific sites were generated for the three comparisons (Figure 1).




Intervention vs Control CpG Samples Common sites
1.0
0.8
0.6
0.4
0.2
0.0
708B meth_ratio
377B meth_ratio
928 1YR meth_ratio
708Y meth_ratio
669 1YR meth_ratio
687 1YR meth_ratio
432 1YR meth_ratio
882 1YR meth_ratio
434 1YR meth_ratio
427 1YR meth_ratio
709B meth_ratio
918 1YR meth_ratio
918 Base meth_ratio
428 1YR meth_ratio
428 Base meth_ratio
928 Base meth_ratio
505B meth_ratio
705B meth_ratio
956B meth_ratio
400B meth_ratio
226B meth_ratio
705Y meth_ratio
505Y meth_ratio
226Y meth_ratio
400Y meth_ratio
709Y meth_ratio
956Y meth_ratio
613Y-C meth_ratio
377Y meth_ratio
669 Base meth_ratio
434 Base meth_ratio
432 Base meth_ratio
882 Base meth_ratio
427 Base meth_ratio
743Y meth_ratio
743B meth_ratio
687 Base meth_ratio
613B-C meth_ratio

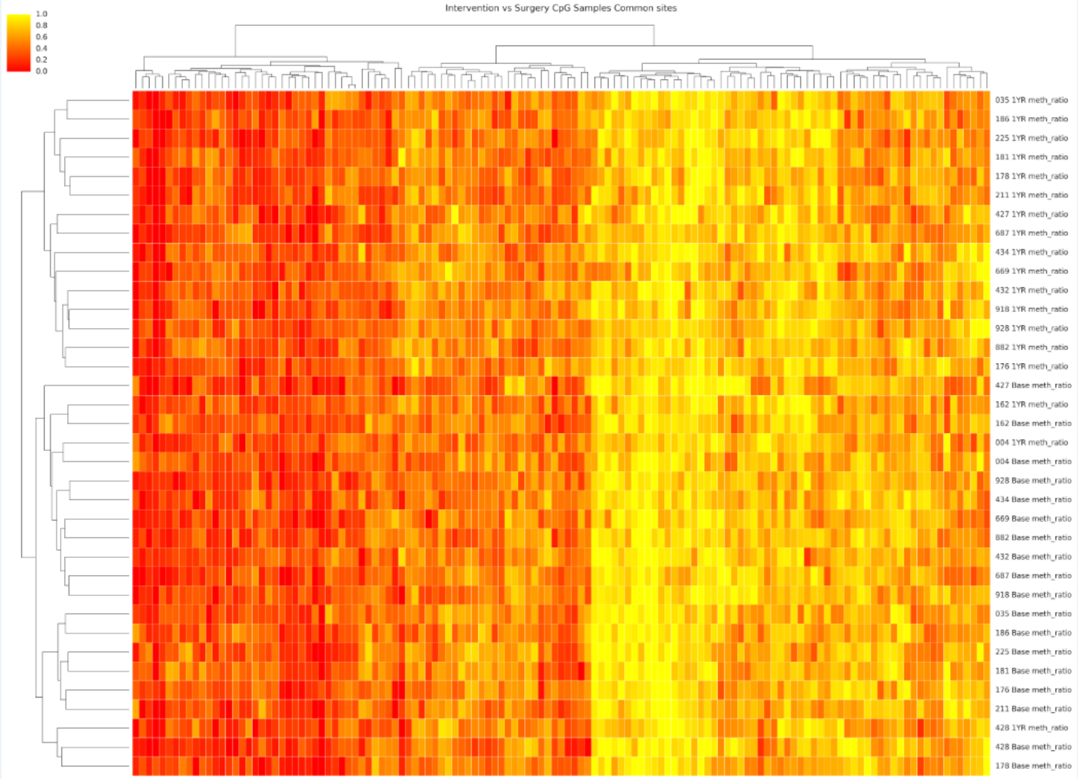

Intervention vs Surgery CpG Samples Common sites
1.0
0.8
0.6
0.4
0.2
0.0
035 1YR meth_ratio
186 1YR meth_ratio
225 1YR meth_ratio
181 1YR meth_ratio
178 1YR meth_ratio
211 1YR meth_ratio
427 1YR meth_ratio
687 1YR meth_ratio
434 1YR meth_ratio
669 1YR meth_ratio
432 1YR meth_ratio
918 1YR meth_ratio
928 1YR meth_ratio
882 1YR meth_ratio
176 1YR meth_ratio
427 Base meth_ratio
162 1YR meth_ratio
162 Base meth_ratio
004 1YR meth_ratio
004 Base meth_ratio
928 Base meth_ratio
434 Base meth_ratio
669 Base meth_ratio
882 Base meth_ratio
432 Base meth_ratio
687 Base meth_ratio
918 Base meth_ratio
035 Base meth_ratio
186 Base meth_ratio
225 Base meth_ratio
181 Base meth_ratio
176 Base meth_ratio
211 Base meth_ratio
428 1YR meth_ratio
428 Base meth_ratio
178 Base meth_ratio

Figure 1. Heat maps showing clustering of patient samples for the following comparisons: (top) LAGB patients vs controls; (middle) lifestyle participants vs controls; (bottom) LAGB patients vs lifestyle participants.

Task #2: Profile metabolic activity in blood and adipose tissue during surgical weight loss

During the project, 280 patients undergoing laparoscopically placed adjustable gastric banding (LAGB) were enrolled in the study. There were 38 dropouts and 242 patients remained active in the study. A summary of time points for which gene expression assays were conducted using RNA from peripheral blood is as follows: baseline pre-surgery (n=117), 5-7 months post-surgery (n=70), ~1 year post-surgery (n=54), ~1.5 years (n=30), ~2 years (n=15), ~2.5 years (n=9), ~3 years (n=5), ~3.5 years (n=13), ~4 years (n=12), ~4.5 years (n=17), ~5 years (n=17), ~5.5 years (n=12), and ~6 years post-surgery (n=10).

Total RNA was isolated from 462 PAXgene peripheral blood samples from 162 patients (Table 6). RNA concentrations were 75.20±43.66 ng/µl (range 0-233.47 ng/µl), OD260/280 ratios were 2.20±0.23 (range 0-4.17), and RIN numbers were 8.20±0.51 (range 6.50-9.30). RNA samples (n=424) were run on Affymetrix gene expression arrays with call rates of 58.99±1.66% (range 51.77-62.47%).

Table 6. Concentrations, purity measures, and call rates on gene expression arrays for 462 RNA samples isolated from whole blood from 162 laparoscopically placed adjustable gastric banding patients.

Sample	Time Point	Concentration (ng/µl)	OD260/280	RIN	Call Rate (%)
131	2 yr 8m	51.70	1.93	7.6	58.20
94	3y1m16d	37.02	2.37	8.3	58.98
14	4yr 6m	126.53	2.23	8.2	58.48
129	2yr	84.91	2.24	8.2	59.89
3	1 yr	47.43	2.03	8.3	60.91
4	1 yr	66.89	2.26	9.0	60.66
79	3yr 5m	122.08	2.20	7.6	58.89
13	4yr 7m	42.20	2.46	8.5	55.56
79	3yr 6m	34.21	2.25	8.3	58.46
107	2yr 5m	27.70	2.16	8.5	58.69
32	1yr	115.04	2.14	8.3	60.66
104	4yr 5m	78.60	2.21	8.3	58.04
73	4yr	127.87	2.22	7.8	57.71

4	18m	85.53	2.22	8.7	60.13
5	5yr 3m	79.62	2.31	7.7	56.42
8	5yr 2m	109.53	2.17	8.1	57.07
62	4yr	128.04	2.22	7.8	56.20
102	3yr 4m5d	122.00	2.30	8.7	56.56
35	1yr	86.30	2.19	8.2	59.93
44	1yr	102.97	2.19	8.8	59.06
46	1yr	115.91	2.17	8.0	54.93
31	1yr	23.18	2.04	---	61.02
77	4yr 27d	134.59	2.20	8.6	54.93
3	6m	39.76	2.26	8.3	58.54
3	2yr	67.36	2.22	8.5	56.62
81	3y 8m	28.78	2.06	8.0	59.16
30	1yr	39.74	2.21	8.9	59.83
97	3yr 6m6d	73.10	2.33	8.4	57.81
18	2yr	37.22	2.40	8.3	---
15	4yr 9m	140.97	2.05	7.6	57.63
47	4yr6m	93.90	2.23	8.2	58.45
5	6m	61.35	2.23	9.2	59.39
105	3yr3m26d	96.15	2.22	8.5	58.33
1	baseline	45.23	2.27	8.7	60.62
3	baseline	70.01	2.14	8.9	58.09
7	6m	54.07	2.26	8.5	58.73
13	5yr1m18d	42.00	2.05	8.3	57.79
3	5yr9m3d	72.07	2.17	7.4	56.56
4	baseline	74.24	2.24	8.6	57.72
21	4yr9m	122.19	2.21	7.5	57.96
137	1yr1m5d	8.63	3.96	---	
6	6m	14.76	2.92	8.5	
93	baseline	116.97	2.22	8.2	60.07
114	3yr	51.94	2.20	8.2	58.32
106	3yr3m18d	63.20	2.31	8.5	58.67
119	3yr1m18d	83.00	2.21	8.2	57.74
31	4yr8m	51.84	2.37	8.6	60.42
79	4yr26d	191.56	2.22	7.9	59.61
21	3m	30.76	2.49	8.8	59.41
14	6m	31.85	2.29	8.9	59.13
127	2yr	12.47	1.93	---	
58	4y4m28d	109.70	2.20	8.2	57.84
47	1yr	81.44	2.23	8.4	58.14
3	18m	22.02	2.85	8.6	57.89
84	3yr10m23d	128.31	2.17	7.1	57.05
5	baseline	91.31	2.22	8.3	59.05

9	18m	114.05	2.20	8.4	58.30
115	2y6m	187.73	2.19	7.6	56.57
49	4yr6m16d	105.10	2.21	8.3	57.19
119	2yr	100.66	2.14	8.2	59.83
55	1yr	136.54	2.17	8.2	58.03
112	2y6m	233.47	2.17	8.1	57.86
122	2yr14m24d	6.15	4.17	---	
14	18m	134.28	2.19	8.9	59.81
8	6m	51.43	2.36	9.1	59.84
4	5yr4m	85.04	2.28	7.7	56.62
50	4yr7m29d	38.35	2.33	8.7	58.83
24	18m	93.49	2.28	8.9	58.22
95	3yr9m24d	47.52	2.25	7.7	56.11
17	18m	145.04	2.20	8.8	60.00
121	2y7m29d	169.07	2.16	7.7	57.48
67	1yr	82.91	2.31	9.0	60.91
131	2yr4m29d	92.31	2.24	7.5	55.05
34	5yr3d	109.32	2.28	7.2	55.40
76	4yr5m27d	54.31	2.42	8.4	58.27
115	3yr3m4d	71.30	2.27	7.9	57.75
44	18m	38.50	2.24	8.5	55.05
6	baseline	57.00	2.18	9.0	58.19
129	2yr5m23d	42.04	2.08	8.8	60.21
142	1yr29d	114.67	2.04	8.4	59.69
7	baseline	38.39	2.31	9.0	58.68
110	3yr4m15d	107.96	2.24	8.2	59.20
128	2yr8m3d	86.99	2.21	8.7	59.00
94	3yr11m21d	40.95	2.33	8.7	58.87
73	18m	64.15	2.38	8.0	56.23
121	2yr11m27d	80.96	2.25	7.4	57.15
15	6m	43.67	2.29	9.1	58.83
14	4y11m21d	79.90	2.23	8.7	59.35
62	1y	71.33	2.23	8.6	58.49
148	1y22d	86.77	2.18	7.9	56.80
136	1y1m24d	61.48	2.18	8.5	57.19
170	4m26d	153.12	2.21	8.5	58.39
72	4y4m15d	76.74	2.00	7.9	54.53
158	1y22d	53.19	2.17	8.0	60.43
36	4y9m24d	87.29	2.09	7.7	56.97
41	4y9m9d	103.99	2.27	7.2	56.93
141	1y4m11d	159.95	2.18	8.7	56.39
8	baseline	33.97	2.66	8.8	59.39
35	5y17d	52.14	2.24	8.5	58.39

