Coronary disease is not associated with robust alterations in inflammatory gene expression in human epicardial fat

Timothy P. Fitzgibbons, … , Stanley K.C. Tam, Michael P. Czech


Background: Epicardial adipose tissue (EAT) is the visceral fat depot of the heart. Inflammation of EAT is thought to contribute to coronary artery disease (CAD). Therefore, we hypothesized that the EAT of patients with CAD would have increased inflammatory gene expression compared to controls without CAD.

Methods: 26 patients referred for cardiac surgery with (n=13) or without CAD (n=13) were consented. Samples of EAT and subcutaneous adipose tissue (SAT) were obtained at the time of surgery. Gene expression analysis was performed using Affymetrix Human Gene 1.0 ST arrays. Differential regulation was defined as a 1.5 fold change (ANOVA p<0.05).

Results: When comparing SAT and EAT of controls, 693 genes were differentially expressed. 805 genes were differentially expressed between SAT and EAT in cases. Expression of 326 genes was different between EAT of cases and controls; expression of 14 genes was increased in cases, while 312 were increased in controls. qRT-PCR confirmed that there was no difference in expression of major inflammatory genes (CCL2, CCR2, TNFα, IL6, IL8, PAI1) between cases and controls. Immunohistochemistry demonstrated that there were more macrophages in EAT than SAT, but that there was no difference in the number or activation state between cases and controls.

Conclusion: In contrast to prior studies, we did not find increased inflammatory gene expression in the EAT of patients with CAD […]

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Coronary Disease is not Associated with Robust Alterations in Inflammatory Gene Expression in Human Epicardial Fat

Timothy P. Fitzgibbons MD PhD, Nancy Lee MD, Khanh-Van Tran MD PhD, Sara Nicoloro, Mark Kelly, Stanley K.C. Tam MD, Michael P. Czech PhD,

1Department of Medicine, UMASS Medical School, Worcester MA, 01655
2Program in Molecular Medicine, UMASS Medical School, Worcester MA, 01655
3Department of Surgery, St. Elizabeth’s Medical Center, Brighton MA, 02135

Running title: Epicardial Fat and Coronary Atherosclerosis

Keywords: epicardial adipose tissue, coronary artery disease, inflammation, nuclear hormone receptor

Corresponding Author:
Timothy P. Fitzgibbons MD, PhD
Division of Cardiovascular Medicine
55 Lake Avenue North
Worcester, MA 01655
Phone: 508-856-6573
Fax: 508-856-4571
Timothy.fitzgibbons@umassmed.edu
Abstract

Background: Epicardial adipose tissue (EAT) is the visceral fat depot of the heart. Inflammation of EAT is thought to contribute to coronary artery disease (CAD). Therefore, we hypothesized that the EAT of patients with CAD would have increased inflammatory gene expression compared to controls without CAD.

Methods: 26 patients referred for cardiac surgery with (n=13) or without CAD (n=13) were consented. Samples of EAT and subcutaneous adipose tissue (SAT) were obtained at the time of surgery. Gene expression analysis was performed using Affymetrix Human Gene 1.0 ST arrays. Differential regulation was defined as a 1.5 fold change (ANOVA p<0.05).

Results: When comparing SAT and EAT of controls, 693 genes were differentially expressed. 805 genes were differentially expressed between SAT and EAT in cases. Expression of 326 genes was different between EAT of cases and controls; expression of 14 genes was increased in cases, while 312 were increased in controls. qRT-PCR confirmed that there was no difference in expression of major inflammatory genes (CCL2, CCR2, TNF α, IL6, IL8, PAI1) between cases and controls. Immunohistochemistry demonstrated that there were more macrophages in EAT than SAT, but that there was no difference in the number or activation state between cases and controls.

Conclusion: In contrast to prior studies, we did not find increased inflammatory gene expression in the EAT of patients with CAD in comparison to controls without CAD. We conclude that specific adipose tissue organ, rather than CAD status, is responsible for the majority of differential gene expression.
Background

Obesity is a known risk factor for type 2 diabetes mellitus (T2DM). T2DM conveys increased risk for the development of cardiovascular disease (CVD). In fact, diabetes mellitus is considered a “risk equivalent” of established CVD itself(1). Obese persons with visceral, rather than subcutaneous adiposity, are at greater risk for the development of T2DM and incident CVD. In the setting of chronic caloric excess, an inflammatory response is initiated in visceral adipose tissue (VAT)(2). The stimulus for this inflammatory response remains unknown. VAT inflammation results in adipocyte dysfunction, including failure to store free fatty acids as triglyceride and increased lipolysis. Excess free fatty acids are released into the circulation and deposited in ectopic tissues, such as the liver, skeletal muscle, heart and pancreas. Ectopic lipid storage results in insulin resistance in target organs(2, 3). This paradigm has dominated our understanding of T2DM since the 1990s(4, 5). It follows that anti-inflammatory therapies would be beneficial in the treatment of T2DM and the prevention of CVD; yet the success of anti-inflammatory therapies in the treatment of cardio-metabolic disorders has been limited(6).

