EVALUATING THE IMPACT OF A COMMUNITY-BASED DIABETES INTERVENTION
ON MONOCYTE EPIGENOMES OF NATIVE HAWAIJANS AND PACIFIC PEOPLES

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ABSTRACT

Diabetes mellitus disproportionally affects Native Hawaiians and Pacific Islanders. To reverse these disparities, the Partners in Care (PIC) community-based diabetes self-management intervention program was established to improve glycemic control. Little is known about the molecular mechanisms associated with the change in glycemic control in the PIC intervention. The objective of this study was to understand how inflammatory states, and underlying epigenetic processes, of monocytes from peripheral blood mononuclear cells (PBMC) respond to behavioral changes implemented by the PIC. To test this hypothesis, PBMCs were isolated from whole blood samples that were collected from sixteen type 2 diabetic patients with A1c > 7% pre- and post-PIC intervention over three months. Monocytes were isolated and their inflammatory response characterized using flow cytometry. Infinium HumanMethylation 450 BeadChip technology was used to survey the DNA methylomes of monocytes pre- and post-intervention. Results showed genome-wide differential DNA methylation patterns in the monocytes. These changes corresponded to a reduction of monocyte inflammatory response and improved A1c level. Differentially methylated loci correlate with inflammatory and glucose metabolism gene networks. Together, these results suggest that epigenetic processes that regulate the pro-inflammatory response of monocytes are linked to glycemic control and can be altered by behavioral changes. The observed DNA methylated loci may be used to monitor an individual’s response to a diabetes self-management program and the potential long-lasting influences of the program on their overall health.
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List of Abbreviations and Symbols

2hrPG: Two Hour Plasma Glucose
A1c: Glycated Hemoglobin
BMI: Body Mass Index
CBPR: Community Based Participatory Research
CCL2: Chemokine Ligand 2
CD14: Cluster Differentiation 14
CD16: Cluster Differentiation 16
CpG: Cytosine phosphate Guanine
CVD: Cardiovascular Disease
DM: Diabetes Mellitus
DNA: Deoxyribonucleic acid
DNMT: DNA Methyltransferase
EWAS: Epigenome Wide Association Studies
FA: Fatty Acid
FPG: Fasting Plasma Glucose
IL-1b: Interleukin 1 Beta
IL-6: Interleukin 6
IL-8: Interleukin 8
LDL: Low Density Lipoprotein
LPS: Lipopolysaccharide
MO: Monocyte
MONO-ICS: Monocyte Intracellular Cytokine Staining
NH/PIs: Native Hawaiians and Pacific Islanders
PBMC: Peripheral Blood Mononuclear Cells
PIC: Partners in Care
POP: Partnership for Improving Lifestyle Intervention ‘Ohana Project
RNA: Ribonucleic Acid
SDSCA: Summary of Diabetes Self-Care Activities
$t_0$: Baseline
$t_3$: Three Months
$t_6$: Six Months
T2DM: Type 2 Diabetes Mellitus
TLR: Toll Like Receptor
TNFa: Tumor Necrosis Factor Alpha
CHAPTER 1: INTRODUCTION

Diabetes Prevalence
The prevalence of Type 2 Diabetes Mellitus (T2DM) diagnosis in the United States has increased 176% over the past 30 years [CDC 2014]. As of 2010, the nationwide prevalence for T2DM was 8.3%, comparable to Hawaii’s 8.2% overall prevalence [CDC 2010]. There is an ethnic disparity for T2DM that persists in Hawaii and is greater among Native Hawaiians and other Pacific Islanders (NH/PIs) as shown by higher morbidity and mortality from T2DM and its associated complications, including cardiovascular disease (CVD) [Nguyen et al., 2013]. As of 2013, 11% of Native Hawaiians were diagnosed with T2DM [Nguyen et al., 2013]. The drastic increase of T2DM correlates with a global rise of obesity and unhealthy lifestyles [Forouhi & Wareham 2014].

Clinical Perspective
The first diagnosis of Diabetes mellitus (DM) dates back to 1500bc. DM is a disease that affects the concentration of blood plasma glucose, due to the body’s inability to properly use or secrete insulin, resulting in starvation of cells and abnormally high glucose concentrations in the blood plasma. Glucose is essential to cells because its catalysis provides the energy necessary for cells to perform their functions, which is important to the overall health of the body. The transport of glucose into the cell is facilitated by insulin, a peptide hormone secreted by the beta cells of the pancreas. Prolonged glucose cell starvation can lead to a reduction in normal body function and the onset of DM symptoms including unexplained weight loss, extreme hunger, and feeling tired much of the time [CDC 2014]. There are three classical types of DM that are defined by the time of DM onset and the basis of the altered insulin mechanism. In T2DM the body becomes resistant to insulin being produced, resulting in a lack of glucose transport into cells [ADA 2014]. The two other classes of DM include early onset DM (Type 1 DM) and
gestational DM. For this study, we focus on T2DM, as it is the most prevalent type of DM experienced.

**Diagnosis**

The concentration of glucose in the blood plasma is used to diagnose DM. There are three standard of care tests used to diagnose DM: (1) fasting plasma glucose (FPG), (2) two-hour plasma glucose (2hrPG), and (3) glycated hemoglobin (A1c) level tests [ADA 2014]. The FPG test examines the body’s ability to process glucose after no caloric intake, fasting, for at least 8 hours. DM is diagnosed in patients with a FPG greater than or equal to 126mg/dL. The 2hrPG tests the body’s ability to tolerate an immediate large influx of glucose, through an oral glucose tolerance test, where 75 grams of anhydrous glucose dissolved in water is consumed, and then plasma glucose is tested after 2 hours. DM is diagnosed in patients with a 2hrPG test value greater than or equal to 200mg/dL. Unlike the FPG and 2hrPG tests, the A1c test is convenient and patients do not need to fast prior to the test. In addition, A1c levels do not fluctuate relative to the patient’s daily lifestyle choices [ADA 2014]. The A1c test measures the percentage of glycated hemoglobin present in blood. Higher A1c levels are indicative of reduced control of blood glucose levels, where DM is diagnosed in patients with A1c levels greater than 6.5% [ADA 2014]. Limitations of the A1c test include problems with interpreting results from patients with abnormal hemoglobin and variation of glycation rates based on race [Ziemer et al., 2010; Kumar et al., 2010].

Several studies have identified a disparity of T2DM prevalence amongst Native Hawaiians and Pacific Islanders in comparison to their Caucasian counterparts [Furbuyashi & Look 2005; McEligot et al., 2010; Wong et al., 2009; Lee et al., 2010]. Race as well as cholesterol and triglyceride levels, physical inactivity, hypertension, and obesity are all risk factors for T2DM [ADA 2014]. There is a clear disparity amongst ethnic populations in Hawaii
for obesity. Seventy-two percent of Native Hawaiians are overweight, defined by BMI 25.0-29.9, or obese, defined by BMI above 30, in comparison to the other ethnic populations in Hawaii with an overweight or obese sub-population of 55.7% for Whites, 52.1% for Filipinos, 47.8% for Japanese and 56.5% for Others [Ngyuen & Salvail 2013]. Multiple mechanistic links between obesity, insulin resistance, and T2DM have been identified but not yet clearly defined [Kahn et al., 2006]. One of the proposed mechanisms linking obesity and insulin resistance is based on microinflammation and the release by macrophages of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), [Yang et al., 2012; Wellen et al., 2005; Fain et al., 2004].

**Inflammatory Response**

Macrophage accumulation in adipose tissue has been associated with obesity [Weisberg et al., 2003]. In obese individuals, adipose tissue is stressed due to excessive amounts of fat. The excess fat globules release inflammatory factors, resulting in a low-grade chronic inflammatory state; see *figure 1* [Odegaard & Chawla, 2012]. Inflammatory factors, such as pro-inflammatory and anti-inflammatory cytokines, regulate inflammation. They are small dynamic signaling proteins that are produced by various cells and have the ability to respond to and elicit a response to cellular signals in the body. When sustained, this low-grade microinflammation causes tissues to become resistant to insulin, the first step to developing T2DM [Odegaard & Chawla, 2012]. When accumulation of macrophages is interrupted, inflammation as well as insulin resistant abnormalities is ameliorated [Vachharajani & Granger, 2009].
Figure 1. A scheme for Adipose Tissue Inflammation and Insulin Resistance

At the sites of inflammation, macrophages differentiate from their precursor cells, monocytes [Ginhoux et al., 2014]. Monocytes are a subgroup of leukocytes in the blood, which originate from hematopoietic progenitor cells of the bone marrow [Shi et al., 2011]. They make up 3-7% of the circulating leukocyte pool, have an average circulation time in the bloodstream of 32 hours and are able to patrol the luminal surface of the endothelium [Van Furth et al., 1972]. Upon detecting recruitment factors that signal an inflammation site, monocytes recruit other immune cells and further differentiate into macrophages at tissue sites [Ginhoux et al., 2014]. Figure 2 shows the production of monocytes in the bone marrow, their transition to the bloodstream, and their recruitment to inflamed tissues where they differentiate into macrophages.
Figure 2. Monocyte Origination, Circulation and Differentiation (adapted from Ginhoux 2014). Hematopoietic progenitor cells (HPCs) of the bone marrow differentiate into monocytes (MO) in the bone marrow, which can migrate into the bloodstream with the help of chemokines. Upon detecting recruitment factors that signal an inflammation site, monocytes recruit other immune cells. Monocytes also migrate into tissues where they can differentiate into macrophages.