102	3y10m9d	137.29	2.27	8.2	58.76
157	1y1m3d	166.94	2.19	8.1	56.26
126	2y10m4d	71.07	2.20	9.1	60.20
110	2y	38.70	2.30	8.5	56.79
129	2y11m14d	147.30	2.11	8.5	59.99
73	4y10m28d	135.50	2.10	8.4	57.89
134	1y	74.97	2.20	8.5	58.92
13	baseline	87.45	2.21	8.2	57.37
104	3y1m2d	101.50	2.16	8.6	59.50
14	baseline	52.30	2.14	8.2	58.96
15	baseline	77.69	2.26	8.9	59.45
138	1y5m29d	13.16	2.24	7.9	
62	4y6m25d	37.63	2.15	8.7	56.16
18	6m	34.02	2.35	9.1	60.64
137	1y6m19d	107.70	2.23	8.4	57.01
27	6m	63.95	2.15	9.0	59.27
91	4y1m2d	128.01	2.22	7.9	58.04
163	1y14d	56.57	2.15	7.4	57.10
16	baseline	43.39	2.24	9.2	60.47
17	baseline	66.86	2.36	8.6	54.46
47	4y11m13d	108.36	2.25	8.3	58.21
28	6m	49.99	2.24	8.4	60.53
49	9m	69.75	2.35	8.8	59.60
114	3y6m12d	86.78	2.17	8.9	60.20
105	3y9m25d	130.80	2.15	8.8	58.85
18	baseline	54.95	2.00	8.6	59.13
23	6m	17.83	2.64	8.7	59.11
44	6m	69.26	2.22	8.6	57.84
142	8m25d	68.27	2.28	7.9	57.61
148	7m	151.94	2.07	7.7	51.77
21	baseline	27.21	2.21	8.1	60.54
136	1y7m16d	56.26	2.33	7.9	60.23
21	5y2m19d	132.96	2.16	8.8	56.91
156	1y2m21d	20.00	1.68	8.3	59.27
15	5y3m16d	73.08	2.27	8.6	57.86
23	baseline	54.21	1.99	8.4	58.24
140	11m	83.44	2.23	8.3	58.01
49	4y11m19d	135.71	2.22	7.7	58.83
77	4y7m2d	224.66	2.21	7.6	58.62
150	1y3m28d	75.90	2.14	8.2	54.44
1	6y2m10d	53.33	2.10	8.2	59.46
24	baseline	136.67	2.22	8.8	56.65
172	8m5d	118.62	2.20	7.8	55.93

47	6m	79.08	2.27	8.9	58.98
138	11m22d	44.51	2.35	8.5	56.96
73	4y4m29d	107.36	2.16	7.5	57.05
28	baseline	94.84	2.22	8.3	57.54
4	5y11m29d	90.20	2.07	8.4	57.54
67	6m	34.88	2.38	8.7	61.32
44	5y1d	75.03	2.29	8.4	57.76
30	baseline	92.52	2.21	8.8	57.64
162	1y	52.86	2.34	8.0	58.17
5	5y8m26d	47.88	2.38	8.6	59.85
31	5y4m20d	0.60	3.23	---	
178	5m26d	30.96	2.33	9.3	60.66
18	5yr3m16d	61.70	2.31	8.8	58.29
218	baseline	113.70	2.20	7.8	54.97
165	1y	174.40	2.24	7.4	57.35
199	6m7d	45.84	2.04	8.9	61.38
160	1yr2m8d	13.12	2.25	8.6	
31	baseline	179.24	2.16	8.5	57.93
146	1y5m1d	12.10	1.39	---	
32	baseline	170.91	2.20	8.6	57.49
43	6m	128.20	2.22	8.9	58.25
157	7m	35.17	2.68	8.7	60.22
33	baseline	126.40	2.18	8.7	59.52
162	6m	42.75	2.14	7.5	60.91
34	baseline	131.06	2.27	9.0	56.98
49	6m	57.60	2.02	8.6	59.22
146	9m29d	58.96	2.14	7.5	61.89
35	baseline	60.27	2.12	8.7	60.51
36	baseline	63.96	2.33	9.0	59.91
45	6m	104.11	2.20	8.7	59.64
55	6m	79.37	2.23	9.0	59.37
143	10m	117.69	2.28	7.9	57.85
44	baseline	98.39	2.25	8.5	55.42
41	baseline	14.91	2.56	---	
43	baseline	153.52	2.25	8.6	58.20
46	baseline	34.36	2.28	9.1	60.00
45	baseline	51.36	2.31	8.7	58.72
75	baseline	68.45	2.24	8.0	57.75
64	6m	72.00	2.28	8.8	59.52
69	baseline	40.48	2.30	9.2	60.73
73	baseline	119.52	2.17	8.7	56.83
49	baseline	112.33	2.26	8.8	58.94
63	baseline	24.90	2.53	8.6	59.28

70	baseline	176.07	2.17	8.8	59.40
48	baseline	11.91	2.59	8.9	
56	baseline	105.81	2.27	9.0	58.89
62	baseline	72.62	2.21	7.8	56.16
81	baseline	75.63	2.30	7.8	57.58
89	baseline	202.06	2.20	8.1	58.48
94	baseline	39.50	2.16	---	58.85
42	baseline	207.70	2.17	8.9	58.81
53	baseline	57.60	2.21	8.2	60.47
58	baseline	76.42	2.20	8.8	60.28
79	baseline	28.50	2.33	8.0	57.88
110	3yr10m27d	55.04	2.29	8.4	59.39
148	1yr6m24d	57.30	2.27	8.6	60.14
138	2yr	35.77	2.45	7.9	61.32
174	11m4d	93.00	2.13	8.1	58.53
13	5yr8m3d	60.50	2.12	7.5	58.55
153	1yr6m5d	52.08	2.31	8.5	59.84
154	1yr6m5d	69.10	2.16	8.8	60.79
14	5yr8m1d	53.30	2.34	8.5	59.08
79	4yr7m13d	193.01	2.18	6.8	57.24
63	6m	105.48	2.22	8.5	60.27
62	6m	57.40	2.15	8.6	57.30
121	3yr7m15d	60.65	2.27	7.8	59.07
141	2yr	111.22	2.27	7.2	57.96
135	1yr10m11d	54.50	2.25	8.2	59.11
167	1yr3m8d	72.83	2.27	8.4	60.06
176	1yr6d	46.32	2.33	8.1	60.30
94	6m	63.66	2.17	7.4	58.81
35	5yr7m11d	54.68	2.34	7.7	60.17
70	6m	218.54	2.16	8.2	60.17
96	6m	52.76	2.26	7.2	58.56
98	4yr6m	54.68	2.31	7.1	58.15
121	baseline	113.46	2.28	6.5	56.27
90	6m	127.80	2.21	7.2	58.75
128	3y2m20d	106.20	2.22	8.0	58.54
178	1yr2d	137.55	2.25	6.7	58.79
47	baseline	51.90	2.17	8.4	59.94
34	5yr6m29d	108.22	2.31	7.3	58.19
50	baseline	112.91	2.10	8.1	59.71
181	1yr	67.02	2.31	7.7	60.61
41	5y6m	77.75	2.26	6.9	57.74
179	11m9d	61.85	2.40	7.3	58.98
177	1y	44.37	2.39	7.3	59.44

114	6m	37.94	2.34	7.1	59.63
92	6m	72.83	2.28	7.2	61.12
123	baseline	71.42	2.22	7.6	60.08
93	6m	28.72	2.40	7.9	61.13
186	1y	46.70	2.13	8.8	60.45
155	6m	77.19	2.23	8.3	60.57
177	baseline	19.30	2.41	7.5	60.72
153	6m	49.50	2.14	8.5	58.48
76	baseline	58.99	2.16	8.5	58.67
165	1y6m	92.00	2.21	8.2	55.08
18	6yr	3.80	2.58	---	
198	1yr	87.73	2.14	7.8	60.58
137	2y	20.60	2.30	7.9	57.73
196	7m5d	145.65	2.17	---	56.61
195	9m3d	174.00	2.14	7.9	61.61
77	baseline	176.10	2.13	8.2	58.63
84	baseline	49.64	2.08	7.4	59.09
174	6m10d	107.36	2.18	8.2	59.31
204	7m6d	158.56	2.12	8.7	59.07
154	6m	38.90	2.14	8.9	58.61
137	6m	27.50	2.04	8.5	57.87
166	1Yr	67.20	2.12	8.7	58.61
150	6M	42.10	2.36	8.9	57.48
138	6M	10.30	2.25	---	
156	base	28.30	2.14	8.7	---
146	base	64.00	2.25	8.4	57.88
129	base	119.20	2.18	8.7	58.29
153	10m	69.50	2.31	8.2	56.55
114	base	83.10	2.26	7.9	55.64
174	base	72.40	2.35	7.9	59.53
22	base	28.00	2.23	8.2	drop out
37	base	122.00	2.12	8.8	drop out
103	3YR 6Mon	58.20	2.14	8.1	drop out
117	3Yr 1Mon	44.90	2.15	8.3	drop out
144	7m	2.70	1.60	---	drop out
161	8m	14.00	2.14	8.0	drop out
164	6m	55.20	2.10	8.6	drop out
168	base	67.60	2.17	8.3	drop out
200	6m	88.90	2.12	8.7	drop out
201	base	136.40	2.17	7.3	drop out
203	base	108.50	2.15	7.8	drop out
209	base	66.10	2.21	7.7	drop out
110	base	194.50	2.16	7.2	53.45

154	base	30.40	2.22	8.2	59.02
157	1Y 10m	65.00	2.20	8.3	60.21
165	6m	76.30	2.23	6.7	55.68
174	1Y 5m	121.30	2.16	8.3	59.92
105	base	67.00	2.16	7.8	59.19
131	3y 3m	18.30	2.22	8.5	
21	5YR 11m	49.40	2.03	8.7	59.84
105	4yr 5mon	55.40	2.03	8.5	60.24
135	6m	89.20	2.09	8.0	57.54
150	base	151.50	2.10	7.1	55.3
73	5yr 4m	40.00	2.22	---	60.47
154	10m	88.70	2.21	8.2	57.63
176	base	75.00	2.19	7.9	56.94
104	3YR 8Mon	43.60	2.16	8.4	60.28
138	base	9.00	2.64	8.5	
148	2YR	35.50	2.23	7.9	57.94
177	6Mon	40.30	2.14	8.6	59.56
62	5YR 5m	18.20	2.14	8.2	59.08
102	base	77.40	2.29	8.8	57.63
114	4YR 3m	89.60	2.19	8.5	61.21
142	2YR	60.60	2.18	8.7	59.43
176	1YR 6m	53.90	2.24	8.4	59.2
185	base	56.10	2.23	8.0	57.42
104	base	43.70	2.13	8.6	58.59
146	1YR 11Mon	64.60	2.12	8.6	57.61
166	base	62.70	2.04	7.7	56.27
153	2YRs	57.90	2.14	9.0	59.78
166	6M	85.50	2.14	8.0	57.05
137	base	12.20	2.20	8.2	
142	base	41.70	2.23	8.0	61.96
156	6Mon	117.30	2.17	8.3	58.92
150	2YRs	82.00	2.16	7.8	58.96
67	base	35.80	2.26	8.7	61.22
148	base	34.00	2.24	8.4	60.82
165	base	4.50	2.25		
178	base	123.70	2.17	7.5	58.33
136	base	39.90	2.16	7.9	58.98
157	base	56.00	2.15	7.8	55.62
162	base	67.60	2.16	8.1	61.54
163	base	55.50	2.24	8.1	60.37
179	base	72.90	2.21	7.6	59.70
194	1YR	60.40	2.11	8.7	58.78
131	base	9.30	2.60		

174	2YRs	63.70	2.23	8.0	59.79
185	1YR	57.40	2.26	8.1	61.08
186	base	81.70	2.17	7.8	58.24
194	base	83.40	2.22	7.4	58.32
156	1YR 10Mon	94.09	2.12	8.4	59.73
193	1YR 2Mon	42.76	2.02	8.0	60.82
135	base	23.62	2.09	9.1	59.81
154	2YRs	118.67	2.14	8.9	60.57
153	base	4.42	1.31	---	
115	base	35.07	2.12	8.6	59.82
128	base	72.31	2.04	8.1	58.05
107	1YR	10.26	1.49	---	
169	2Yrs	163.29	2.15	7.5	58.53
182	base	163.04	2.12	7.2	55.07
184	11m	5.69	1.21	---	
137	3YRs	69.64	2.17	8.7	61.61
193	base	94.23	2.13	7.6	58.45
202	base	0.00	0.00	---	
216	1YR 1M	32.02	1.99	8.4	61.47
232	base	74.42	2.14	8.1	58.55
102	5YRs	47.60	2.13	8.7	61.35
173	Base	49.00	2.23	8.0	60.81
195	1YR	50.30	2.15	8.4	61.66
196	Base	40.40	2.13	7.9	60.21
232	1YR	43.40	2.13	8.6	61.11
102	6M	130.75	2.11	7.5	58.66
119	base	82.94	2.17	7.9	59.42
134	base	21.10	2.12	8.5	62.47
179	6m	60.42	2.13	7.7	59.79
182	1yr 4mon	139.77	2.17	8.3	62.45
204	base	178.69	2.11	6.9	60.98
210	base	95.85	2.17	6.9	58.21
225	6m	75.62	2.17	8.4	61.94
227	1yr	85.45	2.10	8.3	62.32
199	base	40.63	2.12	7.3	58.77
211	base	36.00	2.16	7.9	59.73
215	base	153.75	2.13	7.9	61.34
140	base	48.01	2.12	7.9	59.11
169	base	96.07	2.17	7.3	57.69
176	6m	96.83	2.18	8.1	60.71
181	base	176.02	2.17	7.4	58.95
218	1YR 1M	49.66	2.16	7.9	61.20
90	base	41.49	2.21	8.6	61.32

106	base	48.00	2.15	8.5	60.35
114	5YRs	65.44	2.16	8.5	59.64
178	2YRs	97.35	2.23	8.3	61.70
185	6m	61.23	2.19	8.6	60.74
193	6m	66.03	2.21	8.2	58.40
197	1YR 3m	68.56	2.27	8.6	59.64
208	base	38.00	2.18	8.5	59.73
222	base	56.23	2.16	7.5	58.06
198	base	57.70	2.26	7.5	58.46
204	1YR	58.22	2.24	8.0	61.80
211	5Mon	51.56	2.23	8.5	61.71
214	base	62.19	2.22	8.3	59.98
228	base	61.44	2.23	7.7	60.77
107	base	36.34	2.24	7.7	60.78
154	2YR 6M	35.03	2.07	7.7	61.29
155	base	77.51	2.17	7.5	59.85
166	1YR 6M	62.12	2.19	7.9	58.43
175	base	57.10	2.11	8.1	60.57
177	1YR 6M	86.19	2.10	7.4	60.06
192	base	104.49	2.16	7.9	59.81
193	1YR 6M	83.65	2.13	8.4	59.56
218	6M	44.07	2.30	7.8	61.65
229	base	40.06	2.23	8.1	61.73
135	2Yr 6M	28.28	2.13	7.8	58.75
172	base	33.72	2.23	8.1	60.42
181	6m	138.61	2.15	7.8	59.48
199	1Yr	43.22	2.20	8.1	58.49
236	base	194.96	2.15	7.9	58.85
95	base	40.59	2.22	8.2	59.95
173	6mon	43.49	2.26	8.4	60.76
186	6mon	54.35	2.20	8.3	60.89
197	base	67.26	2.22	7.3	58.88
210	1YR	51.32	2.40	8.2	61.38
216	base	62.32	2.22	7.5	59.62
158	base	81.37	2.20	8.4	59.27
192	1YR	40.33	2.24	9.1	61.74
194	6M	104.74	2.17	8.2	57.71
208	1YR 6Mon	77.65	2.18	8.6	61.80
216	6M	79.05	2.19	8.6	61.80
225	base	72.82	2.14	7.8	58.57
97	base	33.91	2.06	7.7	57.54
173	1YR 6Mon	76.49	2.16	8.4	61.38
182	6Mon	56.59	2.10	8.0	59.88

195	base	81.92	2.12	8.0	60.09
222	6mon	37.33	2.09	7.8	61.59
244	base	66.81	2.13	7.8	59.74
163	6Mon	37.41	2.29	7.9	60.50
167	Base	64.83	2.25	6.8	55.41
192	6Mon	40.58	2.53	7.7	60.25
198	1YR 6mon	85.09	2.31	7.7	60.51
212	base	176.70	2.23	7.4	58.63
227	base	74.35	2.33	7.9	59.28
232	6Mon	111.35	2.28	8.0	59.60
238	base	0.00			
91	base	113.52	2.20	8.4	60.09
115	4YR 6m	25.39	2.19	8.0	60.48
166	2yr	37.08	2.10	8.3	61.35
197	7mon	112.88	2.19	8.2	61.50
211	1yr	12.42	2.16	8.7	61.65
225	1yr	21.95	2.48	8.1	61.54
77	5YR 2mon	42.45	2.34	8.1	60.53
112	base	106.94	2.15	7.6	58.23
175	6m	143.33	2.20	8.4	60.00
181	1YR 6m	50.91	2.22	7.9	60.23
210	5Mon	111.05	2.19	7.7	60.04
253	base	81.66	2.22	8.2	58.16
31	5YR 10m	22.97	1.64	8.7	61.35
49	5YR 8m	9.99	1.87	7.7	
53	5YR 6m	172.84	2.14	8.0	57.65
98	base	3.80	0.88	6.7	
157	2YR 4m	175.34	2.17	8.7	59.39
175	1YR 6m	144.48	2.15	9.1	59.82
212	1YR	89.43	2.11	8.2	60.52
229	6m	23.92	2.00	8.3	59.03
244	1YR	29.95	2.07	8.5	60.63
73	5YR 10m	44.15	2.17	8.2	59.10
167	7Mon	70.29	2.13	8.1	60.40
169	1YR 8m	65.40	2.10	8.3	59.40
210	1YR 5m	76.97	2.11	7.8	61.14
212	5Mon	114.51	2.13	8.0	60.07
214	6Mon	63.73	2.16	8.4	60.71
215	6Mon	181.52	2.14	7.8	58.40
222	1YR 4mon	80.87	2.15	8.4	59.61
228	6Mon	41.57	2.19	---	61.20
229	1YR	129.28	2.13	8.3	60.43
236	1YR 1m	58.69	2.09	8.3	57.82

244	6Mon	71.47	2.14	7.7	59.21
5	6YR	76.35	2.11	7.8	55.83
50	5YR 8Mon	69.06	2.11	8.8	59.97
53	6YR	63.09	2.10	8.2	59.59
94	5YR	43.44	2.09	8.5	60.03
126	base	94.63	2.11	7.9	60.07
214	1YR 3Mon	60.66	2.12	8.7	59.53
227	7Mon	64.34	2.17	8.4	59.16
228	1YR	35.40	2.07	8.5	59.73
236	6Mon	68.59	2.11	7.8	57.93
253	1YR	65.01	2.11	8.5	59.45
258	Base	66.59	2.10	8.3	57.75
50	6YR	73.74	2.10	8.7	59.62
126	3YR 6Mon	29.55	2.25	8.6	58.87
258	1YR	67.80	2.22	8.6	60.82
49	6YRs	143.10	2.19	7.6	58.97
84	5YRs	68.90	2.12	7.8	59.03
185	1YR 6Mon	62.80	2.17	8.1	60.69
192	1YR 6Mon	93.90	2.15	8.3	59.67

Analysis of Gene Expression

Several quality control assessments were performed on the gene expression array results from LAGB patients in order to determine their quality and suitability for subsequent data analysis. Gene expression arrays from patients with no follow-up time points and patients who officially dropped out of the study were excluded from the QC analysis. Results of the QC analysis found that 377 arrays were suitable for subsequent analysis, while only 4 arrays (05-03-003 2YR, 05-03-046 1YR, 05-03-077 4YR, 05-03-110 Base) did not pass the comprehensive QC assessment (Figure 2).

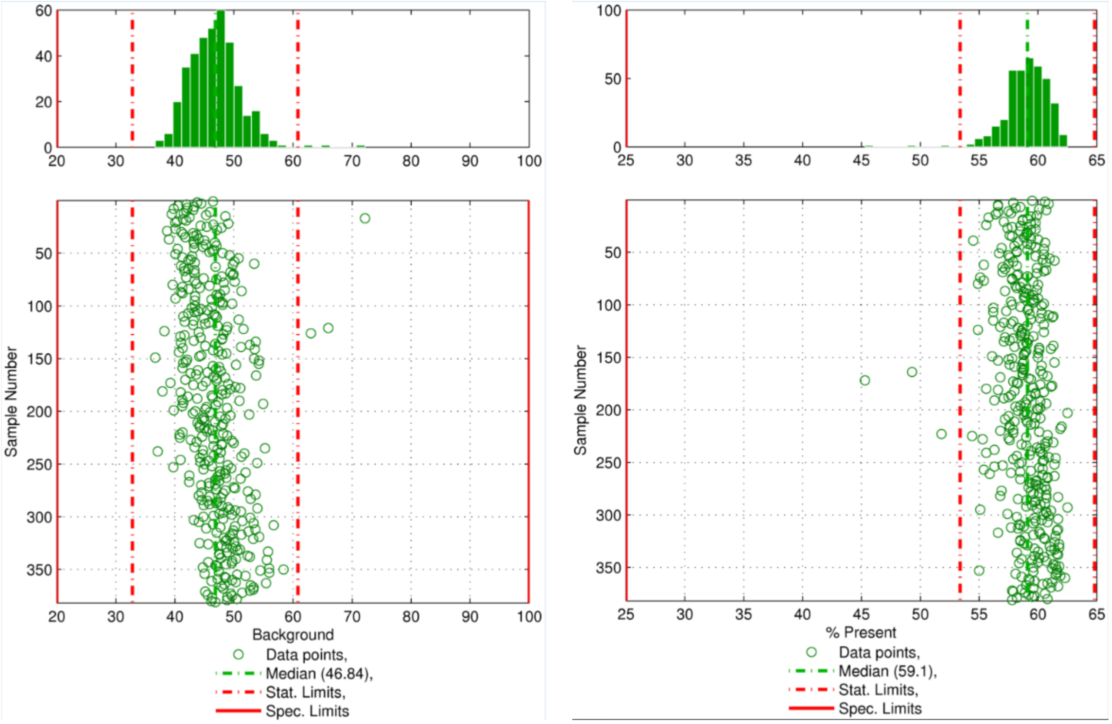


Figure 2. Quality control results from analysis of background intensity (left) and percent present calls (right). The average background value or average percent present calls for each chip is represented by a green circle. Specified limits are shown as solid red lines; statistical limits are shown as red dash-dotted lines.

Quality control parameters were checked against externally defined lower, upper, or both limits for a variety of variables: background intensity, raw noise (Q) value, percent present calls, scaling factor, and GAPDH 3' to 5' ratio. For background intensity, there are no official Affymetrix® guidelines regarding background values; however, Affymetrix® has found that typical background values range from 20 to 100 for arrays scanned with GeneArray® scanners, calibrated to the new PMT setting (10% of maximum). Arrays being compared should ideally have comparable background values, assessed using mean absolute deviation. Raw noise is a measure of pixel-to-pixel variation of probe cells on a GeneChip® array. The two main factors that contribute to noise are electrical noise of the GeneArray® scanner and sample quality. Each GeneArray® scanner has a unique inherent electrical noise associated with its operation. Arrays being compared, which were scanned on the same scanner, should ideally have comparable raw Q values, assessed using mean absolute deviation. The number of probe sets called "present" relative to the total number of probe sets on the array is calculated as a percentage. These values depend on a multitude of factors including cell and tissue type, biological or environmental stimuli, probe array type, and overall quality of RNA. Samples should have comparable percent present values, assessed using mean absolute deviation, and should demonstrate percent present calls of greater than 25%. Scaling methods are applicable to large arrays such as the Human Genome U133 Plus 2.0 GeneChip®, as only a fraction of the transcripts represented on the array are changing from chip to chip. As the majority of transcripts are non-changing, the average intensity across all the arrays should be similar. In order to evaluate scaling factors, we set an arbitrary target intensity. All intensity values on the array were transformed so that the average intensity of the array equaled that of the pre-defined target intensity (typically set at 100). This was then carried out for all chips. The scaling factors between chips should be comparable, assessed using mean absolute deviation. According to Affymetrix® guidelines, the maximum scaling factor should be not more than three times the minimum scaling factor. The GAPDH and beta-actin housekeeping genes were used to assess the RNA sample and assay quality for each GeneChip® array. The signal values for the 3′ probe sets were compared to the 5′ probe sets for both GAPDH and beta-actin. For Affymetrix 2-cycle-processed samples the GAPDH and /or Actin ratio should be less than 4.

Large-scale RNA Sequencing

Throughout this project, supply issues prevented us from being in full production mode for generating RNA sequence data on the HiSeq machine.