Monocytes can be classified into several subsets defined by their expression of lipopolysaccharide (LPS) co-receptors CD14 and CD16. The three well-known subsets of human monocytes are: 1) Classical monocytes (CD14++ CD16-) that are characterized by high expression of CD14 and no expression of CD16, 2) intermediate monocytes (CD14++ CD16+) that express high levels of CD14 and CD16, and 3) non-classical monocytes (CD14+ CD16++) that express lower levels of CD14 than classical and intermediate monocytes but higher levels of CD16 on their surface [Ziegler-Heitbrock et al., 2010]. CD16 expressing monocytes have been characterized as pro-inflammatory cells [Ziegler-Heitbrock, 2000] that have been shown to have a high capacity for the uptake of oxidized LDL and that secrete pro-inflammatory cytokines, TNFα and IL-1β [Cros et al., 2010; Mosig et al., 2008]. TNFα and IL-1β have been shown to
inhibit insulin sensitivity of adipocytes as well as sustain activation of adipose tissue macrophages [Chawla et al., 2011]. Together, macrophages and monocytes play a central role in the human immune system defending from infection as well as contributing to inflammation.

One mechanism that links obesity, inflammation and insulin insensitivity is via the cross talk between adipose tissue macrophages and adipocytes. Obesity results in the recruitment of monocytes and macrophages into the adipose tissue, and promotes adipose tissue inflammation and insulin resistance. Cell surface receptors of macrophages are activated by fatty acids as well as pro-inflammatory cytokines in the adipose tissue, resulting in the activation of inflammatory signaling cascades. These inflammatory pathways induce the production of pro-inflammatory cytokines such as the TNFα and IL-1β. The pro-inflammatory cytokines reduce insulin sensitivity of adipocytes. Once initiated, these pro-inflammatory cascades are perpetuated by crosstalk between the inflamed adipocytes and classically activated adipose tissue macrophages via the production of various pro-inflammatory factors. Chemokine secretion, such as CCL2, leads to the recruitment of inflammatory monocytes, which differentiate into classically activated adipose tissue macrophages and enhance adipose tissue inflammation. This is a cycle that links obesity, insulin insensitivity and inflammation, which is depicted in figure 3 [Chawla et al., 2011]. Recently, it has been shown that production of pro-inflammatory cytokines is under epigenetic regulation [Tekpli et al., 2013].
Figure 3. Molecular Pathways of Obesity, Insulin Insensitivity and Inflammation (adapted from Chawla, 2011). Toll-like receptors (TLR) are activated by fatty acids in adipose tissue macrophages, resulting in the activation of the inflammatory signaling cascades. These pathways induce the production of pro-inflammatory cytokines such as TNFα and IL-1β. The pro-inflammatory cytokines inhibit the insulin sensitivity of adipocytes. Once initiated, these pro-inflammatory cascades are perpetuated by crosstalk between the inflamed adipocytes and classically activated adipose tissue macrophages via the production of various factors. CCL2 leads to the recruitment of inflammatory monocytes, which differentiate into classically activated adipose tissue macrophages to enhance adipose tissue inflammation. The inflammatory cycle is perpetuated by saturated fatty acids and pro-inflammatory cytokines from adipocytes, which sustain the activation of adipose tissue macrophages.

Epigenetics

Epigenetics is the study of heritable changes in gene function that cannot be explained by changes in DNA sequence [Russo et al., 1996, Waterland et al., 2006]. Pluripotent genes of embryonic stem cells have been shown to be influenced by epigenetic processes, leading to cell type differentiation [Farthing et al., 2008]. The epigenetic process is dynamic, with the ability to alter gene expression and influence disease states [Lancaster et al., 2009, Garcia et al., 2005, Sharma et al., 2010]. Environmental factors such as poor diet, stress and smoking have been
associated with influencing epigenetic marks, chemical tags that decorate the genome [Ordovas & Smith, 2010]. Research on the epigenome, the underlying DNA sequence and the epigenetic marks, focuses on understanding epigenetic modifications as it relates to development and disease. There are three general types of epigenetic modifications, two of which use chemical epigenetic marks to alter gene expression and include post-translational histone tail modifications and DNA methylation, as well as RNA interference. RNA interference mechanisms utilize small non-coding RNA molecules to regulate transcriptional and post-transcriptional gene expression [Carthew et al., 2009; Cui et al., 2009; Rakyan et al., 2011]. DNA methylation is the best-characterized epigenetic mechanism and is the focus of this thesis.

DNA methylation, similar to histone modification, is an important feature of chromatin remodeling that influences gene activity by altering DNA accessibility; see figure 4 [Holiday and Pugh 1975, Riggs 1975, Jaenisch, R. & Bird 2003]. DNA methylation is mediated by DNA methyltransferases (DNMTs), which are responsible for methylating the 5'-carbon position of the nucleic acid cytosine figure 5 [Leonhardt et al., 1992; Pradhan et al., 1999]. In mammals, cytosine methylation usually occurs adjacent to a guanine nucleic acid at a so-called CpG site [Bird, 2002]. Areas of DNA that stretch greater than 500 bases in size with a CpG content greater than 65% are called CpG islands [Takai & Jones 2002]. Changes in DNA methylation states at CpG islands relative to normal cells, causing either hypermethylation (abnormally high) or hypomethylation (abnormally low) within the promoter region and within the gene body, have been associated with disease states [Weber et al., 2007; Maunakea et al., 2010; Jones & Baylin 2002; Laird, 2010]. For this and other reasons, DNA methylation is a well-suited epigenetic mark for epigenome wide association studies (EWAS); large-scale systematic studies focused on
mapping disease-associated methylations across the genome with specific diseases [Rakyan et al., 2011, Toperoff et al., 2012].

Figure 4. Epigenetic Modifications (adapted from Baccarelli and Ghosh 2012). Histone modifications, such as changes to the charge of the protein, impact chromatin coiling. DNA methylation can alter transcription factor binding, which affects downstream gene transcription.

Figure 5. Cytosine methylation. Cytosine is methylated at the 5'-carbon position. The methyl group from S-Adenosyl Methionine (SAM-CH3) is transferred to cytosine through enzymatic activity catalyzed by DNA methyltransferase (DNMT).

A T2DM EWAS comparing peripheral blood of T2DM patients with non-DM participants was performed by Toperoff et al., 2012. Although the study defined an inter-
individual T2DM DNA methylation profile, it did not address the EWAS issues of DNA methylation, such as cell type specificity and individual participant variations [Toperoff et al., 2012, Rakyan et al., 2011]. A recent case study examined promoter region DNA methylation of adipose and skeletal muscle tissues of discordant monozygotic twins associated with T2DM [Ribel-Madsen et al., 2012]. However, differences in promoter region methylation between the twins were relatively small, most of them not significantly related to T2DM [Ribel-Madsen et al., 2012]. Environmental stimuli, such as diet and exercise, and its association with DNA methylation have revealed DNA methylation patterns and potential epigenetic biomarkers of obesity [Milagro et al., 2011, Ronn et al., 2013]. These studies have established a potential DNA methylation profile associated with T2DM and its ability to be altered by environmental factors. However, no study to date has been able to track whether changes in T2DM condition would relate to modifications of DNA methylation in peripheral cells.
CURRENT STUDY RATIONALE

Environmental factors, including poor diet and physical inactivity, impact onset of T2DM and have been associated with the modification of epigenetic marks [Ordovas 2010]. This type of dysregulation of epigenetic marks can be detected in peripheral blood [Baccarelli & Ghosh 2012]. The peripheral blood, specifically intermediate monocytes, of dialysis patients have been shown to independently predict cardiovascular disease [Rogacev et al., 2012]. In patients with T2DM, non-classical monocytes have been shown to contribute to microinflammation [Yang et al., 2012]. Furthermore, a preliminary study of cytokine production upon lipid stimulation of monocytes identified an inverse correlation between the production of pro-inflammatory cytokines, IL-1β and IL-8, and insulin sensitivity [Barbour et al., unpublished]. These studies suggest a potential link between environmental stimuli and microinflammation of monocytes that may be regulated by epigenetic modifications, which could be the underlying molecular mechanism associated with changes to insulin sensitivity leading to onset of T2DM.
OBJECTIVES

The objective of this thesis was to evaluate the impact of a community-based diabetes intervention on monocyte epigenomes of Native Hawaiians and Pacific Islanders. We hypothesize that the intervention improves glycemic control by influencing the epigenetic regulation of genes involved in the inflammatory states of monocytes. The specific aims of the research are to 1) evaluate and compare the inflammatory response of monocytes from T2DM participants in a NH/PI community pre- and post-intervention, 2) characterize and compare the DNA methylomes of sort-purified monocytes among participants pre- and post-intervention, and 3) integrate the results of the study to provide perceivable information for the community.
CHAPTER 2: EVALUATION AND COMPARISON OF THE INFLAMMATORY RESPONSE OF MONOCYTES FROM T2DM PARTICIPANTS IN A NH/PI COMMUNITY PRE- AND POST-INTERVENTION

Rationale
To address the obesity-related disparities amongst Native Hawaiians and Pacific Islanders, a community-based participatory research (CBPR) partnership was established in 2005 via the Partnership for Improving Lifestyle Intervention ‘Ohana Project (POP) [Nacapoy et al., 2008]. The POP program is structured on a standard behavioral weight loss maintenance program but is culturally tailored for Native Hawaiians and Pacific Islanders. Results have shown the POP program to be more effective for NH/PIs than standard behavioral weight loss maintenance programs [Kaholokula et al., 2012]. The POP program has established a positive relationship with the NH/PI communities through active engagement, communication and inclusion of NH/PI culture.