Samples to be run for the TruSeq stranded total RNA sequencing project using the Illumina HiSeq2000 were selected from two different study cohorts in the Integrative Cardiac Health Program. All intensive lifestyle participants and available sample time points were organized and sorted. Participants with gender- and age-matches in the non-intervention control group and the LAGB patient group, as well as both baseline and one year time points were prioritized. Samples from all age- and gender-matched patients were then checked for available volume and concentration of the RNA samples. Concentrations were measured using the Nanodrop spectrophotometer. Matched samples that had one of the matches fail to meet criteria were re-matched with other patient samples where possible until all matches were exhausted.

Once the sample pool was complete, samples were organized into individual RNA sequencing runs. Each run (of six total runs) consisted of 12 patients with two time points each (baseline and one year) for a total of 24 samples. Six patients from each study cohort were randomly selected from the master list with the only criteria being that both baseline and one year time points as well as their matching participant was included in the same run to reduce the potential for batch effects. We had a total of 144 RNA samples divided among 6 sequencing runs with 24 total samples per run.

For library preparation, an aliquot of each globin-cleared RNA sample was normalized to 50 ng of RNA in 10 μl of water and used for preparing Illumina TruSeq stranded total RNA with ribo-zero globin libraries. The library preparation kit contains reagents for rRNA reduction, mtRNA reduction, and globin reduction to reduce the amounts of these unwanted transcripts and allow for more coverage of transcripts of interest. The 2-3 day long library preparation protocol consisted of RNA denaturation, RNA fragmentation, cDNA library generation, labeling with individual barcodes or index identifiers, and amplification. Index barcodes attached to each library were chosen based on the previously designed sample groupings. Each sample contained a unique individual index/barcode, which allowed them to be run in the same flow cell lane and then later be individually identified when analyzing the sequencing data.

The completed cDNA libraries were assessed for quality and length distribution via the Bioanalyzer (Agilent Technologies) and for quantity via the Qubit (Life Technologies). All Bioanalyzer traces from the 144 libraries exhibited a well-defined peak in the 200 bp to 450 bp range (Figure 3). Average size across all libraries was 311 bp, well within our desired range. Any libraries that exhibited a sharp narrow peak in the 100 bp to 150 bp region were processed through another round of size selection to remove the unwanted fragments. Fragments in the 100 bp to 150 bp region are index adapter dimers that are detrimental to sequencing data quality.

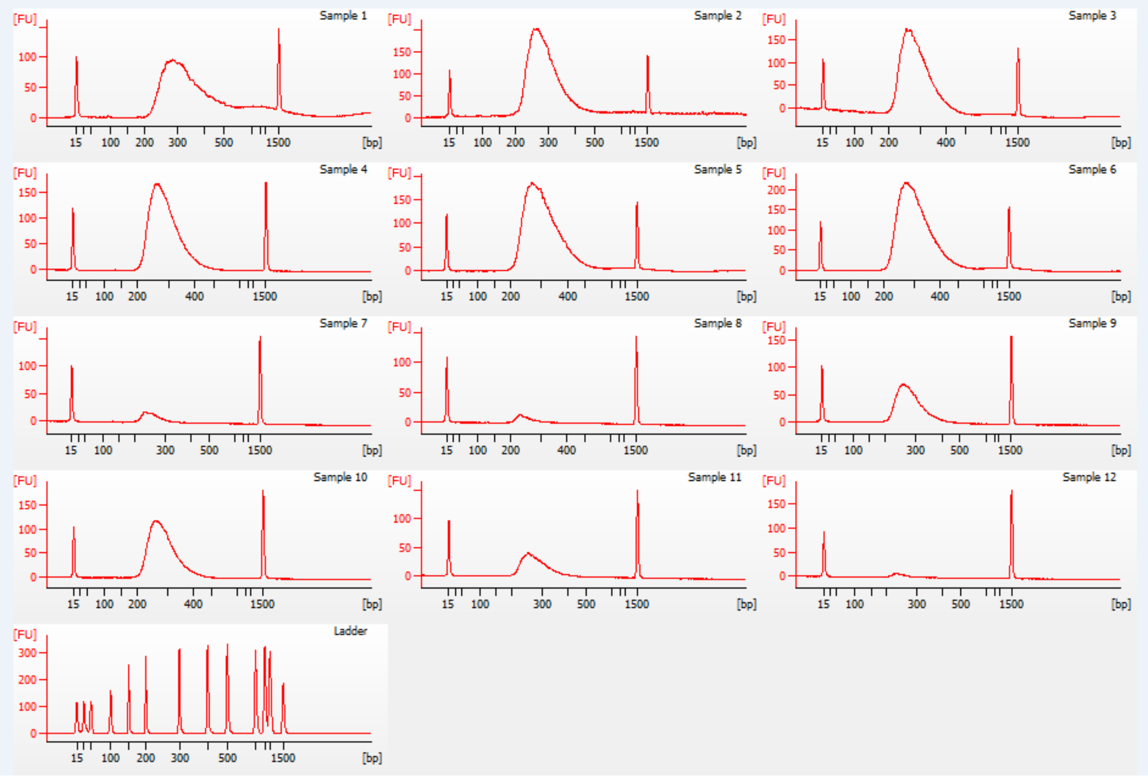


Figure 3. Example Bioanalyzer traces from libraries used for RNA sequencing.

The Qubit is able to distinguish single-stranded from double-stranded cDNA giving a more accurate measurement of the overall usefullness of the library. Quantity of each library was assessed on the Qubit using quantitative fluorometrics and the broad range dsDNA assay (first 48) or the high sensitivity dsDNA assay (last 96). A wide range of concentrations among the 144 libraries was apparent. The average concentration of all libraries was 36.7 ng/μl.

The concentration and average fragment length for each library were used to calculate the concentration of each library, which would later be used for normalizing and diluting the libraries to the desired clustering concentration. Library concentrations were calculated using the following formula: [concentration in ng/μl ÷

(660 daltons x fragment length)] x 10^6. We observed good consistency in concentrations among libraries with few outliers; the average nM concentration for all libraries was 176.3 nM (minimum usable concentration is 4 nM).

An aliquot of each library was normalized to a concentration of 4 nM, and the normalized libraries were pooled using previously determined groupings of six libraries per pool. After pooling, libraries were denatured using 0.1 molar NaOH and further diluted using HT1 buffer to a final cluster concentration of 8 pM to 13 pM. We determined that a final cluster concentration of 8 pM calculated from data obtained via the Qubit high sensitivity dsDNA assay yielded the best sequencing performance.

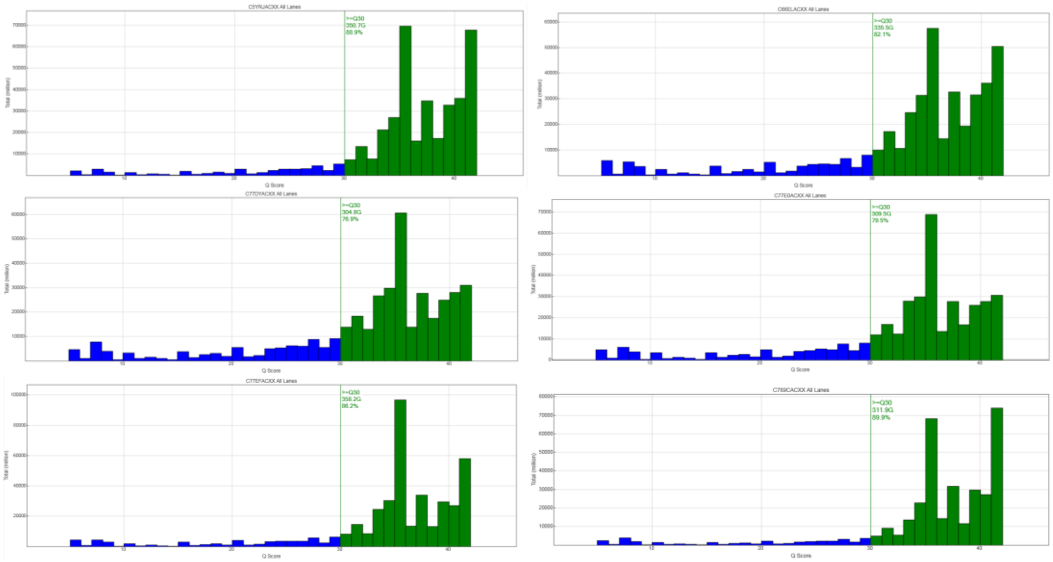


Figure 4. Q-score graphs for the six RNA sequencing runs. Top row: Run #1 (left), Run #2 (right); Middle row: Run #3 (left), Run #4 (right); Bottom row: Run #5 (left), Run #6 (right).

Run 1

Twenty-four libraries were normalized to 4 nM for flow cell clustering. Libraries were then pooled in four groups of six libraries in each group. Samples were then denatured and diluted to a cluster concentration of 12.5 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. For all eight lanes, cluster density ranged from 852,000 clusters/mm^2 to 1,104,000 clusters/mm^2 (average 944,000 clusters/mm^2). The percentage of clusters passing the image filter ranged from 86.9% to 93.3% (average 91%). The number of reads per lane per read that passed filter ranged from 214.26 to 264.63 million (average 235.90 million reads). The percentage of reads in each lane that were >Q30 ranged from 88.2 to 90.4 (average 89.9%) (Figure 4). Each lane produced on average 23.5 billion bases of sequencing data per read length. The paired end 200 cycle sequencing run yielded 4.16 billion reads and generated ~388.0 billion bases of sequence (Table 7).

Run 2

For Run #2, 24 libraries were normalized to 4 nM for flow cell clustering. Libraries were then pooled in four groups of six libraries in each group. Samples were then denatured and diluted to a cluster concentration of 12.0 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. For all eight lanes, cluster density ranged from 951,000 clusters/mm^2 to 1,050,000 clusters/mm^2 (average 1,004,000 clusters/mm^2). The percentage of clusters passing the image filter ranged from 86.4% to 90.4% (average 88.4%). The number of reads per lane per read that passed filter ranged from 235.93 to 250.86 million (average 244.40 million reads). The percentage of reads in each lane that were ≥Q30 ranged from 79.8 to 87.8 (average 83.9%) (Figure 4). Each lane produced on average 24.5 billion bases of sequencing data. The paired end 200 cycle sequencing run yielded 3.91 billion reads and generated ~402.76 billion bases of sequence (Table 7).

Run 3

Twenty-four libraries in Run #3 were normalized to 4 nM for flow cell clustering. Libraries were then pooled in four groups of six libraries in each group. Samples were then denatured and diluted to a cluster concentration of 12.5 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. The average cluster density across all eight lanes ranged from 1,153,000 clusters/mm^2 to 1,171,000 clusters/mm^2 (average 1,161,000 clusters/mm^2). The percentage of clusters passing the image filter averaged 73.9% (range 69.16% to 76.18%). The number of reads per lane per read passing filter averaged 237.25 million reads (range 223.8 to 244.31 million). The percentage of reads in each lane that were ≥Q30 ranged from 79.8 to 82.8 (average 81.52%) (Figure 4). Each lane produced on average 23.62 billion bases of sequencing data. The paired end 200 cycle sequencing run yielded 3.796 billion reads and generated ~390.98 billion bases of sequence (Table 7).

Run 4

In Run #4, 24 libraries were again normalized to 4 nM for flow cell clustering. The libraries were then pooled in four groups of six libraries in each group. Samples were then denatured and diluted to a cluster concentration of 12.0 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. The cluster density for all eight lanes ranged from 1,116,000 clusters/mm^2 to 1,163,000 clusters/mm^2 (average 1,139,00 clusters/mm^2). The percentage of clusters passing the image filter ranged from 63.52% to 79.36% (average 74.3%). Number of reads per lane per read that passed filter ranged from 201.32 to 247.61 million (average 232.8 million reads). The percentage of reads in each lane that were ≥Q30 ranged from 80.8 to 84.2 (average 82.7%) (Figure 4). Each lane produced on average 23.12 billion bases of sequencing data. The paired end 200 cycle sequencing run yielded 3.726 billion reads and generated ~383.79 billion bases of sequence (Table 7).