One of the outcomes of the POP partnership is Partners in Care (PIC). PIC is a NH/PI culturally adapted intervention for DM self-management, which was found to improve glycemic control and reduce DM-specific emotional distress in NH/PI communities across the state of Hawaii [Sinclair et al., 2013]. The PIC intervention focuses on educating participants on positive behavioral choices including exercise and diet. Nutritional factors have been shown to influence inflammatory states in monocytes of DM patients [Riek et al., 2012]. Monocytes from DM patients contribute to microinflammation and can independently predict CVD events [Yang et al., 2012, Rogacev et al., 2012]. In order to gain a better understanding of the underlying molecular mechanisms associated with the improvement of glycemic control in the PIC study, I chose to analyze monocytes of PIC participants’ pre- and post- intervention. Based on previous studies, I hypothesize that the pro-inflammatory response driven by monocytes is reduced in patients responsive to the PIC intervention, which may underlie their improved glycemic control.
Methods

Partners in Care (PIC) Intervention

The PIC intervention was implemented following the methods described in Sinclair et al., 2013. Clinical measurements were taken at baseline (t₀), 3 months (t₃) and 6 months (t₆). The measurements include weight, BMI, A₁c, cholesterol (LDL & HDL), triglycerides, and diastolic and systolic blood pressure. Behavior was measured by examining ‘Problem Areas in Diabetes’ which measured feelings related to living with diabetes and treatment, higher scores indicating greater diabetes-related emotional stress; ‘Diabetes Care Profile’ which measured the understanding of aspects of diabetes self-care, higher scores indicating better understanding. Finally, ‘Summary of Diabetes Self-Care Activities’ (SDSCA) assessed the frequency with which an individual follows a diabetes self-care routine; higher scores indicating more frequent self-care.

Participant selection

A total 16 participants were drawn from a parent PIC intervention cohort study from the same community, Papakolea, and were a part of the Kula no Nā Poʻe Hawaiʻi community organization. Each of the participants self-reported physician-diagnosed T2DM, were 18 years of age or older, were self-reported Native Hawaiian, Filipino, or of other Pacific Islander ethnic background, were English-speaking, and had a baseline A₁c >7%. Ten of the participants were male and six were female. The age of the group was 50.3 +/- 5.04 years, with a median of 51.5 and range of 26-65.

A sample size of 16 individuals was chosen based on sample size calculations with Type I error, α= 0.05, two sided, and Type II error, β=0.2 or power=80%. The sample size selection
was also supported by preliminary data. All of participants provided written informed consent to the study’s purpose and procedures. The informed consent was approved by two Institutional Review Boards: University of Hawai‘i and Papa Ola Lokahi Native Hawaiian Health Board.

Blood biospecimen collection and PBMC isolation

Licensed phlebotomists gathered 20ml of anti-coagulated blood from each participant at the community organization’s site before and after completion of PIC. Peripheral blood mononuclear cells (PBMCs) from whole blood were separated via gradient centrifugation with Ficoll-Paque™ (Miltenyi Biotech) and the PBMCs were cryopreserved, in liquid nitrogen immediately after separation. No remaining blood or its components were stored and samples were de-identified upon delivery for the PBMC isolation.

Assessment of cell population purity, monocyte purification, and functional viability

Cryopreserved PBMCs were thawed followed by a resting overnight period at 37°C and 5% CO2 in a polypropylene plate, a protocol optimized by Jalbert et al., 2013 to avoid monocyte differentiation in culture, and to preserve ex vivo surface expression of CD14 and CD16. The cells were stimulated with LPS (100ng/ml) or media alone (unstimulated) for 6 hours in the presence of brefeldin-A (5µg/ml) and monensin (5µg/ml). Cells were then surface-stained with CD3 (V500), CD14 (Qdot605), CD16 (Alexa700), CD56 (PE-Cy7), CD19 (PE-Cy7), CD20 (PE-Cy7), HLA-DR (APC-H7) antibodies, and with Live/Dead fixable yellow dead cell stain (yellow amine reactive dye: YARD). Cells were subsequently fixed, permeabilized (BD FACS Permeabilizing Buffer II) and stained with conjugated antibodies against IL-1β (PE), IL-8 (FITC), IL-6 (APC) and TNFα (PerCP-Cy5.5). All antibodies were from BD Biosciences, except for
CD14 Q605 and TNFα (eBiosciences). The Live/Dead fixable yellow dead cell stain was from Life Technologies. Data were acquired on a custom 4-laser BD LSRFortessa, and all compensation and gating analyses were performed in FlowJo (TreeStar) to select for monocytes, as seen in figure 6. Data were analyzed based on gating strategies described by Heine et al., 2012 and Jalbert et al., 2013.

![Figure 6. Monocyte Enrichment Diagram.](image)

Monocytes were enriched for using the EasySep protocol. Cell populations were phenotyped pre- and post-enrichment. Based on CD14 antibody fluorescent tagging and gating strategies monocyte populations were defined and quantified. DNA was extracted from sample monocytes enriched using the EasySep protocol. Monocytes for the functional viability were defined and quantified from PBMCs post-stimulation.
Results
Clinical and Behavioral Measurements

Clinical and emotional measurements were taken at baseline (t₀), 3 months (t₃) and 6 months (t₆) based on the PIC intervention protocol to quantify the changes associated with PIC. For this cohort of PIC participants, there was an average statistically significant reduction in several study parameters after three months, including weight (Δχ=−2.06kg), BMI (Δχ=−0.76), total cholesterol (Δχ=−15.38mg/dL), and Problem Areas in DM (Δχ=−12.5), see Table 1. When comparing the group average A1c levels the reduction (Mean -0.16+/−1.07) was not statistically significant (p=0.5538).

However, when the participants were stratified into “early” improvers, defined as A1c reduction between t₀-t₃, and “late” improvers, defined as A1c reduction between t₃-t₆, there was a statistically significant reduction in A1c levels within these subgroups (early improvers Δχ=−0.65, p<0.008; late improvers Δχ=−0.68, p<0.06; see figure 8) suggesting potential implications of PIC improvement results seen in participants with lower baseline A1c before those with higher A1c at baseline. Similar to Sinclair et al., 2013, this PIC study aided with the reduction of T2DM symptoms as seen by the reduction of weight, BMI, LDL, cholesterol, and triglycerides. PIC also helped in reducing emotional stress associated with DM.
| Variable                  | N  | N Miss | Mean  | Std Dev | Median | Max  | P > |t| |
|---------------------------|----|--------|-------|---------|--------|------|-----|---|
| Weight (kg)               | 16 | 0      | -2.06 | 2.71    | -1.6   | 1.7  | 0.0084** |
| BMI                       | 15 | 1      | -0.76 | 0.84    | -0.65  | 0.69 | 0.0037*  |
| A1c                       | 16 | 0      | -0.16 | 1.07    | -0.3   | 2.7  | 0.5538   |
| LDL (mg/dL)               | 7  | 9      | -10.71| 22.56   | -7     | 18   | 0.2556  |
| HDL (mg/dL)               | 11 | 5      | 1     | 9.01    | -1     | 16   | 0.7205  |
| Total Cholesterol         | 16 | 0      | -15.38| 28.56   | -14    | 36   | 0.048*  |
| Triglycerides             | 16 | 0      | -20   | 209.13  | -35    | 412  | 0.7074  |
| Systolic BP               | 15 | 1      | 6.57  | 19.82   | 0      | 68.5 | 0.2203  |
| Diastolic BP              | 15 | 1      | 6.9   | 10.24   | 5      | 37   | 0.0205* |
|                           |    |        |       |         |        |      |      |
| Problem Areas in Diabetes | 16 | 0      | -12.5 | 20.56   | -8.75  | 22.5 | 0.028*  |
| Diabetes Care Profile     | 16 | 0      | 0.68  | 1.01    | 0.42   | 3    | 0.017*  |
| SDSCA score               | 16 | 0      | 0.38  | 6.32    | 1.5    | 14   | 0.8157  |

Table 1. Clinical and Emotional measurements. $t_3$ to baseline difference for clinical and emotional measurements. Mean ± 1 SD; * $p < .05$, ** $p < .01$. N: sample size. N miss: number of study participants among the 16 not included in measurement. Mean: average change in sample group, $t_3$-$t_0$. Std Dev: average change in standard deviation, $t_3$-$t_0$. Median: change in sample group median $t_3$-$t_0$. Max: maximum change in sample group, $t_3$-$t_0$.  


Figure 7. Pre- and post-PIC A1c levels. Early improvers (n=11) had a statistically significant reduction in A1c (p < 0.008) level between Baseline, 3 months (3M) and 6 months (6M). The median baseline A1c for early improvers was 8%. Late improvers (n=5) had a statistically significant reduction in A1c (p < 0.06) level between 3M and 6M with a median baseline A1c of 9.9%.
Monocyte Inflammatory Response

Cytokine production, specifically that of pro-inflammatory cytokines, is associated with inflammation. In order to characterize the inflammatory response of the monocytes, we performed an intracellular cytokine staining assay (Mono-ICS). Inflammatory responses of monocytes were measured by production of cytokines IL-1β, IL-6, IL-8 and TNFα. Figure 9 displays the gates used to determine cytokine positivity in total monocytes and shows that stimulating with LPS activates cytokine production. The monocyte inflammatory response was strongly attenuated post-PIC; see figure 10. IL-1β (p=0.0040) and IL-6 (p=0.0020) production were significantly reduced post-PIC upon LPS stimulation. There was a significant reduction in production of IL-8 (p=0.0282) upon stimulation, however it was not significant when comparing the change, stimulation to no-stimulation, pre- and post- PIC. There was no change seen in TNFα response. Figure 11 shows the inflammatory response when using re-PIC and post-PIC, paired samples (n=3). Due to sample integrity issues, only three of the participants’ samples were used for pre-PIC versus post-PIC cytokine production comparisons. As suggested by the analysis of unpaired samples, the paired samples confirmed the statistically significant reduction in production of IL-1β (p=0.004) and IL-6 (p=0.02).

The monocyte inflammatory response post-PIC suggests that the PIC intervention strongly attenuates pro-inflammatory cytokine production. While these results warrant further investigation, they suggest that following PIC, diabetic patients harbor monocytes that are intrinsically in a lower inflammatory status than prior to PIC.
Figure 8. Cytokine production gating strategy. Intracellular cytokines (IL-1β, IL-6, IL-8 and TNFα) produced in total monocytes were detected without stimulus (No Stim) or in response to lipopolysaccharide (LPS).
Figure 9. Monocyte production of pro-inflammatory cytokines is strongly attenuated post-PIC. Frequency of monocytes expressing pro-inflammatory cytokines, the change (Δ) in cytokine response between no stimulation (NS) and stimulation (LPS). Upon stimulation post-PIC (n=10) showed reduced cytokine (IL-1β, IL-6, IL-8 and TNFα) production in comparison to pre-PIC (n=4). There was a statistically significant reduction in production of IL-1β (p=0.0040) and IL-6 (p=0.0020).
Figure 10. Paired Inflammatory Response. Monocyte inflammatory response with paired samples (n=3). The change (Δ) in cytokine response between no stimulation (NS) and stimulation (LPS), in the enriched monocytes both pre- and post-PIC intervention, as seen in Figure 11. There was a statistically significant reduction in production of IL-1β and IL-6.
Discussion

Previously, the PIC intervention showed a statistically significant decrease in A1c levels of participants with T2DM [Sinclair et al., 2013]. Using the same PIC intervention approach, our results initially differed, which is probably due to the smaller sample size (n=16). As we demonstrated with A1c measurement, when looking at the entire cohort of study participants, there was no statistically significant change in A1c levels. However, when the participants were stratified into “early” (A1c improvement between t₀ and t₃) and “late” (A1c improvement between t₃ and t₆) improvers, there was a significant difference between pre- and post-PIC. We found the stratification of A1c improvement to be correlated with the baseline A1c level. This suggests that baseline A1c may predict PIC participants’ responsiveness to the intervention, with those at a higher baseline A1c improving their glycemic control 3 or more months after the end of the intervention. Behavioral measurements associated with the PIC intervention and understanding of DM care showed a change toward less stress as it pertains to DM, and better understanding of the disease. These behavioral measurements may correlate with reduction of inflammatory markers based on the mitigation of stress pathways, such as the hypothalamic-pituitary-adrenal axis, which plays a role in obesity and metabolic disease.

Similar to Yang et al., 2012, we found that monocytes of the PIC participants may contribute to microinflammation as seen by the strong attenuation of pro-inflammatory cytokines post-PIC. All four of the cytokines tested decreased post-PIC, however only IL-1β and IL-6 were statistically significantly reduced. It has been shown that obesity triggers IL-1β production through the activation of inflammasomes in adipose tissue [Stienstra et al., 2010; Vandanmagsar et al., 2011; Wen et al., 2011]. A reduction in IL-1β production post-PIC suggests inflammasome activation may be reversed by upstream signals associated with obesity. IL-6
production is associated with the ability to reduce insulin sensitivity by hampering insulin signaling in hepatocytes [Klover et al., 2003; Kanemaki et al., 1998] and IL-6 is known to contribute functionally to obesity and CVD by decreasing the activity of lipoprotein lipase, which in turn increases macrophage uptake of lipids. IL-6 also stimulates the HPA axis, which plays a role in obesity, hypertension and insulin resistance [Yudkin et al., 2000]. Although TNFα and IL-8 concentrations have been implicated in improper insulin signaling and obesity, we did not see a significant decrease post-PIC [Moller 2000; Nymo et al., 2014]. The minimal TNFα and IL-8 change could be due to TNFα’s dependence on LDL production [Bekkering et al., 2014], which similar to TNFα was not significantly reduced post-PIC.

The behavioral choices made during PIC intervention positively influenced the clinical characteristics associated with T2DM, including BMI, A1c and total cholesterol. Congruent with recent studies, nutritional alterations influence the inflammatory states of monocytes in DM patients [Riek et al., 2012]. Our results show that pro-inflammatory responses, mediated by the cytokines IL-1β and IL-6, are driven by monocytes as seen in participants responsive to the PIC intervention. The implications of this study are that changes in diet, exercise and educational regimes can alter the inflammatory response of monocytes, which may have larger implications on clinical measurements.
CHAPTER 3: CHARACTERIZE AND COMPARE THE DNA METHYLOMES OF MONOCYTES AMONG PARTICIPANTS PRE- AND POST-PIC

Rationale

DNA methylation of monocytes has been shown to be involved in disease conditions, such as atherogenesis [Yideng *et al.*, 2008]. Evidence also points to DNA methylation as the underlying mechanism for inflammatory phenotypes of immune cells [Kim *et al.*, 2012]. More importantly, T2DM EWASs have identified a T2DM DNA methylation profile [Toperoff *et al.*, 2012, Ribel-Madsen *et al.*, 2012], and provide evidence for specific genes that may be regulated by DNA methylation. However, the previous studies failed to address the cell type-specificity of epigenetics as a confounding factor [Rakyan *et al.*, 2011].

In addition to DNA methylation influencing the functions of monocytes and inflammation, previous studies suggest that DNA methylation is under the influence of external factors [Milagro 2011, Ronn 2013]. Exercise has been associated with acute gene activation triggered by DNA hypomethylation [Barres *et al.*, 2012]. Another lifestyle factor, playing a large role in obesity is diet and in both mouse and human studies, changes in diet have been associated with changes in DNA methylation and gene expression [Dolinoy *et al.*, 2008; Chmurzynska, 2010].

In this part of the study, I examined the effects of behavioral choices on DNA methylation profiles of the monocytes. I hypothesized that significant changes in the DNA methylation profiles of loci associated with pro-inflammatory responses, as driven by monocytes, occurs in participants responsive to the PIC intervention. To address this hypothesis, I characterized and compared the DNA methylomes of monocytes among participants pre- and post-PIC intervention.
Methods

Monocyte population purification

Peripheral blood mononuclear cells are composed of different cell types at frequencies that range per individual including T cells (45-70%), B cells (up to 15%), NK cells (up to 15%), dendritic cells (1-2%) and monocytes (10-30%) [Miyahira, 2012]. Cryopreserved PBMCs obtained by venous blood draw, were thawed in serum-free media (AIM V Medium, Life Technologies) containing 10ug/ml of DNase (Sigma). Polypropylene plates and tubes were used to prevent cell differentiation. Monocytes were enriched from the PBMCs using the EasySep Human Monocyte Enrichment Kit without CD16 depletion, according to the manufacture’s recommendations (StemCell) and monocyte enrichment was confirmed via CD14 expression as seen in figure 6.

DNA Extraction

Genomic DNA was isolated from monocytes using the AllPrep DNA/RNA kit (Qiagen) according to the manufacturer’s recommendations immediately after monocyte enrichment. DNA concentration was determined using the Qubit Instrument (Life Technologies).