Run 5

Twenty-four libraries were normalized to 4 nM for flow cell clustering. Run #5 libraries were then pooled in four groups of six libraries in each group. Samples were denatured and diluted to a cluster concentration of 12.0 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. For all eight lanes, cluster density averaged 1,017,000 clusters/mm^2 (range 880,000 clusters/mm^2 to 1,088,000 clusters/mm^2). The percentage of clusters passing the image filter averaged 88.98% (range 87.95% to 91.3%). The number of reads per lane per read that passed filter ranged from 221.21 to 263.1 million (average 248.68 million reads). The percentage of reads in each lane that were ≥Q30 averaged 88.14% (range 86.5 to 90.4) (Figure 4). Each lane produced on average 24.87 billion bases of sequencing data. The paired end 200 cycle sequencing run yielded 3.979 billion reads and generated ~409.83 billion bases of sequence (Table 7).

Run 6

Twenty-four libraries were normalized to 4 nM for flow cell clustering in Run #6. Libraries were then pooled in four groups of six libraries in each group. Samples were then denatured and diluted to a cluster concentration of 8.0 pM. Following clustering, libraries were subjected to a 200 cycle single indexed, paired-end sequencing run. For all eight lanes, cluster density ranged from 760,000 clusters/mm^2 to 849,000 clusters/mm^2 (average 817.5 clusters/mm^2). The percentage of clusters passing the image filter ranged from 91.09% to 93.0% (average 92.1%). The number of reads per lane per read that passed filter ranged from 194.81 to 213.8 million (average 207.47 million reads). The percentage of reads in each lane that were ≥Q30 ranged from 91.4 to 93.9 (average 92.56%) (Figure 4). Each lane produced on average 20.62 billion bases of sequencing data. The paired end 200 cycle sequencing run yielded 3.319 billion reads and generated ~341.9 billion bases of sequence (Table 7).

Table 7. Cluster densities and quality scores for the six RNA sequencing runs on the HiSeq 2000.

Lane	Density (K/mm^2)	Clusters PF (%)	Reads (M)	Reads PF (M)	% ≥ Q30
Run #1					
1	835±108	94.1±1.6	461.5	433.5	92.5
2	843±110	93.5±1.6	465.9	434.7	91.1
3	1104±85	86.9±4.2	610.7	529.3	87.0
4	998±95	89.7±2.6	551.7	493.8	87.3
5	826±130	94.1±1.7	456.6	428.5	92.0
6	852±105	93.3±1.6	470.9	438.3	89.5
7	1103±80	86.7±3.8	609.9	527.5	85.9
8	991±107	89.5±2.8	548.3	489.1	86.9

Run #2					
1	963±104	89.7±3.8	532.7	476.1	85.9
2	961±110	90.4±2.7	531.1	478.4	85.5
3	1045±118	86.9±3.3	577.8	500.3	80.3
4	1049±97	86.8±3.1	579.9	501.7	79.4
5	971±99	89.8±2.7	537.2	481.3	83.6
6	951±125	90.0±2.6	526.0	471.9	82.8
7	1050±101	86.4±3.2	580.6	500.1	77.9
8	1042±98	87.1±3.1	576.2	500.5	80.1
Run #3					
1	1153±51	76.1±11.9	637.6	484.9	78.5
2	1167±50	69.2±18.6	645.4	447.6	74.7
3	1171±43	73.2±15.8	647.6	474.9	77.6
4	1157±53	75.1±12.8	639.6	479.6	77.8
5	1153±47	73.2±12.5	637.3	466.8	74.6
6	1164±47	74.5±11.8	643.5	479.3	75.6
7	1162±54	73.7±13.4	642.3	474.1	76.6
8	1162±56	76.2±10.7	642.5	488.6	77.6
Run #4					
1	1163±48	77.2±10.5	643.3	495.2	78.1
2	1133±81	77.9±7.6	626.4	485.6	80.6
3	1143±68	74.7±11.1	632.0	469.7	80.1
4	1134±70	73.1±9.6	627.1	456.0	82.4
5	1149±57	63.5±30.8	635.6	402.6	76.0
6	1125±81	75.0±9.2	621.9	464.0	79.3
7	1149±67	73.7±9.9	635.3	465.9	78.8
8	1116±78	79.4±11.7	617.1	487.2	80.7
Run #5					
1	1076±152	88.0±3.8	595.2	521.0	85.4
2	1046±150	88.2±3.4	578.2	507.2	85.5
3	1045±164	89.1±3.4	578.0	512.1	86.5
4	880±151	91.3±2.1	486.4	442.4	88.7

5	1049±150	88.4±3.3	580.2	510.2	84.9
6	1040±155	88.2±3.2	574.8	504.6	84.2
7	1088±152	88.0±3.8	601.6	526.2	84.8
8	911±168	90.7±2.5	504.0	455.2	87.9
Run #6					
1	766±137	93.0±1.8	423.5	392.6	91.4
2	824±126	92.3±1.8	455.9	419.4	90.6
3	822±139	92.1±1.8	454.4	417.1	89.7
4	840±138	91.6±1.7	464.6	424.4	88.3
5	760±128	93.0±1.6	420.0	389.6	91.3
6	830±144	92.4±1.9	458.7	422.2	90.0
7	849±146	91.1±2.6	469.7	426.3	88.8
8	849±143	91.4±1.8	469.4	427.7	88.2

Data Analysis

Run #1 was queued for sequence analysis by de-multiplexing, trimming, and aligning reads to the human genome. Each sample had ~37 million total left reads and ~37 million total right reads. On average, ~28 million left reads mapped to the human genome (79% mapping rate for left reads) and ~11 million right reads mapped to the human genome (33% mapping rate for right reads). The overall mapping rate for all samples and reads was 56.34%; 32.96% of mapped reads showed multiple alignments. Of the mapped left reads, ~35% showed multiple alignments (range 14% to 77%) while ~32% of the mapped right reads showed multiple alignments (range 6% to 80%). For all samples and pairs of reads, the average discordant alignments accounted for 11.38% of the mapped reads and the concordant pair mapping rate was 29.12%.

Run #1 was repeated and queued for analysis as above. For the repeat run, each sample had ~38 million total left reads and ~38 million total right reads. On average, ~36 million left reads mapped to the human genome (93% mapping rate for left reads) and ~36 million right reads mapped to the human genome (94% mapping rate for right reads). The overall mapping rate for all samples and reads was 93.56%; 32.54% of mapped reads showed multiple alignments. Of the mapped left reads, ~33% showed multiple alignments (range 11% to 80%) and ~33% of the mapped right reads showed multiple alignments (range 11% to 80%). For all samples and pairs of reads, the average discordant alignments accounted for 2.43% of the mapped reads and the concordant pair mapping rate was 87.83%.

Gene counting and differential expression analysis discovered 13,281 differentially expressed genes among samples at a nominal level of significance. Using a False Discovery Rate (FDR) $p<0.05$ there were 1,252 differentially expressed genes.

Further processing of Run #2 showed that, on average in each lane, 99.3% of the pass filter reads were identified and matched to a particular sample. Each lane contained six indexed libraries and the distribution of reads in each lane for each library was very good at ±3.5% of the ideal distribution of 16.66% for each sample. All but four samples were within ±1.5%.

For Run #3, 98.8% of all pass filter reads were identified and matched to a particular sample. The distribution of reads per sample per lane ranged from 11.6% to 18.5% across all samples.

In Run #4, 98.7% of all pass filter reads were identified and matched to a particular sample. The distribution of reads per sample per lane averaged 16.6% and ranged from 11.5% to 21%.

For Run #5, on average, 99.3% of the pass filter reads in each lane were identified and matched to a particular sample. The distribution of the six pooled libraries in each lane ranged from 12% to 20%.

For Run #6, 99.1% of the pass filter reads in each lane were identified and matched to a particular sample. The distribution of the six pooled libraries in each lane ranged from 12.4% to 19.3%.

Task #3: Use whole transcriptome analysis in the CRC to examine expression of previously identified genes

From the Integrative Cardiac Health Program, 264 participants were enrolled in the Cardiovascular Risk Clinic. Participants were randomly assigned to the intervention arm (n=144) or to the control arm (n=120). Of those, 205 remained active in the program, while 59 participants dropped out. After one year as a control, 34 of the control group transitioned to the intervention arm.

Total RNA was isolated from 492 peripheral blood samples from 163 participants (Table 8). RNA concentrations were 89.32 ± 41.90 ng/μl (range 2.00-326.25 ng/μl), OD260/280 ratios were 2.17 ± 0.10 (range 1.83-3.24), and RIN numbers were 8.4 ± 0.5 (range 2.0-9.4). RNA samples (n=454) were run on Affymetrix gene expression arrays with call rates of 60.64 ± 1.42% (range 56.05-64.30%). Individual time points were: 153 baseline (T1), 63 control waiting period complete (T2C), 92 intervention complete (T2V), 142 six months after intervention (T3), and 4 one year after intervention (T4).

Table 8. Concentrations, purity measures, and call rates on gene expression arrays for RNA isolated from whole blood from Cardiovascular Risk Clinic lifestyle participants.

Sample	Time Point	Concentration (ng/μl)	OD260/280	RIN	Call Rate (%)
194	T1	108.31	2.16	8.9	60.08
	T2V	54.90	2.12	8.1	61.13
	T3	57.70	2.26	8.5	60.16
669	T1	42.73	2.20	7.8	58.90
	T2V	91.57	2.44	8.1	60.71
	T3	64.50	2.12	8.0	58.68
521	T1	90.46	2.26	8.6	61.13
	T2V	172.36	2.16	6.8	61.19
	T3	64.83	2.15	8.8	60.63
641	T1	94.69	2.27	8.6	61.46
	T2V	92.46	2.24	8.8	57.01
	T3	181.54	2.22	7.3	56.64
882	T1	37.56	2.10	8.5	60.09
	T2V	47.89	2.35	8.5	61.47
	T3	49.79	2.17	8.7	62.09
533	T1	115.75	2.08	8.2	57.21
	T2V	8.57	1.83	---	---
	T3	52.62	2.15	8.7	60.95
534	T1(364 T5)	163.14	2.19	7.9	58.14
	T2V	98.87	2.23	8.6	60.50
	T3	64.05	2.22	8.8	59.26
530	T1	51.88	2.22	8.6	60.72
	T2V	54.16	2.44	8.6	60.65
	T3	29.20	2.16	8.9	59.34
323	T1	64.58	2.36	8.2	59.88
	T2V	133.58	2.29	8.4	59.28
	T3	60.00	2.26	8.8	60.43
649	T1	88.97	2.13	8.2	60.62
	T2V	49.46	2.30	8.4	59.78
	T3	29.73	2.03	9.4	58.09
608	T1	96.13	2.29	8.4	59.42
	T2V	174.90	2.21	8.4	59.04
	T3	69.20	2.07	7.8	60.10
219	T1	67.63	2.12	9.2	60.93

	T2V	112.47	2.10	8.0	59.66
	T3	100.04	2.08	8.8	58.90
25	T1	32.90	2.32	8.4	59.59
	T2V	90.55	2.15	8.3	60.36
	T3	51.59	2.26	8.8	61.00
200	T1	38.68	2.24	8.2	61.03
	T2V	73.71	2.38	9.2	61.56
	T3	65.09	2.15	8.7	62.24
50	T1	84.54	2.12	8.1	59.75
	T2V	67.95	2.08	8.1	59.48
	T3	95.11	2.18	8.7	58.13
15	T1	77.59	2.08	8.8	62.31
	T2V	66.33	2.15	9.0	60.67
	T3	77.79	2.15	9.2	61.89
932	T1	111.89	2.27	8.5	59.97
	T2V	141.50	2.17	8.6	60.31
	T3	105.10	2.15	8.3	58.22
481	T1(167 T4)	129.23	2.11	8.0	59.40
	T2V	32.77	2.19	8.6	59.56
	T3	35.24	2.29	8.6	---
874	T1	107.87	2.07	8.4	58.66
	T2C	114.07	2.11	8.8	62.97
	T3	28.26	1.96	---	61.66
39	T1	58.07	2.19	8.3	59.24
	T2V	57.70	2.13	8.7	59.88
	T3	90.07	2.13	8.1	58.93
326	T1	98.58	2.25	8.7	56.40
	T2V	156.69	2.17	8.9	61.15
	T3	194.69	2.13	8.5	58.99
778	T1(718 T3)	91.89	2.11	8.5	59.46
	T2V	106.87	2.21	8.4	58.71
	T3	74.37	2.14	8.0	59.86
204	T1	123.00	2.16	8.4	59.26
	T2V	110.88	2.21	8.8	58.62
	T3	50.36	2.11	8.7	60.38
683	T1(176 T3)	198.51	2.15	8.5	58.79
	T2V	53.09	2.16	8.3	58.99
	T3	90.48	2.13	8.7	61.46
887	T1	38.37	2.15	8.5	59.88
	T2V	48.19	2.35	8.7	59.85
	T3	53.50	2.08	8.7	62.19
751	T1	171.94	2.17	8.4	58.60
	T2V	78.84	2.20	8.7	61.08