DNA Methylation Analysis on Illumina 450k Array

500 ng of DNA per sample was bisulfite converted using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer’s instructions. Bisulfite-converted DNA (4 µl per sample) were randomly assigned to a chip wells of the Infinium HumanMethylation450 BeadChip, amplified, hybridized onto the array, stained and imaged with the Illumina iScan SQ instrument to obtain raw image intensities. Array IDAT intensity data were preprocessed in R using the RnBeads 0.99.10 pipeline analysis package [Assenov et al., 2014]. Methylation beta-
values ranging from 0 -1 (corresponding to unmethylated to methylated signal intensity) for each sample were normalized using the methylumi package and subset-quantile within-array normalization (SWAN) method options. Data were filtered for missing probes, SNP-enriched probes, and low detection $P$-value probes (detection $P$-value > 0.05). A total of 485,311 (485,764 - 453) were left after filtering. [Corley et al, 2015]

Gene Ontology

Gene ontology analysis was performed using GREAT: Genomic Regions Enrichment of Annotations Tool (http://bejerano.stanford.edu/great/public/html/index.php) as described in McLean et al., 2010. The human genome was used as background and the level of significance was set to $p<0.05$. The top 5 enriched pathways are described for the differentially methylated genes. The genomic regions were associated with genes using basal plus extension set at proximal 2.0kb upstream, and 1.0kb downstream, plus a distal region of up to 1000.0kb.

Results

Monocyte Enrichment

In order to evaluate cell type-specific inflammatory responses, we enriched for monocytes from PBMCs. The quality of enrichment was quantified using flow cytometry, based on methods by Jalbert et al., 2013. Pre-intervention (pre-enrichment: 11.34+/−5.58% of PBMC; post-enrichment: 64.66+/−25.79% of PBMC) and post-intervention (pre-enrichment: 17.47+/−5.46%; post-enrichment: 71.79+/−8.71%) monocytes were effectively separated from the other cell types such as T cells, B cells and NK cells. Table 2 shows average enrichment percentages. We enriched for CD14 expressing monocytes using the Human Monocyte enrichment kit without CD16 depletion. Based on cell type-specific phenotyping, an increase in monocytes post-enrichment indicated that the majority cell type enriched for was indeed monocytes.
The quality of the enriched monocyte samples in comparison to PBMC populations based on the DNA methylation states distinguishing the different populations was assessed (figure 12). Linear regression was used to define the strength of the correlation between sample DNA methylation profiles and PBMC-specific or monocyte-specific DNA methylation profiles as described in Koestler et al., 2013. The published monocyte-specific methylation profiles versus sample methylation profiles positively correlated (Pearson r=0.84, *p*<0.0001) with the percentage of enriched monocytes as measured by flow cytometry. In comparison, the PBMC-specific methylation profiles versus the study sample methylation profiles correlated negatively with the percentage of enriched monocytes (Pearson r=−0.82, *p*<0.0001). This quality assessment was used to filter the data based on the monocyte cell population purity; samples with a methylation profile versus a monocyte-specific methylation profile with a coefficient of determination (R^2) less than 0.500 were deemed of poor quality (8/30 of the samples) and were excluded from further analysis; see figure 13.

<table>
<thead>
<tr>
<th></th>
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</tr>
<tr>
<td>Paired n</td>
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<td>14</td>
</tr>
<tr>
<td>Pre Avg.</td>
<td>11.34±5.58</td>
<td>64.66±25.79</td>
</tr>
<tr>
<td>Post Avg.</td>
<td>17.47±5.46</td>
<td>77.79±8.71</td>
</tr>
<tr>
<td>Total Avg.</td>
<td>14.41±6.26</td>
<td>71.22±20.06</td>
</tr>
</tbody>
</table>

Table 2. Monocyte Enrichment percentage based on flow cytometry. Mean ± 1 SD.
Figure 11. Quality Assessment of Monocytes & Methylomes Example graphs of monocyte purity calculations based on DNA methylation was identified by comparing monocyte and PBMC sample DNA methylation profiles to publicly available PBMC and Monocyte DNA methylation profiles.
<table>
<thead>
<tr>
<th>PID</th>
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<th>Monocyte Post-enrichment %</th>
<th>R^2</th>
<th>PBMC mCpG</th>
<th>Monocyte Post-mCpG</th>
<th>R^2</th>
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<td>92.8</td>
<td>0.2615</td>
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</table>

*Coefficient of determination R^2 > 0.5000

**Figure 12** Monocyte population quality assessment; DNA methylation profile vs. % Monocyte enrichment. Pearson correlation analysis of all samples pre- and post-PIC between the coefficient of determination enrichment values versus DNA methylation values.
The relative methylation patterns of the top genes with differential DNA methylation profiles pre- and post-PIC are illustrated in a heat map made by Dr. Michael Corley in the Maunakea lab. 453 CpGs were differentially methylated, with 431 (95%) hypomethylated and 22 (5%) hypermethylated. A comparison was made between the individual participants’ A1c change from t₀ to t₃ and their DNA methylation profile. Two non-diabetic individual’s DNA methylation profiles were added as a comparison of CpG sites associated with non-diabetics and the T2DM participants; see figure 14. The gene location of the 453 differentially methylated sites was identified to be in the promoter (26.8%), the gene body (41%), the 3’ untranslated region (5.1%) and the intergenic region (27.1%), see figure 15.

Figure 13. Characterization of Monocyte DNA Methylation. Heatmap of 453 differentially methylated, either hyper- or hypomethylated, regions with correlation to A1c level changes at t₃ of PIC intervention from pre- and post-intervention samples as well as non-diabetic (donor) samples.
Figure 14. Location of differentially methylated regions on the gene. The upper part of the figure describes the gene region distribution of all methylation CpG loci on the 450K array, and the lower part the distribution of the 453 differentially methylated loci in the monocytes pre- and post-PIC.

To address the issue of cell-type specificity of methylation, we characterized DNA methylation of unsorted PBMCs from five individuals pre- and post-PIC. All PBMC samples had a PBMC specific methylation profile versus study sample PBMC methylation profile coefficient of determination greater than 0.500, figure 16A. Only 48 CpGs were differentially methylated: 14 (29.2%) were hypomethylated and 34 (70.8%) were hypermethylated, see figure 16B.
A) Table of regression analysis for PBMCs vs. Monocyte (MO) methylation profiles.

<table>
<thead>
<tr>
<th>PID</th>
<th>PBMC R^2</th>
<th>MO mCpG R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-01 Pre</td>
<td>0.77</td>
<td>0.01</td>
</tr>
<tr>
<td>30-02 Pre</td>
<td>0.85</td>
<td>0.06</td>
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</tr>
<tr>
<td>30-05 Post</td>
<td>0.83</td>
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</table>

B) Heatmap of differentially methylated PBMC samples pre vs. post-PIC.

**Figure 15. Characterization of PBMC DNA methylation.** Heatmap of 48 differentially methylated, either hyper- or hypomethylated, regions from pre- and post- intervention samples. A) Table of regression analysis for PBMCs vs. Monocyte (MO) methylation profiles. B) Heatmap of differentially methylated PBMC samples pre vs. post-PIC.
Functional enrichment analysis was performed for all of the 453 differentially methylated CpG sites of the pre- and post-PIC monocytes. Using the GREAT: Genomic Regions Enrichment of Annotations tool, which predicts functions of cis-regulatory regions, we identified the top hits for the biological process, disease ontology, molecular pathways and molecular functions associated with the 453 differentially methylated sites [McLean et al., 2010]. Three of the four top hits (-log10(6.29)<p<-log10(3.29)) for the biological process are associated with inflammation and immune response. The fourth hit for biological process was regulation of the protein serine/threonine kinase activity (p=-log10(3.89)). The diseases associated with the differentially methylated genes included leukemia and cardiovascular disease/atherosclerosis (-log10(5.54)<p<-log10(4.41)). Common molecular pathways that were affected include purinergic G-protein coupled receptors and cell surface interaction at the vascular wall as well as signaling by interleukins. The associated molecular function areas include histone deacetylase binding (p=-log10(6.57) and kinase binding (p=-log10(4.52)), see figure 17.
Figure 16. Classification of Functional Genes. Gene ontology was performed using GREAT [McLean et al., 2010]. The human genome was used as background and the level of significance was set to p<0.05. The top ten hits, either Biological Process, Disease, Pathway or molecular function, are described for genes having significantly differentially methylated loci.
Discussion

This study addressed the issue of cell-type specificity, a typical confounding factor in EWAS and other epigenetic studies using blood cells. Using the DNA methylation profiles, we assessed the cell populations’ post-monocyte enrichment. We found that changes in methylation profiles were cell type-specific; there was a shift in global monocyte methylation patterns post-PIC, *figure 13*, whereas no specific change was seen for the methylation patterns in the PBMCs, *figure 16*. This finding has significant implications on future DNA methylation studies such that specific cell types should be isolated prior to using blood cell methylation as a marker for disease.

A common occurrence in DNA methylation is the aberrant hypermethylation of promoter regions that often overlap with CpG islands leading to the silencing of genes [Baylin, 2005]. However, ninety five percent (95%) of the 453 CpG sites that we observed were hypomethylated post-PIC. This suggests that the corresponding genes associated with the T2DM disease state are hypermethylated and may work by silencing genes that are necessary for proper glycemic control. This explanation could be typically used for the methylated CpGs in the promoter regions. In contrast, studies have shown a correlation between increased gene body methylation and increased transcription genome wide [Ball *et al.*, 2009]. Therefore, methylation of the CpGs in the gene body and intergenic regions may be associated with increased gene transcription.

Although there were no genes that directly regulated the post-PIC intervention attenuated pro-inflammatory cytokines, IL-1β or IL-6, there were genes that suggest indirect pathways that lead to a reduction in inflammatory genes, such as the LY86 gene, lymphocyte antigen 86, that was hypomethylated post-PIC. This gene is of interest due to its associated role as a negative regulator of TLR in response to LPS [Divanovic *et al.*, 2005]. LY86 has also previously been suggested as a DNA methylation marker for obesity, insulin resistance and inflammation [Su *et
To understand the transcriptional activity of the specific genes, they were examined by using gene ontology analysis, which revealed prospective molecular processes, diseases, and pathways associated with the methylation-regulated genes. Gene ontology analysis pointed towards immune response as associated with the methylation-regulated genes. Diseases correlated with the methylated genes included chronic leukemias that include inflammation and cardiovascular diseases, for which DM is a risk factor. This suggests that T2DM methylation profiles may be correlated with inflammation and cardiovascular disease methylation profiles.

We observed an overall shift in the DNA methylation profiles of the participants’ monocytes post-PIC. The post-PIC monocyte DNA methylation profiles resemble the DNA monocyte methylation profiles of non-DM donor samples. This suggests that the PIC intervention may be influencing methylation states of participants towards a non-DM state pattern. At the three month time point, a majority of the participants’ monocyte methylation profiles shifted towards the non-DM state, however, their A1c levels were not yet reduced. Taken together, the data suggest that DNA methylation may be influencing the change in A1c level seen at a later time point. Similarly, the changes in A1c may be much slower and downstream of the inflammatory changes so a longer evaluation period may be needed. This suggests that DNA methylation profiles of monocytes could be used as a diagnostic and prognostic tool for T2DM.
CHAPTER 4: INTEGRATION OF THE STUDY RESULTS TO PROVIDE PERCEIVABLE INFORMATION FOR THE COMMUNITY

Rationale

Previous unethical research practices such as the Havasupai diabetes project has generated mistrust in some indigenous communities [Pacheco et al., 2013]. Historically, indigenous groups have not played a large role in the decision making process as it relates to their health and wellness [Burhansstipanov et al., 2011, LaVeaux et al., 2009]. However, the importance of biospecimen collection from diverse populations in disease disparity research has now been established [Vaught et al., 2011; DeSouza & Greenspan 2013]. Clarity and education regarding use, collection, and storage of biospecimen is necessary in order to ethically gather and utilize biospecimens from diverse populations for basic science research.

Distrust between indigenous communities and academic investigators, as it pertains to research, stems also from past experiences with “helicopter research”, where outside investigators flew into indigenous communities for a short period of time, gathered data and left nothing of immediate, perceivable value for the communities. There are documented cases of helicopter research projects in which biospecimen were inappropriately collected to produce culturally insensitive and statistically invalid results that were published without review or consent [Mello 2010; LaVeaux 2009]. Community-based participatory research (CBPR) is a research methodology that focuses on collaborative efforts involving equally academic scholars and community members [Minkler and Wallerstien, 2003]. CBPR addresses previous mistrust and broken promises with indigenous communities by establishing a new, positive relationship for future collaborative efforts.

CBPR involves gaining permission from communities and actively engaging in research with and for the community. Community organizations like The Center for American Indian
Health, and the Association of Hawaii Civic clubs educates both community members and academic researchers on understanding the importance and respect for each other’s efforts as it pertains to history, culture, values and wishes [Pacheco et al., 2013; Fong et al., 2003].

This study addressed the issue of research in indigenous communities and collection of the biospecimen. In this specific aim, we worked with the community to develop an ethical, meaningful research experience for both the community and the academic researchers. It was hypothesized that through CBPR, educating and engaging the community at all phases of the study would produce relevant, perceivable information for the study’s participants that would add value to decision making and continuing practices associated with the PIC intervention.

Methods

The principal investigator of this study, Dr. Alika Maunakea met with community organization leaders several times to determine the most appropriate protocol for educating participants, obtaining informed consent, providing incentives, collecting samples, and debriefing once the study was complete. The informed consent process included a full disclosure of the collection, use, and disposal of samples, which was limited to the study’s specific research questions. Informational meetings for community participants were held prior to the study. This project was reviewed and approved by two Institutional Review Boards: University of Hawai’i and Papa Ola Lokahi Native Hawaiian Health Board, a non-profit consortium of Native Hawaiian organizations and public institutions working to improve the health and well-being of Native Hawaiians and other Native peoples.

Focus Group

Preliminary results were presented to the participants using a short PowerPoint presentation as well as a one-page handout, which included A1c, inflammatory response and
methylation data, by the principal investigator of the study. Immediately after the presentation of results, a one-hour focus group was conducted with 8 (50%) of the 16 participants, five males and three females, to discuss basic issues regarding the study. The focus group was declared an exempt study by the University of Hawaii Committee on Human Subjects' IRB. Dr. Gregory Maskarinec led the study. All eight participants provided signed consent approving the audio recording of the session, which was then transcribed.

**Results**

All of the participants expressed satisfaction with both the epigenomic study as well as the "PIC" (Partners in Care) classes, and welcomed the presentation of the findings with enthusiasm. As one of them said: "I like the results of these studies because they show that whatever the sixteen people are doing, it's working, so now, keep doing what you're doing, it will get better." Another agreed: "The results show that we're moving in the right direction, you just got to be consistent." "Right, it shows that you're doing what you need to be doing, so if you keep it up, you'll get better," said another, while a fourth commented on the overall PIC program, including the epigenomics study: "I'm glad that Papakolea and you guys make this program for us Hawaiians, and others, to be more aware, to live a little longer, I have *mo'opunas* [H: grandchildren], I like to see them graduate." Asked whether any of them had any concerns, whether psychological, spiritual, physical, social or financial, regarding the blood samples, none of the eight participants had any issues with the blood draws: "I didn't have anything negative about it, I was hoping that what he’s doing is for the good of this program, that's the main thing," said one, while another observed: "Sharing the *koko* [H: blood] is a gift." Two other participants admitted being scared by needles and blood draws but agreed that if it is for a good reason, they are willing to do it, while two others routinely require regular blood draws to maintain their
current jobs and saw giving blood for an epigenomics study as not significantly different from this. None were concerned that the study was invasive, though one did joke that although it was okay, "... main thing, I no want to see another me," humorously referring to his DNA being used for cloning. Another did comment, however, that it is not just the blood draw, but the time involved, that discourages participation.

All did agree that the results should be disseminated widely, with a message, and one suggested: "Tell them, train your mind, you like live or you like go early?" Another summed up the information session: "Because it comes out from the blood, it can come out, it gives hope, the blood can actually change to get better, and the lifestyle can be more better, not carry all kind of opala [H: waste], we need to get this out." All agreed with the participant who concluded: "You can look at this, this is proof, how much more proof you need, but for stubborn guys, they going say, nah, I'm not that bad. This is proof, it is working. If this isn't motivating for them, I don't know what else can motivate them. You can only help so much, you know, it's like anything in life, it is really up to the person to change." [Maskarinec Summary].

Appendix A contains the complete transcript of the focus group study.
Discussion

In engaging with the community at all phases of the study we were able to learn about the people and what they value. We found that it is easy to work with the community when there is clear communication from all parties involved. Utilizing community leaders as a source of knowledge and guidance pushed this portion of the project to new levels. We were able to work with the community members more efficiently by having leaders work directly with the researchers as the point of contact. The community leaders contributed largely to this study because they were able to initiate and motivate participants to engage in the study and maintain participation throughout the study.

It can be clearly seen through the responses of the participants that they are not afraid of the collection of biospecimens, as long as these are used for their rightful purpose. The participants understood that we, the researchers, were there to help them and their future generations to understand T2DM as it pertains specifically to them. The participants clearly stated that the DNA methylation data is motivational because it shows that they are moving in the right direction even though current tests, like A1c tests or weight, do not yet show a change. This information from the participants could aid in promoting DNA methylation tests as a potential diagnostic test for diseases like T2DM.

With the guidance of POP, we have initiated a change in perspective of basic science researchers and NH/PI communities. This unique portion of this study has laid the foundation for future basic scientists to engage in research with NH/PI communities in Hawaii. This study was relatively small but its impacts are great on the NH/PI community because they now understand the implications of the study--they are in charge of their health and it is their lifestyle choices that are going to make them healthy or not.
CHAPTER 5: CONCLUSIONS

Summary of Findings

Clinical and Inflammatory Response to the Intervention

In order to gain a better understanding of the underlying molecular mechanisms associated with the improvement of glycemic control in the PIC study, the inflammatory response of monocytes of PIC participants was examined pre- and post-intervention. The inflammatory response was assessed through monocyte intracellular cytokine staining in vitro by specifically looking at the single cell monocyte production of pro-inflammatory cytokines, IL-1β, IL-6, IL-8 and TNFα. Similar to previous studies on the PIC intervention, there was a significant reduction in weight, BMI, cholesterol and emotional distress post-PIC intervention. The A1c levels of the participants were also reduced when the data was stratified based on the time of A1c improvement, t3 or t6. Using repeated measures, it was determined that the pro-inflammatory cytokines were strongly attenuated post-PIC intervention. Specifically, the production of IL-1β and IL-6 was reduced post-PIC suggesting their possible role in a microinflammation state associated with obesity and DM.