	T3	141.62	2.17	8.1	58.92
262	T1	77.55	2.12	8.4	58.74
	T2V	72.89	2.19	8.6	59.37
	T3	143.51	2.19	8.1	57.40
687	T1	89.94	2.08	8.0	58.64
	T2V	124.80	2.21	8.7	57.38
	T3	42.91	2.16	8.7	57.45
645	T1(775 T3)	209.90	2.17	8.4	59.35
	T2V	103.21	2.19	8.6	57.19
	T3	308.49	2.09	8.3	56.09
698	T1	126.59	2.14	8.7	62.05
	T2V	71.70	2.27	8.8	60.85
	T3	57.51	2.04	9.0	---
135	T1	110.00	2.16	8.7	59.64
	T2V	60.58	2.24	8.4	59.68
	T3	81.28	2.19	9.0	61.52
604	T1(784 T3)	88.59	2.13	8.3	60.31
	T2V	36.38	2.19	8.2	61.18
	T3	72.63	2.25	8.8	60.18
676	T1	133.78	2.11	7.8	59.69
	T2V	85.82	2.12	7.9	60.08
	T3	127.03	2.12	7.0	57.97
705	T1	95.73	2.07	8.4	60.29
	T2V	119.50	2.13	8.6	58.25
	T3	326.25	2.16	---	60.87
755	T1	58.06	2.09	8.7	58.76
	T2V	48.65	1.94	8.6	59.13
	T3	23.85	2.17	8.5	61.12
412	T1	119.01	2.21	8.3	59.68
	T2V	59.33	2.13	9.2	60.98
	T3	147.74	2.15	8.5	59.61
250	T1	73.43	2.16	8.9	60.38
	T2V	48.08	2.30	8.2	61.57
	T3	68.23	2.30	8.5	59.46
870	T1	51.71	2.04	8.5	61.82
	T2V	51.32	2.02	8.2	59.75
	T3	68.63	2.17	8.6	61.31
898	T1(737 T4)	149.73	2.17	8.5	60.61
	T2V	113.73	2.16	8.3	60.22
	T3	93.83	2.10	8.5	58.62
76	T1(756 T4)	43.92	2.30	9.0	60.46
	T2V	95.68	2.14	8.9	61.69
	T3	72.35	2.13	9.1	60.47

195	T1	107.80	2.14	7.6	58.18
	T2V	81.00	2.21	7.9	58.39
	T3	45.81	2.33	8.6	58.80
989	T1	78.41	2.25	8.8	59.49
	T2V	115.62	2.27	8.7	58.29
	T3	101.35	2.23	8.6	60.03
471	T1	58.76	2.24	8.5	61.42
	T2V	150.62	2.21	8.0	58.97
	T3	56.99	2.12	8.2	60.95
919	T1(834 T3)	86.76	2.21	8.5	59.68
	T2V	60.78	2.15	8.8	59.13
	T3	122.09	2.16	8.3	59.81
302	T1	185.07	2.10	8.0	57.71
	T2C	208.36	2.09	7.7	61.07
	T3	107.24	2.28	8.6	62.16
	T4	78.06	2.06	8.8	61.29
962	T1	52.01	2.00	8.0	60.17
	T2C	46.90	2.06	8.5	61.59
	T3	105.94	2.23	8.3	61.18
767	T1	114.22	2.12	7.9	60.21
	T2C	103.73	2.12	8.0	61.37
	T3	---	---	---	---
182	T1	101.23	2.20	8.7	60.11
	T2V	120.05	2.12	7.6	58.69
	T3	52.41	2.13	8.2	60.96
073	T1	26.04	2.00	8.8	60.15
	T2V	---	---	---	---
496	T1	58.37	2.11	8.9	61.02
	T2C	135.80	2.08	8.2	60.91
	T3	158.41	2.20	8.5	58.53
850	T1	121.41	2.14	7.7	57.99
	T2C	145.93	2.21	8.3	58.63
	T3	175.47	2.18	7.8	59.11
046	T1	126.41	2.10	8.2	59.25
	T2V	68.28	2.14	8.3	60.19
	T3	114.30	2.10	8.0	60.66
525	T1	111.73	2.12	8.3	61.00
	T2C	126.66	2.12	7.9	60.67
	T3	60.27	2.26	---	60.80
026	T1	51.24	2.22	8.7	61.86
	T2C	52.40	2.12	8.8	60.42
	T3	95.60	2.16	8.4	60.52
173	T1	43.69	2.11	8.7	60.12

	T2C	134.50	2.17	8.4	59.73
	T3	39.50	2.25	8.6	59.90
251	T1	52.89	2.22	8.1	58.36
	T2C	73.60	2.20	8.3	60.01
	T3	60.60	2.15	8.1	60.02
375	T1	49.78	2.21	8.1	61.18
	T2C	44.10	2.02	8.0	62.08
	T3	101.90	2.15	8.0	63.19
424	T1	84.00	2.17	8.1	63.24
	T2C	104.49	2.14	8.0	59.78
	T3	71.70	2.07	8.1	59.41
754	T1	58.90	2.15	8.2	61.31
	T2C	76.30	2.11	8.6	61.42
	T3	157.32	2.16	7.4	61.43
366	T1	69.50	2.19	---	59.86
	T2C	74.70	2.12	8.4	60.91
	T3	66.60	2.10	8.0	60.39
005	T1	62.60	2.00	8.2	63.95
	T2C	40.62	2.23	8.1	60.16
	T3	77.70	2.18	8.3	60.46
006	T1	75.91	2.18	8.2	62.11
	T2C	94.30	2.05	8.6	60.94
	T3	47.10	2.25	8.7	60.12
009	T1	131.42	2.25	7.5	61.06
	T2C	119.29	2.23	8.7	61.77
	T3(672 T1)	68.01	2.12	8.5	60.42
017	T1	53.40	2.14	8.5	59.64
	T2C	83.05	2.24	7.7	61.07
	T3(792 T1)	52.47	2.27	8.4	---
163	T1	84.91	2.21	8.0	60.40
	T2C	109.40	2.05	8.5	62.83
	T3(508 T1)	40.50	2.18	8.7	60.34
176	T1	129.20	2.17	8.4	60.79
	T2C	87.96	2.20	8.3	57.88
	T3(683 T1)	---	---	---	---
036	T1	112.40	2.07	7.9	62.70
	T2V	75.20	2.09	8.6	61.18
	T3	45.10	2.16	8.6	---
089	T1	103.90	2.06	8.4	63.01
	T2V	51.11	2.17	8.4	61.63
	T3	124.70	2.14	8.5	57.95
735	T1	94.28	2.15	8.1	61.74
	T2C	132.19	2.20	8.6	60.44

	T3	47.30	2.10	8.1	60.14
212	T1(614 T5)	143.72	2.19	8.7	60.96
	T2V	45.36	2.39	9.0	59.73
	T3	56.50	2.05	8.8	63.52
333	T1	128.50	2.16	8.1	58.89
	T2V	89.15	2.22	8.1	57.24
	T3	78.80	2.15	8.1	57.70
360	T1(427 T3)	58.10	2.23	8.1	59.93
	T2V	56.00	2.14	8.2	60.62
	T3	78.16	2.17	7.4	60.21
439	T1	48.38	2.17	8.4	59.82
	T2V	60.30	2.13	8.6	61.76
	T3	41.70	2.12	8.7	60.47
455	T1	124.06	2.11	8.3	60.29
	T2C	117.19	2.10	8.3	62.57
	T3	56.44	2.12	8.6	61.05
949	T1	108.77	2.16	9.0	62.10
	T2V	190.11	2.11	8.8	60.87
	T3	---	---	---	---
165	T1	92.96	2.14	8.8	62.00
	T2V	82.65	2.28	8.9	63.16
	T3	80.84	2.25	8.5	60.61
532	T1	94.68	2.17	8.6	60.50
	T2V	77.88	2.22	7.9	59.31
	T3	125.38	2.16	8.7	60.53
100	T1	96.23	2.14	8.3	62.10
	T2V	---	---	---	---
414	T1	88.65	2.08	8.6	62.50
	T2C	62.58	2.07	8.8	59.48
	T3	72.21	2.07	9.2	61.04
119	T1	69.99	2.13	8.8	62.20
	T2C	91.55	2.10	8.0	60.45
	T3	---	---	---	---
614	T1	126.34	2.08	8.5	61.40
	T2C	123.23	2.13	7.9	59.53
	T3	58.70	2.21	8.4	61.24
215	T1	85.78	2.11	9.0	61.80
	T2V	83.47	2.20	6.4	---
	T3	146.12	2.18	8.3	58.56
303	T1	102.70	2.10	8.7	62.80
	T2V	89.79	2.06	8.5	60.88
	T3	67.00	2.16	8.4	61.48
737	T1	162.45	2.17	8.0	61.90

	T2C	169.67	2.12	9.0	59.26
	T3	106.55	2.19	8.1	60.99
862	T1	134.57	2.11	8.2	61.23
	T2V	121.63	2.10	8.4	60.40
	T3	96.32	2.18	8.8	59.34
639	T1	114.74	2.09	8.6	61.31
	T2C	123.32	2.08	8.6	59.58
	T3	57.50	2.15	8.3	60.65
777	T1	53.50	1.91	8.2	61.09
	T2C	82.10	2.06	8.6	61.56
	T3	45.80	2.18	8.3	62.18
814	T1	107.87	2.07	8.4	60.57
	T2V	114.07	2.11	8.8	59.39
	T3	88.60	1.92	8.0	61.25
500	T1(725 T3)	82.20	2.05	8.3	61.56
	T2V	52.60	2.11	8.6	60.64
	T3	104.00	2.15	8.3	60.67
550	T1	52.30	2.17	8.3	60.61
	T2V	112.70	2.10	7.4	60.79
	T3	60.80	2.16	8.5	61.50
595	T1	133.00	2.17	8.4	60.19
	T2V	40.30	2.22	8.7	61.23
	T3	55.70	2.09	8.7	62.20
672	T1(009 T3)	---	---	---	---
	T2V	108.90	2.17	8.4	60.17
	T3	89.40	2.07	8.6	61.68
792	T1(017 T3)	---	---	---	---
	T2V	72.20	2.13	8.6	61.13
	T3	39.70	1.99	8.1	62.72
811	T1	114.40	2.14	7.6	60.96
	T2V	152.30	2.14	7.9	61.23
	T3	81.80	2.19	7.9	60.73
427	T1	34.27	2.22	8.4	60.49
	T2C	75.67	2.13	8.7	61.54
	T3(360 T1)	---	---	---	---
241	T1	70.50	2.33	8.3	61.95
	T2C	75.61	2.25	6.6	59.89
	T3(457 T1)	56.10	2.01	8.4	61.39
387	T1	162.95	2.23	8.6	57.73
	T2C	22.03	2.26	---	61.60
	T3(926 T1)	112.00	2.18	8.6	58.03
206	T1	70.75	2.28	8.2	59.06
	T2C	119.70	2.17	8.3	61.08

	T3(938 T1)	115.00	2.17	8.5	61.08
498	T1	51.71	2.33	8.3	62.24
	T2C	50.25	2.01	9.0	62.59
	T3(822 T1)	56.00	2.24	8.0	61.89
477	T1	77.30	2.09	8.2	60.09
	T2C	44.70	2.17	9.1	64.30
	T3(149 T1)	92.10	2.13	8.7	62.70
822	T1(498 T3)	---	---	---	---
	T2V	46.70	2.14	8.1	58.18
	T3	54.80	2.24	8.4	60.24
575	T1	68.94	2.09	8.5	60.51
	T2C	67.56	2.28	8.8	61.51
	T3(755 T1)	---	---	---	---
578	T1	65.40	2.13	8.6	62.41
	T2C	155.30	2.23	8.1	62.50
	T3 (211 T1)	142.70	2.11	8.0	62.11
718	T1	63.60	2.09	8.6	59.88
	T2C	94.29	2.28	2.0	60.32
	T3(778 T1)	---	---	---	---
725	T1	56.91	2.24	7.7	61.14
	T2C	82.88	2.17	8.2	61.33
	T3(500 T1)	---	---	---	---
756	T1	63.81	2.33	7.6	60.52
	T2C	112.48	2.29	9.0	61.19
	T3(076 T1)	---	---	---	---
772	T1	121.30	2.09	8.3	59.61
	T2C	76.30	2.21	7.8	59.55
	T3(706 T1)	30.40	2.16	8.6	62.36
775	T1	187.90	2.23	8.2	59.76
	T2C	179.17	2.25	7.9	61.29
	T3(645 T1)	---	---	---	---
784	T1	80.95	2.18	8.6	63.21
	T2C	118.17	2.29	8.8	59.87
	T3(604 T1)	---	---	---	---
815	T1	32.00	2.16	8.5	59.97
	T2C	61.90	2.11	8.3	61.31
	T3(502 T1)	74.30	2.13	8.4	59.93
834	T1	105.37	2.31	8.3	62.08
	T2C	133.11	2.23	8.1	58.98
	T3(919 T1)	---	---	---	---
940	T1	65.36	2.29	8.9	60.55
	T2C	107.49	2.07	8.9	61.79
	T3(881 T1)	65.10	2.18	8.5	61.09