DNA methylomes

To determine a possible molecular mechanism leading to the change in monocyte cellular production of the pro-inflammatory cytokines, the DNA methylation profiles of PIC participants were compared pre- and post-intervention. Both the monocyte and PBMC DNA methylation profiles of 5 of the PIC participants were studied to address the issue of tissue-specificity associated with the epigenetic profile. The monocyte DNA methylation profiles showed an overall change in the DNA methylation profile in contrast to the PBMC profile suggesting that DNA methylation profiles are cell type-specific. Using repeated measures, a general shift in
monocyte DNA methylation profiles of T2DM participants was seen when pre- and post-intervention profiles were compared. When the PIC participant DNA methylation profiles were compared to non-DM donor DNA methylation profiles, the post-PIC intervention profiles correlated with non-DM donor profiles. This finding suggests that the PIC intervention may be influencing the DNA methylation profiles of participants to shift towards a non-DM DNA methylation profile state. Utilizing the DNA methylation data, the genomic region of the methylation also examined. Like previous studies, methylation was observed not only in the promoter regions of the genes but majority of the methylation occurred in the gene body. DNA methylation in the gene body suggests that methylation events may play a larger role in gene regulation other than the classical promoter region methylation, resulting in gene silencing, but also other roles for example in alternative splicing [Baccarelli & Ghosh 2012; Maunakea et al., 2010]. In looking at the possible implications of the associated disease and other molecular mechanisms using a computer based gene grouping program, we found that the differentially methylated genes are associated with inflammatory and cardiovascular disease gene networks. The cardiovascular disease network should be further examined because of its association with T2DM as one of its risk factors.

Community Implications

In this study we addressed the issue of indigenous communities engaging in basic science research especially as it pertains to the collection and use of biospecimen. We methodically planned and executed each step of the study by engaging the community in all steps of the research process from the study design to data analysis and interpretation. In using CBPR we were able to utilize the strengths of the community, allowing us, as basic science researchers, the
opportunity to further understand what is important to the community and how they truly perceived this specific study. The community participants overwhelmingly supported the study, which has laid a foundation for future collaborations between basic scientists and the Native Hawaiian/Pacific Islander communities in Hawaii.

**Overall Conclusions**

We have identified potential underlying molecular mechanisms of action associated with the PIC intervention participant improvement in glycemic control. Our findings suggest that T2DM is a result of chronic inflammation due to pro-inflammatory cytokines, which are likely to be regulated by DNA methylation in the intergenic regions. Environmental influences including lifestyle choices, such as diet and exercise regimes, impact the DNA methylation profiles associated with T2DM. Our study provides evidence that epigenetics is reversible within a relatively short timeframe, such as three months. DNA methylation changes on the global scale, but is cell type-specific, as shown by the difference in methylation-regulated genes in PBMCs versus monocytes. In conclusion, our study shows that lifestyle choices associated with the PIC intervention are shifting the DNA methylation profiles of T2DM participants to a non-DM methylation profile state. The genes associated with methylation could be linked to cardiovascular disease and other immune diseases through methylation regulated molecular pathways.

**Limitations of the study**

The sample size was statistically powered at 16 participants. However, due to external factors, not all of the samples were of highest quality in the various experiments. Using both computer based and molecular filters we defined new quality parameters for each of the
experiments. Due to time and financial constraints, the study was limited to a three month time period resulting in minimal data collection and lack of a longevity study. Monocyte enrichment was not 100% effective therefore cell-type specificity was a confounding factor. Inter-individual participant variations were also limitations.

**Future Directions**

In order to acquire a full understanding of the functionality of the methylation-regulated genes, RNA expression experiments need to be conducted. Expression experiments such as reverse transcriptase-PCR and RNA-Seq experiments would give a holistic picture of which genes are functional and responsive to modification of DNA methylation states. The genes may correlate with the gene ontology data that would provide evidence for T2DM as a risk factor for cardiovascular disease.

This study could also be repeated with a longer duration or follow up with participants at later time points to see the impact of the PIC intervention on monocyte DNA methylation profiles at later times. The longer study would be able to examine the DNA methylation profile changes of individuals and its implications on disease onset. Examining each of the differentially methylated genes and comparing them to their downstream functions could conduct further analysis of the DNA methylation dataset.

DNA methylation profiles could be used as a more sensitive diagnostic tool for potential T2DM patients. However, in order to use DNA methylation profiles for diagnosis an EWAS developed from a large diverse population of T2DM patients would need to be established. The burden of obtaining perceivable methylation data needs to be addressed via streamlined data processing from the molecular bench work to bioinformatics data analysis.
APPENDIX

Complete Transcript of Focus Group Study
Papakolea, 22 July 2014
Focus Group led & transcribed by Dr.GG Maskarinec

8 Participants, assigned individual letters here. Bolded material currently included in the 500 word summary.

Q1) Please describe your experience participating in this project.

K: My diabetes is more controlled, it's me, I got to change, I got to do it myself.

S: The results show that we're moving in the right direction, you just got to be consistent.

J: It forces you to think more about what you're doing, where you're going, and how you want your health to be, because it is all about control, and you, if you want to change or not, it up to you.

E: I've been in this program for the past three months, I am very happy, so glad that I joined this program, because, I knew what I was supposed to do, what to eat, but I was really out of control, knowing when I come here now I'm more disciplined, I know I should be eating well, I think we need to meet each other more often. The program really does help you to think right, do right, we got to control our diabetes, it helped doing this research on us, thank you.

P: This class has really opened my eyes, for my dad and them before there was more hush hush, denial that they get diabetes, growing up, you're big you're healthy, first I was kind of skeptical, but coming to this program really help me, because I'm a driver, too, we have to control my diabetes to go on the road, if our sugar's too high or too low could slip into a coma and cause a lot of deaths on the road, coming to this program really help me balance my sugar, intake of foods, taking the test keeps me aware where my sugar is, not only if my sugar too low, drink Pepsi, or take several tablets, taking the tablets much better than drinking one Pepsi, they tell me you drink one Pepsi you supposed to drink one small little cup, don't like to waste, you spend money, drink the whole can, that was another thing that was hard too, getting away from that, every time I drink water, I need another taste, sometimes I put lemon and stuff, only good for a little while then the lemony taste is gone, but gotta keep focus, sometimes I fall off, like one day I just came home from work I better check my blood, it was around 375, highest I ever see a reading, from the time I see that, ooh, better get back, every hour I was checking it...with this program it really helps, it helps, it is good for everyone, not only for Polynesians, for every race, we all have our different ways, I'm glad that Papakolea and you guys make this program for us Hawaiians, and others, to be more aware, to live a little longer, I have mo'opunas [H: grandchildren], I like to see them graduate.

A: Basically the same thing brudda said, its like a roller coaster, fortunately for us, me and my wife joined the class, and we learned a lot, thanks, I'm very fortunate to have a wife, my wife, 'cause she kinda keeps me on track too, if I eat too much, I like that, I get upset, some of us Hawaiians you know, are like, ah, I don't have the attitude like you only live once, just go for it,
by taking this class you gotta control, we gotta level out a bit, its us, we got to it for ourselves, eat right, exercise, ah, me and my wife we try, we go out walk a couple evenings, but the next week we come home from work we tired, we're trying real hard, we try to read labels also, that helped a lot, it's up to us, we got to change our lifestyle and habits, exercise.

M: I'm here because there's a hope that diabetes can be manageable, and if you lose the hope but it's doable, no fear of weeping legs, no fear of amputation, so I was in it encouraging, it's chronic, when I realized that my neighbor was the same, think of families that have died from this disease, but it's doable.

H: first of all thank you all for this program, at first it was very stressful, we have families, it kind of makes you scared because we have, most of us, young children, its very educational and I learn a lot, still struggling but, if we can continue to help each other out to stay on the path, there's special days, gatherings, you tend to want to eat what you're supposed not to eat, but then you get back on track, I really like this program because I learn a lot.

Q2) Did you have, or do you still have, concerns about this particular project, giving the blood sample, and how your blood was used? (Any psychological, spiritual, physical, social or financial concerns?)

P: [jokes] Main thing, I no want to see another me, you taking DNA in the blood. I mean, if it's going to help others, everyone has different kind of blood, if it's going to help others, if it's going to help science, then I'm down for it.

H: I didn't have anything negative about it, I was hoping that what he's doing is for the good of this program, that's the main thing.

A: I think it is good for generations coming up, if we can train them more it will be more preventative for them, so its more a learning process for us, but it's for our children, so that things like diabetes, high blood pressure, large heart, scoliosis, things you get when you get old, if we can prevent these things

M: Sharing the koko [H: blood] is a gift.

E: I'm a scaredy-cat when it come to needles, I really try to hide and everything, but when it comes to this kind of testing, when it has to be done, I'm for it. So it's okay.

K: Every three months I have to draw blood, for my captain license, and it costs me $27 every time, every year they want a copy, they want it below 8, I'm lucky, I've been keeping it below 7.2, but I gotta do it, just to keep my license.

S: I got to feel optimistic, with the results, with the reversal of the epigenetic, Hawaiians still, the word diabetes, they don't mention it, there's a lot more people we can touch, maybe, we can recruit people to come to the next one, education is a, the sky is the limit, the benefits is to know the effects, we need programs like that, for this disease, lot of promise. I would do it again.
J: Yeah, it was good, the blood draw is good as long as there's benefits, not just for me, but also for the study.

Q3) Please comment on the results that you have just heard. (Do they make sense to you? Is there more you would like to know?) & Q4) Does learning the results presented change your attitude about Partners in Care (PIC)? Why or why not?

[W - not a study participant]: I think its would make more sense if the participants at least was given their information, I think it is important, not for the purpose of diagnostics but so the individual knows where they are, because some people may not have the monitoring every three months of getting lab draws, which is a great idea, but in the real world that's not the way it is. It would be good if the individual was given the results, it would make a little more sense, it would be more personable, I would be more prone to participate if I knew my own results, that's just my opinion.

J: We did it as a group, right?

A: Doing it as a community, whatever program is sponsoring you guys, you set the parameters, we just got to understand that.

P: Not just the blood, the time, too, that's the problem with today, everybody so busy they got no more time, but when something happen to you it's final and you're serious, then you wish went to this class, like me, I got family members that have diabetes, all we can do is just tell them, what I learned, what I do, but it is really up to them. I seen my colleague, he's got to drain his legs, not taking his meds, but me, I'd rather take meds, I hate needles, when they’re going to draw blood I'm going to look someplace else, you know, ...
[long digression about his brothers and fellow workers, plate lunches, you get that craving, we gotta drink shakes...] Tell them, train your mind, you like live or you like go early? Before, when I'd come over here, my blood sugar was high, but it never registered, but now I now what I gotta do.
[exercising during the TV commercials with his grandkids] I'm really thankful for the training, when I found about diabetes, I thought that was the end of the world already, a lot of friends say, oh yeah, they cut your toes, ...
just train yourself, that's the hard part...

M: It shows that there was improvement, this shows it works on the molecular almost, you can have hope.

A: Just get on the right track, only you can do what you gotta do, only you can, don't just offset, that used to be the word with me and my friends, offset with more pills, I'm going to eat sugar, cake and all that, tonight, and going to offset it with insulin, more pills, that no work, it's not gonna work, you gotta stay on the track you got to be moderate, try to stay away from the sweets, sugar,

E: You guys going to be a study on gout? Plenty local have gout,
P: need more water, watch how we drink, only thing that do it for arthritis is take the pain killers, but I don't like to take them, some of them make you drowsy, but I used to that, to offset, overload and then offset,

K: I like the results of these studies because they show that whatever the 16 people are doing, it's working, so now, keep doing what you're doing, it will get better.

[C: they're so proud to be one of the sixteen]

E: Awesome.

Q5) Do these results help to motivate you further in your lifestyle change?

S: You gotta go further, like the lecturer said, only you can help yourself, its good knowledge, he said some guys improve earlier and some improve later, but all 16 improved.

J: Right, it shows that you're doing what you need to be doing, so if you keep it up, you'll get better, like my doctor always tells me, if you lose the weight, you won't need to take some much meds, I'm trying, he's very dramatic, he shows me a 5 pound bag of rice and then a 20 pound bag, now carry this around, now put it down, you wouldn't have that extra weight on you, for me, that works, if I can see it, and I can understand it, touch it, then I know it.

Q6) Would you recommend others to participate in these types of projects (both PIC as well as the epigenomic study)?

P: I would show them the results from the doctor, the paper, this is what I had before and this is what I came down, when coming to this program, it really helped me realize that you can do something about diabetes, by doing right, by eating right, you're not only helping diabetes, you're helping clear your heart, your kidneys and all your stuff there, you like to see results, but sometimes I going up and down but I can get control, for my trucking license I got to be in the sevens, I'm glad they say 7 not 5, so, it's like everything else, it's really up to them, if they like to learn, because you know, stubbornness, yeah, a lot of them, they stubborn, my cousin is one of them, the Hawaiians, you drink diet Pepsi, that's two can of sugar, drink water that's the best, put lemon inside if you like more taste, but then he get cut, now he like it, now he's watching what he eats, what he drinks, plenty water,

H: You can share what you learn and it's up to them, because a lot, like our families they have a busy schedule, don't have the time, just a few of them have diabetes, but whatever I learn from this class I share with them, about, how much portion, what you should eat, quarters plate, half plate starch, but sharing information with them, so hopefully they can think about diabetes.

[W: I think too seeing your own health improve, and other people seeing that you're healthier, they say, what are you doing, and you can share that, role model to make the change, people are doing to wonder, what have you been up to, your health is better, you're looking great, its really the nutrition]
A: Sometimes you gotta get them motivated, you got to carry them to the class, come, and once they come to the class, they kind of realize oh, I'm not the only person, it kind of motivates them to make the effort, the extra effort, like brudda says, oh, shoulda went to the class, I want to learn more, later.

[W: will there be other classes?]

Q7) What suggestions do you have to improve any future projects of a similar sort?
M: Sometimes you cannot participate when you bring the A1C down too much, if it is too low you cannot participate, but it still helps you to be in the class.

A: A good motivational speaker once in a while, to speak about their experience,

P: Like, it helped me too it will help others.

J: If your A1C improves, you're out, but if it goes back up.

K fall off the wagon just to get back on the wagon.

[W: there should be a maintenance program]

[C: comments about setting the parameters for PIC]

[A: if we didn't do this study, would you still be motivated? If you didn't have this information, or if you are one of the individuals whose A1C isn't changing, or your weight isn't changing, would that encourage you more?]

E: We would still come if we didn't have this information.

A: It just shows, right on this scale right here, that it is working, that would give me more motivation, last week I had surgery on my eye, it was because of diabetes, they found the vessel and did it right there,

H: Knowing the results of this study it makes me more motivated, it gives me hope, at first, I thought like, ah man, but getting these results make me feel better, gives me hope, I think this is good information and I'm glad that we did this PIC, just by learning from the A1C, but to do this means more to me and I'm glad, there is improvement

J: It's like what she said, if there wasn't any changes in A1C, I would have to ask what can I do to get the numbers down, 'cause you're exercising, you're losing maybe not that much weight, maybe a pound, two pounds, and I could be curious of what I need to do to change the number, I'm tired of being stuck at the same number everytime I come see you, like 748 or 842 or something, then I would be forced to try to ask what physically I need to eat, get into portion sizes and stuff, and then for him to come back to me, to try to be like yeah, you're eating this
much, now you know that's too much, right? and then you would yeah, try eating half of that, or a quarter of that.

Q8) What suggestions do you have to disseminate these results to the local and wider communities?

P: This would motivate, you know some people don't have insurance,

A: Sometime people with no money shame come, I mean, for them, this would give them more hope, because they can see the progress, and for guys with medical, if we never have this, you can still see the progress, so its a motivation that you're doing something right, it's proof, so you get hope, you don't have to be weary any more, I know there's a lot of people without insurance, seeing this, there is hope fighting diabetes, we can always go out there and talk to people, yeah, tell your neighbors, convince your friends, or I see someone's legs, and I say, if you don't mind my asking, let me tell you, ...I don't know how you can get the word out, word of mouth, maybe one DVD or something

J: More action, nobody wants a DVD or something,

K: By learning about this program and sharing with others,

[Alika: would this information help others?]

P: Definitely. You can look at this, this is proof, how much more proof you need, but for stubborn guys, they going say, nah, I'm not that bad. This is proof, it is working. If this isn't motivating for them, I don't what else can motivate them. You can only help so much, you know, it's like anything in life, it is really up to the person to change.

A: It's gonna need more than just this, I mean, this is a big part of it, but then, education is the number one thing, they all need to be educated.

J: This is a big part of it, more education, what to eat, more exercise.

M: at the kupuna level, because it comes out from the blood, it can come out, it gives hope, the blood can actually change to get better, and the lifestyle can be more better, not carry all kind of opala [H: waste], we need to get this out, I would put it in front of the senate, this can happen, we're going to clean our blood, we're going to make it sugar free, diabetes free, so on the next visit, you're sugar free, when you feed him right you're not that old blood sugar,

P: You know, my first thought, I never knew anything about these things, this thing called diabetes, now, [people say?] you're at the risk of being diabetic, as the generations go by, things change, you know, oh yeah, I never know anything about that, diabetes, you gotta check your blood and stuff, but now I hearing plenty, you know, my niece says, good thing, you're checking your blood, oh, you diabetic? no, because I'm pregnant, I could be diabetic, so don't drink so much soda, ... so much I hear it now.

9) Please add any additional remarks you would like to share.
K: Are we going to have more of these, more blood draws?

J: if you come up with the result, to show that it continues, the sixteen,

A: We want to say mahalo for coming down, thank you for coming down, everybody, opening everyone's eyes that we can help

E: When I was first diagnosed diabetic, I was in my 50s, and I wasn't too serious about it, everybody too sweet you know what I mean, but then, coming to these classes, I got serious, really wanted to learn more, how to control and everything, but people are no biggie, but thank you, it's almost like cancer in a way
P: Yeah, diabetes is not okay you hear that word cancer, you hear cancer, oooh,

E: but how so much cancer, so much diabetic, diabetes,

[ends as people get up to take food]
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