964	T1	154.09	2.22	8.8	62.20
	T2C	123.12	2.25	8.8	62.32
	T3(135 T1)	---	---	---	---
967	T1	37.98	2.36	8.2	63.54
	T2C	126.61	2.11	---	61.22
	T3(588 T1)	155.50	2.12	8.4	62.22
588	T1(967 T3)	---	---	---	---
	T2V	62.50	2.03	8.2	62.97
	T3	35.10	2.21	8.7	60.29
745	T1	73.90	2.11	7.7	60.77
	T2C	32.10	2.24	8.5	58.73
	T3(679 T1)	56.30	2.10	7.6	60.48
738	T1	70.27	2.32	8.0	61.61
	T2C	---	---	---	---
	T3(854 T1)	125.00	2.11	7.9	60.19
854	T1(738 T3)	---	---	---	---
	T2V	83.40	2.09	7.7	62.51
	T3	59.10	2.21	8.4	61.38
728	T1	142.90	2.10	7.7	58.71
	T2C	103.50	2.12	8.5	61.08
	T3	68.80	2.08	7.8	60.79
167	T1	48.52	2.13	9.1	62.45
	T2C	68.51	2.15	8.9	63.53
	T3	19.83	2.92	9.3	61.31
024	T1	147.09	2.24	8.5	62.28
	T2C	138.93	2.22	8.4	62.87
	T3	139.00	2.05	7.9	62.23
016	T1	107.58	2.27	7.9	60.69
	T2C	113.09	2.29	7.5	62.15
	T3	73.70	2.06	7.2	59.99
069	T1	64.40	2.31	9.0	61.09
	T2C	87.32	2.25	9.1	62.17
	T3	82.80	2.17	8.4	59.86
	T4	55.39	2.17	8.2	61.48
276	T1	120.78	2.25	8.7	60.51
	T2C	163.09	2.20	8.0	60.37
	T3	52.70	2.15	7.9	60.27
	T4	132.88	2.21	7.9	61.14
601	T1	35.66	2.44	8.5	63.79
	T2C	56.22	2.24	8.6	63.87
	T3	73.40	2.17	8.6	61.69
057	T1	85.19	2.13	8.5	60.39
	T2C	88.61	2.12	7.8	59.84

	T3	90.27	2.29	8.4	61.08
392	T1(057 T5)	88.17	2.11	8.1	61.35
	T2V	38.80	2.29	8.7	61.59
	T3	67.00	2.20	8.1	60.39
364	T1	96.97	2.18	8.7	61.88
	T2C	75.22	2.12	8.3	63.11
	T3	2.00	---	---	---
	T4	126.89	2.25	8.5	61.17
	T5(534 T1)	---	---	---	---
142	T1(302 T5)	56.78	2.04	8.6	60.49
	T2V	14.00	2.09	7.9	---
	T3	126.50	2.16	8.3	59.38
791	T1	71.68	2.20	8.8	63.83
	T2C	63.84	2.38	9.0	63.68
	T3	77.83	2.20	8.9	62.61
709	T1	85.70	2.12	8.3	59.71
	T2C	68.92	2.03	8.3	59.32
	T3	104.24	2.16	8.4	61.92
774	T1	127.71	2.10	7.5	60.98
	T2C	44.48	2.09	8.4	62.05
	T3	41.25	2.13	8.7	63.06
933	T1	164.06	2.11	8.4	61.25
	T2C	115.38	2.12	8.1	61.52
	T3	107.10	2.23	8.1	61.93
961	T1	190.33	2.10	8.1	62.84
	T2C	270.14	2.11	7.4	62.35
	T3	143.89	2.14	8.7	60.83
844	T1	119.59	2.07	8.7	62.84
	T2C	101.63	2.17	8.9	61.01
	T3	35.70	1.98	8.7	62.72
881	T1(940 T3)	---	---	---	---
	T2V	67.20	2.17	8.9	61.35
	T3	68.30	2.18	7.9	61.39
587	T1	119.81	2.08	8.3	62.69
	T2V	129.08	2.11	8.0	62.32
	T3	52.20	2.12	8.5	61.38
615	T1	38.60	2.10	8.2	60.80
	T2V	43.70	2.17	8.6	61.44
	T3	65.90	2.19	8.0	60.42
404	T1	125.70	2.11	7.3	57.37
	T2V	58.30	2.18	8.9	61.90
	T3	66.50	2.13	8.1	60.14
905	T1	42.59	2.09	8.6	---

	T2V	95.94	2.08	8.2	63.03
	T3	64.80	2.14	8.1	61.00
056	T1	60.90	2.14	8.5	61.09
	T2V	42.30	2.14	8.2	59.75
	T3	67.10	2.06	6.9	56.05
211	T1(587 T3)	---	---	---	---
	T2V	85.30	2.12	8.2	58.99
	T3	90.00	2.17	8.9	62.48
425	T1	53.90	2.16	8.3	62.65
	T2V	72.10	2.12	8.6	63.02
	T3	118.10	2.11	9.0	61.54
740	T1	104.60	2.11	7.7	58.14
	T2V	123.00	2.16	8.0	60.14
	T3	74.90	2.14	8.1	60.76
926	T1(387 T3)	---	---	---	---
	T2V	87.20	2.09	8.0	60.99
	T3	31.70	2.09	8.8	59.16
042	T1	40.60	2.04	8.6	62.22
	T2V	86.25	2.28	8.8	62.61
	T3	100.80	2.08	7.3	60.25
342	T1	88.13	2.20	8.5	61.48
	T2V	97.35	2.20	8.5	60.86
	T3	91.90	2.15	8.4	60.71
386	T1	90.34	2.19	8.5	61.75
	T2V	154.98	2.16	7.0	58.25
	T3	80.20	2.13	7.7	59.34
390	T1	83.15	2.24	8.7	62.18
	T2V	123.57	2.37	8.3	61.81
	T3	120.20	2.12	8.6	60.75
856	T1	73.60	2.09	9.0	62.43
	T2V	112.69	2.24	8.1	61.64
	T3	34.50	2.19	8.7	60.71
934	T1	65.00	2.12	8.7	58.91
	T2V	80.27	2.27	8.2	60.64
	T3	72.70	2.15	7.4	58.74
941	T1	60.64	2.21	8.9	62.15
	T2V	120.95	2.21	8.3	61.70
	T3	47.00	2.09	8.3	62.27
966	T1	68.10	2.17	8.8	62.06
	T2V	96.96	2.28	8.0	61.31
	T3	85.70	2.11	8.5	61.16
062	T1	71.24	2.29	8.6	62.74
	T2V	5.67	3.24	---	---

	T3	73.77	2.25	8.1	61.55
245	T1	---	---	---	---
	T2V	81.05	2.29	8.8	---
	T3	162.19	2.19	8.9	---
257	T1	100.46	2.24	9.0	60.39
	T2V	58.37	2.29	7.8	61.09
	T3	195.95	2.12	7.8	59.06
338	T1	104.61	2.16	7.9	61.03
	T2V	35.68	2.39	8.0	60.83
	T3	92.80	2.08	8.7	61.24
567	T1	77.43	2.23	9.0	60.88
	T2V	83.73	2.24	7.9	61.58
	T3	106.68	2.22	8.4	61.48
557	T1	82.57	2.19	8.0	62.11
	T2V	63.35	2.30	7.8	62.55
	T3	94.30	2.09	8.4	60.43
140	T1	61.82	2.10	8.8	61.48
	T2V	100.25	2.16	8.1	58.98
	T3	52.59	2.06	9.0	61.18
290	T1	155.20	2.13	8.0	60.83
	T2V	142.48	2.09	7.7	58.25
	T3	145.41	2.12	8.6	62.22
579	T1	188.29	2.12	8.3	58.25
	T2V	117.85	2.11	8.1	61.22
	T3	177.10	2.11	8.4	60.42
760	T1	82.86	2.06	8.2	63.17
	T2V	100.78	2.16	8.2	62.25
	T3	86.71	2.19	7.0	60.84

Task #4: Investigate gender and patient subgroup differences in molecular response

Gender Differences

For an analysis of changes in gene expression by gender, differential gene expression analysis was conducted over three months and one year in the intensive lifestyle intervention. ANOVA with patient identification number as the random effects factor and time point as fixed effects factor was used for the following subsets of samples: female lifestyle participants (Table 9), female controls, male lifestyle participants (Table 10), and male controls. This analysis allowed paired comparisons through random effects factors and time point contrasts in the same model. Significance of the ANOVA p-values was further adjusted for multiple testing by False Discovery Rate (FDR) correction, and gene lists were generated through combined filtering with both differential p-values and fold changes (1.1 fold). The results indicate that differences in gene expression between women and men occur during participation in a lifestyle modification intervention.

The results were as follows:
Female lifestyle participants – 0 differentially expressed genes at 3 months
19 differentially expressed genes at 1 year
Female controls – 2 differentially expressed genes at 3 months
0 differentially expressed genes at 1 year

Table 9. Genes differentially expressed in FEMALE lifestyle participants after one year of intervention

Probe ID	Gene Symbol	Gene Name	Fold-Change
202018_s_at	LTF	lactotransferrin	-1.57
217878_s_at	CDC27	cell division cycle 27 homolog (S. cerevisiae)	-1.35
200615_s_at	AP2B1	adaptor-related protein complex 2, beta 1 subunit	-1.32
213926_s_at	AGFG1	ArfGAP with FG repeats 1	-1.21
203609_s_at	ALDH5A1	aldehyde dehydrogenase 5 family, member A1	-1.20
208476_s_at	FRMD4A	FERM domain containing 4A	-1.19
209616_s_at	CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	-1.18
214869_x_at	GAPVD1	GTPase activating protein and VPS9 domains 1	-1.17
212804_s_at	GAPVD1	GTPase activating protein and VPS9 domains 1	-1.17
203922_s_at	CYBB	cytochrome b-245, beta polypeptide	-1.17
202067_s_at	LDLR	low density lipoprotein receptor	-1.15
200832_s_at	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	-1.15
204393_s_at	ACPP	acid phosphatase, prostate	-1.14
222059_at	ZNF335	zinc finger protein 335	+1.10
202617_s_at	MECP2	methyl CpG binding protein 2 (Rett syndrome)	+1.12
204021_s_at	PURA	purine-rich element binding protein A	+1.12
213018_at	GATAD1	GATA zinc finger domain containing 1	+1.14
222018_at	NACA /// NACAP1	nascent polypeptide-associated complex alpha subunit /// nascent-polypeptide-ass	+1.14
204663_at	ME3	malic enzyme 3, NADP(+)-dependent, mitochondrial	+1.15

Male lifestyle participants – 12 differentially expressed genes at 3 months
0 differentially expressed genes at 1 year
Male controls – 0 differentially expressed genes at 3 months
0 differentially expressed genes at 1 year

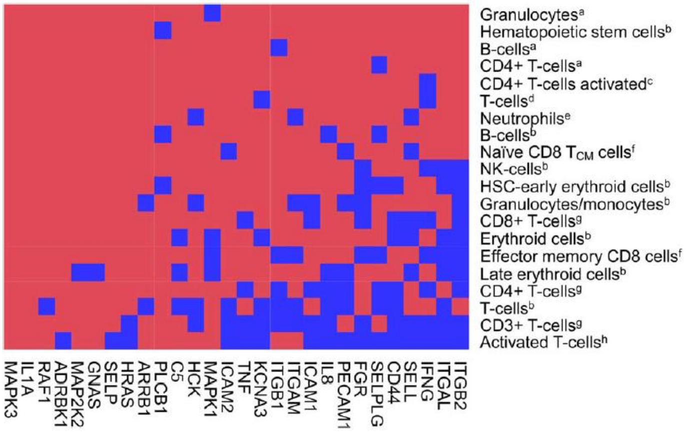
Table 10. Genes differentially expressed in MALE lifestyle participants after three months of intervention

Probe ID	Gene Symbol	Gene Name	Fold-Change
202018_s_at	LTF	lactotransferrin	-1.94

206676_at	CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	-1.80
212768_s_at	OLFM4	olfactomedin 4	-1.68
207802_at	CRISP3	cysteine-rich secretory protein 3	-1.57
210244_at	CAMP	cathelicidin antimicrobial peptide	-1.39
207329_at	MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	-1.33
220570_at	RETN	resistin	-1.30
209771_x_at	CD24	CD24 molecule	-1.28
219496_at	ANKRD57	ankyrin repeat domain 57	-1.14
219818_s_at	GPATCH1	G patch domain containing 1	-1.12
211113_s_at	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.21
203505_at	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	+1.27

Weight Loss

Our molecular studies have shown that lifestyle interventions are useful for cardiovascular risk reduction and weight loss. Weight loss is associated with improved endothelial function and the amount of weight loss may affect improvement in inflammation and vascular health. As part of our ongoing research, we sought to examine relationships between weight loss through changes in lifestyle and peripheral blood gene expression profiles. We conducted a prospective nonrandomized trail conducted over 1 year in participants undergoing intensive lifestyle modification to reverse or stabilize progression of coronary artery disease. Cardiovascular risk factors, inflammatory biomarkers, and gene expression as a function of weight loss were assessed in 89 lifestyle participants and 71 retrospectively matched controls undergoing usual care. We found that substantial weight loss (-15.2±3.8%) in lifestyle participants (n=33) was associated with improvement in selected cardiovascular risk factors and significant changes in peripheral blood gene expression from pre- to post-intervention: 132 unique genes showed significant expression changes (false discovery rate corrected P-value <0.05 and fold-change >1.4). Altered molecular pathways were related to immune function and inflammatory responses involving endothelial activation (Figure 5). Many genes comprising the differentially expressed pathways, which were related to immune and inflammatory responses, were expressed in a variety of leukocyte types, but mainly in activated T-cells and other T-cell populations (Figure 5). In contrast, participants losing minimal weight (-3.1±2.5%, n=32) showed only minor changes in cardiovascular risk factors and markers of inflammation, and no changes in gene expression compared to non-intervention controls after 1 year. Results of this study show that weight loss (>10%) during lifestyle modification is associated with down-regulation of genetic pathways governing interactions between circulating immune cells and the vascular endothelium and may be required to successfully reduce CVD risk.



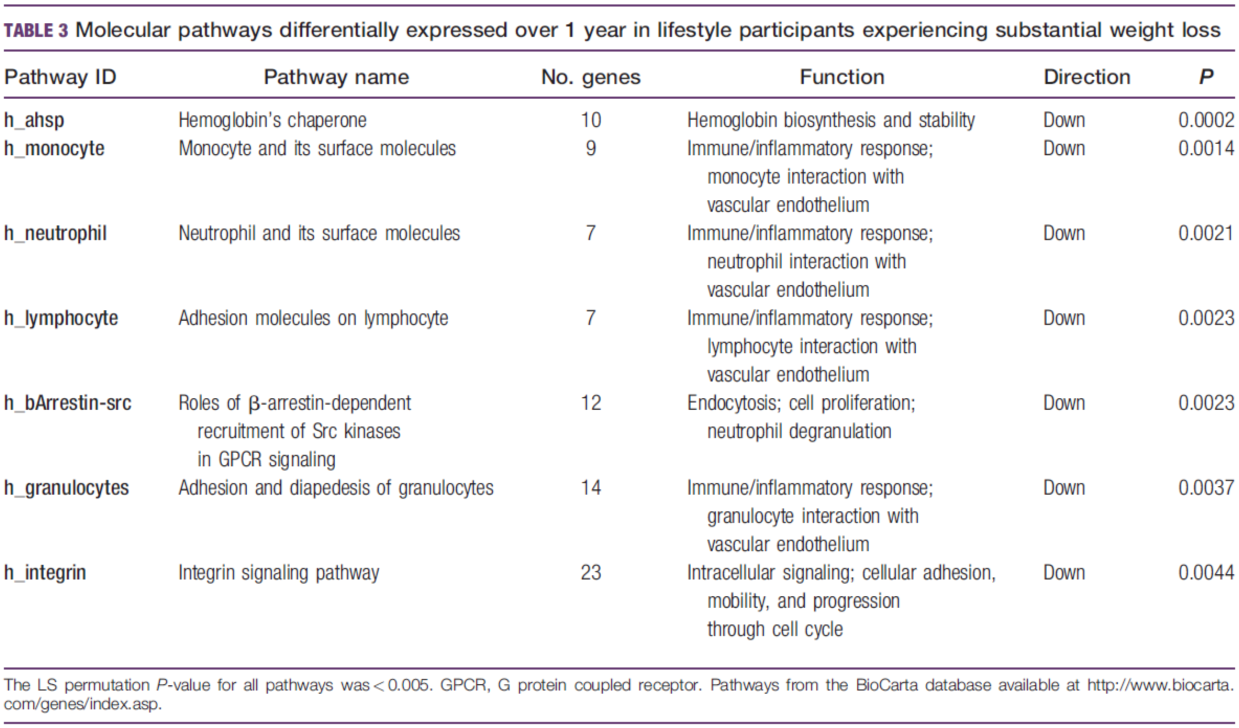


TABLE 3 Molecular pathways differentially expressed over 1 year in lifestyle participants experiencing substantial weight loss

Pathway ID	Pathway name	No. genes	Function	Direction	P
h_ahsp	Hemoglobin's chaperone	10	Hemoglobin biosynthesis and stability	Down	0.0002
h_monocyte	Monocyte and its surface molecules	9	Immune/inflammatory response; monocyte interaction with vascular endothelium	Down	0.0014
h_neutrophil	Neutrophil and its surface molecules	7	Immune/inflammatory response; neutrophil interaction with vascular endothelium	Down	0.0021
h_lymphocyte	Adhesion molecules on lymphocyte	7	Immune/inflammatory response; lymphocyte interaction with vascular endothelium	Down	0.0023
h_bArrestin-src	Roles of β-arrestin-dependent recruitment of Src kinases in GPCR signaling	12	Endocytosis; cell proliferation; neutrophil degranulation	Down	0.0023
h_granulocytes	Adhesion and diapedesis of granulocytes	14	Immune/inflammatory response; granulocyte interaction with vascular endothelium	Down	0.0037
h_integrin	Integrin signaling pathway	23	Intracellular signaling; cellular adhesion, mobility, and progression through cell cycle	Down	0.0044

The LS permutation *P*-value for all pathways was < 0.005. GPCR, G protein coupled receptor. Pathways from the BioCarta database available at http://www.biocarta.com/genes/index.asp.

Figure 5. (top) Blue squares denote genes comprising immune and inflammatory pathways, which were significantly down-regulated during substantial weight loss in this study, that are expressed in various subpopulations of human leukocytes. (bottom) Molecular pathways that were altered during lifestyle modification that are related to immune function and inflammatory responses involving endothelial activation.

Task #5: Discover new genetic influences on heart disease by profiling micro-RNAs and rare RNA transcripts

Expression profiling of microRNAs and rare RNA transcripts was completed in Tasks #2 and #3 using LAGB patients and participants in the lifestyle modification intervention.

Task #6: Develop systems biology approach to integrate various types of risk factor data

Near the end of the funding period, we began the systems biology approach to integrating various types of genetic, biochemical, and risk factor data collected in the Integrative Cardiac Health Program. We hope to derive maximum information from this data by collaborating with scientists who have expertise in systems biology to integrate all of the different types of data. This approach will allow us to uncover inter-relationships and patterns within the data that may not be apparent when each modality is analyzed independently. We hope to conduct this analysis using funding from other sources.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. Completed first ever analysis of changes in genome-wide patterns of DNA methylation in response to lifestyle modification and weight-loss surgery.
2. Published paper describing how intensive cardiovascular risk reduction induces sustainable changes in the expression of genes and pathways that are important to healthy vascular function.
3. Published paper on the importance of losing a substantial amount of weight (>10%) in order to alter gene expression during cardiovascular lifestyle modification.

5. CONCLUSION: Substantial molecular changes occur following LAGB surgery and during lifestyle modification for cardiovascular risk reduction.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Publications

1. Voeghtly LM, Neatrour DM, Decewicz DJ, Burke A, Haberkorn MJ, Lechak F, Patney HL, Vernalis MN, Ellsworth DL. Cardiometabolic risk reduction in an intensive cardiovascular health program. Nutr Metab Cardiovasc Dis 2013;23:662-669.
2. Ellsworth DL, Croft DT Jr, Weyandt J, Sturtz LA, Blackburn HL, Burke A, Haberkorn MJ, McDyer FA, Jellema GL, van Laar R, Mamula KA, Vernalis MN. Intensive cardiovascular risk reduction induces sustainable changes in expression of genes and pathways important to vascular function. Circ Cardiovasc Genet 2014;7:151-160.
3. Blackburn HL, McErlean S, Jellema GL, van Laar R, Vernalis MN, Ellsworth DL. Gene expression profiling during intensive cardiovascular lifestyle modification: Relationships with vascular function and weight loss. Genom Data 2015;4:50-53.
4. Ellsworth DL, Mamula KA, Blackburn HL, McDyer FA, Jellema GL, van Laar R, Costantino NS, Engler RJ, Vernalis MN. Importance of substantial weight loss for altering gene expression during cardiovascular lifestyle modification. Obesity 2015;23:1312-1319.
5. Ellsworth DL, Costantino NS, Blackburn HL, Engler RJM, Kashani M, Vernalis MN. Lifestyle interventions differing in intensity and dietary stringency improve

insulin resistance through changes in lipoprotein profiles. Obes Sci Pract 2016; (in press).

Presentations

1. Ellsworth DL, Croft DT Jr, Burke A, Haberkorn MJ, Patney HL, Mamula KA, Vernalis MN. The importance of weight loss for effecting molecular change during intensive cardiovascular risk reduction. Obesity 2012: 30th Annual Scientific Meeting, September 20-24, 2012, San Antonio, TX.
2. Miller EJ, Mamula KA, Leng L, Piecychna M, Vernalis MN, Bucala R, Ellsworth DL. Cardiovascular disease risk factor modification decreases HS-CRP and Macrophage Migration Inhibitory Factor (MIF): Influence of gender. American Heart Association Scientific Sessions 2012, November 3-7, 2012, Los Angeles, CA.
3. Decewicz A, Hicks M, Mamula KA, Burke A, Haberkorn MJ, Patney HL, Vernalis MN, Ellsworth DL. SNPs associated with plasma triglyceride levels influence response during intensive cardiovascular risk reduction. American Society of Human Genetics, November 6-10, 2012, San Francisco, CA.
4. Blackburn HL, Mamula KA, Haberkorn MJ, Burke A, Slavik JE, Sann NJ, Marley KR, Vernalis MN, Ellsworth DL. Differential effectiveness of laparoscopically-adjustable gastric banding versus lifestyle modification for modifying plasma lipoprotein profiles. Obesity 2013: 31st Annual Scientific Meeting, November 11-16, 2013, Atlanta, GA.
5. Ellsworth DL, Mamula KA, Blackburn HL, Engler RJM, Vernalis MN. Cardiac lifestyle interventions differing in dietary stringency improve insulin resistance through changes in lipoprotein profiles. American College of Cardiology 2015, March 14-16, 2015, San Diego, CA.
6. Vernalis M, Engler R, Mamula K, Blackburn H, Villines T, Kashani M, Ellsworth D. Weight loss impact on insulin resistance as a measure of pre-diabetes: a novel lipoprotein insulin resistance index (LP-IR) identifies differing phenotypes of response to lifestyle-dietary interventions. Military Health System Research Symposium, August 17-20, 2015, Fort Lauderdale, FL.
7. Ellsworth DL, Costantino NS, Blackburn HL, Engler RJM, Vernalis MN. Cardiac interventions differing in lifestyle modification intensity improve insulin resistance through changes in lipoprotein profiles. American Heart Association EPI/Lifestyle 2016 Scientific Sessions, March 1-4, 2016, Phoenix, AZ.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS: Nothing to report.

10. REFERENCES: Nothing to report.

11. APPENDICES: Nothing to report.