The ability of VAT to stimulate this cycle of inflammation and insulin resistance is underscored by the syndrome of “normal weight” obesity; patients with normal BMIs, but increased waist circumference, and disproportionately increased VAT(7). Despite normal BMI these patients are prone to insulin resistance and T2DM due excess VAT. These observations have led to interest in the direct adverse effects of VAT on neighboring organs. For example, epicardial adipose tissue (EAT) is the visceral fat depot of the heart(8). Modest amounts of EAT are present in normal humans; in overweight humans the amount of EAT increases proportionately with obesity and VAT(9). EAT is contained within the visceral pericardium and supports the epicardial coronary arteries and great cardiac veins(8). Early studies by Mazurek et. al. demonstrated that EAT surrounding atherosclerotic coronary arteries had increased expression of inflammatory genes and dense inflammatory cell infiltrates compared to SAT(10). This observation suggested that inflammation of EAT may contribute to or exacerbate
atherosclerosis in the underlying coronary artery. Many other groups have replicated these results, finding increased inflammatory gene expression in the EAT of patients with CAD(11-14).

However, there are some limitations to these prior studies. First, control patients without CAD were not routinely included. This is important, because inflammation in VAT is typically greater than SAT even in normal conditions(15). Second, it impossible to discern the temporal relationship between the development of CAD and inflammation in adjacent EAT. Therefore, in order to address some of these limitations, we chose to directly compare gene expression in the EAT of patients with and without CAD.

We hypothesized that inflammatory gene expression would be greater in the EAT of patients with CAD. Contrary to our hypothesis, we found a greater number of genes differentially expressed in the EAT of patients without CAD. In particular, we found increased expression of all three members of the NR4A subfamily of orphan nuclear hormone receptors (NR4A1, NR4A2, NR4A3) in the EAT of those without CAD. Members of this subfamily have been found to suppress inflammatory gene expression(16, 17). For example, whole body and macrophage specific deletion of Nr4a1 in mice results in a worsening of atherosclerosis(17, 18). Therefore, we conclude that the development of CAD may be associated with decreased anti-inflammatory gene expression in EAT. Promotion of anti-inflammatory effects via pharmacological activation of the orphan nuclear hormone receptors may be advantageous in the treatment of CAD and other cardio-metabolic disorders and will be the focus of future studies.

Methods

Subject Recruitment

Adult patients referred for elective cardiac surgery were screened for enrollment and informed consent was provided. This study was approved by the UMASS IRB (Docket #H-14436). Patients with active infection or cancer were excluded. All patients had pre-operative coronary angiography. Controls patients were referred for elective valve surgery and had no significant CAD (any single lesion >50%) on pre-operative coronary angiograms. Only three
controls had single lesions of 30%, otherwise all had normal coronary arteries or minor luminal irregularities. Cases were patients referred for coronary artery bypass surgery due to significant CAD. The severity of CAD was determined by single blinded review of coronary angiograms and calculation of the Gensini score as previously described(19). The Gensini score is a continuous variable that increases with the severity of coronary atherosclerosis (e.g. a score of 0=none, 100=severe).

Sample Collection and Preparation

At time of surgery a sample of EAT (0.5 gm) adjacent to the right coronary artery was obtained, a section was taken and stored in 10% formalin, and the remainder was immediately snap frozen in liquid nitrogen (-80°C). Next a sample of SAT (0.5 mg) from thoracic subcutaneous fat was obtained, a section was taken and stored in 10% formalin, and the remainder was immediately snap frozen in liquid nitrogen (-80°C).

Microarray Analysis

RNA was prepared from aliquots of frozen fat samples using the QIAGEN mini Lipid RNA Extraction Kit. RNA quality was analyzed on an Agilent Bioanalyzer. Samples with a RNA integrity number (RIN) below 7.5 were excluded. Samples that passed QC analysis were sent to the UMASS Genomics Core for cRNA preparation and hybridization to Affymetrix Human Gene 1.0 Arrays. Expression analysis was performed using Affymetrix Transcriptome Analysis Software. The raw data was deposited in the NCBI Gene Expression Omnibus (GEO) website with accession number GSE120774 (https://www.ncbi.nlm.nih.gov/geo/).

qRT-PCR

qRT-PCR was performed using a cDNA synthesis kit, SyberGreen Master Mix, and a CFX 96 thermocycler (BIORAD, Hercules, CA) as previously described(20). Primer sequences were obtained from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and were as follows:

CCR2 forward CCACATCTCGTTCTCGGTTTATC, CCR2 reverse CAGGGAGCACCCTGTAATCATAATC, CCL2 forward CAGCCAGATGCAATCAATGCC, CCL2
reverse TGGAATCCTGAACCCACTTCT, GAPDH forward GGAGCGAGATCCCTCATAAAT,
GAPDH reverse GGCTGTGTCATACTTCTCATGG, IL8 forward
TTTTGCCAAGGAGTGCTAAAAGA, IL8 reverse AACCCTCTGCACCCAGTTTTC, IL6 forward
ACTCACCCTTCAGAAGCAATAG, IL6 reverse CCATTTTGGGAAGGTCCAGTTG, ITLN1
forward ACGTGCCAATAAGTCCTCC, ITLN1 reverse CCGTTGTCACTCCAACACTTTC, NNAT
forward ACTGGGTAGGATTCGCTTTTC, NNAT reverse ACACCTCACTTCTCGCAATGG,
NR4A1 forward ATGCCCTGTATCCAAGCCC, NR4A1 reverse GTGTAGCCGTCCATGAAGGT,
NR4A2 forward GTTCAGGCGCAGTATGGGTC, NR4A2 reverse
CTCCCCAAGGAGTGTAAGTGT, NR4A3 forward TGGCTCCAAGCCCAATAGGC, NR4A3
reverse GGTGTATTCCCGAGCTGT, PAI1 forward ACCGCAACGTTGTGGTCTCA,
PAI1 reverse TTGAATCCCATAGCTGTCGAAAT, PTGS2 forward
CTCGCGTCCTAGCCTCCAACTAG, PTGS2 reverse CGCACTTTATACTGGTCAAATCCC, SOCS3
forward CCTGCGTCCTAGCCTCCAACTAG, SOCS3 reverse GTCACTCGGTCCGAGTAGAA, TNFα
forward CCTCTCTCTTAACGTCTCGCTTTG, TNF α reverse GAGACCTGGGAGTAGATGAG.
Expression relative to GAPDH was determined using the 2Δct method of Livak et al (21).

Histology and Immunohistochemistry

Formalin fixed sections were embedded in paraffin and sectioned by the UMASS DERC
Morphology core. Adipocyte sized was determined on H&E stained sections using the program
Adiposoft as previously described(22). To quantify blood vessels, unstained sections were
incubated with rabbit anti-human Von Willebrand Factor (1:300) dilution (Abcam ab6994) and
then a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Pierce).
Negative controls utilizing secondary antibody alone were also included. To quantify M1 or M2
macrophages, cryosections were stained with rabbit anti-human CD11c (1:1000) dilution
(abcam ab52632) and rabbit anti-human MRC1(1:1000) dilution (abcam ab64693) antibodies,
and then a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Three
representative fields at 20X of SAT and EAT of each patients were used to quantify CD11c+
and MRC1+ cells.

Statistics
Clinical characteristics were compared with the two-tailed Students t test for continuous
variables or the Chi squared test for dichotomous variables. A p value less than 0.05 was
considered significant. Adipocyte size and endothelial cell numbers were compared with the
Student’s t test. For the qRT-PCR experiment, expression values relative to GAPDH were
compared using 2 way ANOVA and Tukey’s Multiple comparison test. The Affymetrix
Transcriptome Analysis Console was used to analyze microarrays using ANOVA and multiple
comparison testing.

Results
Clinical Characteristics
The clinical characteristics of subjects are shown in Table 1. There was no difference in age,
gender, BMI, co-morbid conditions, or pertinent medication use. The mean age of controls was
67.1 ± 9.9 years and cases 65.3 ± 13.0 years (p=0.70). There was a trend for more female
patients in the control group (46% vs. 23% for cases, p=0.41). The mean Gensini score was
0.7 ± 1.3 in controls and 53.7 ± 27.2 in cases (p<0.001). Therefore, with the exception of the
presence of CAD, our study groups were well matched.

EAT Contains Smaller Adipocytes and Is More Vascularized
We first compared the histological characteristics of SAT and EAT. Both SAT and EAT
contained uni-locular white adipocytes (Figure 1); no brown adipocytes were visualized. Control
SAT adipocytes were significantly larger than those in EAT (5213 ± 1024 vs. 3904 ± 1241 μm²,
p<0.01)(Figure 1, top panel). This relationship was also seen in cases (5136±1058 vs. 3920 ±
612 μm², p<0.01). There was no difference between cases and controls in depot specific
adipocyte size. We then utilized an endothelial specific anti-body (Von Willebrand Factor) to
compare the number of blood vessels in SAT and EAT (Figure 1, bottom panel). EAT had a
A greater number of blood vessels than SAT; this was true for cases (2.2 ± 1.67 vs. 0.66 ± 0.58 per HPF, p<0.01) and controls (2.2 ± 1.29 vs. 0.89 ± 0.72 per HPF, p<0.01). There was no difference between cases and controls in depot specific blood vessel number.

*Microarray Analysis Demonstrates Predominantly Depot Specific Differences In Gene Expression*

We then compared gene expression in SAT and EAT in control patients. Despite the absence of significant CAD there were large differences in gene expression between SAT and EAT (Figure 2, Supplemental Table 1). In total, 693 genes were differentially expressed (FC>1.5, p<0.05). Expression of 383 genes was increased in EAT and 310 in SAT. We then performed gene set enrichment analysis (GSEA) of differentially expressed genes in both depots. SAT was enriched for expression of genes encoding proteins related to the Renin-Angiotensin System, Neuroactive Receptor-Ligand Interactions, and many biochemical pathways (Insulin signaling, Starch, Sucrose, and Butanoate metabolism)(Figure 2C). In contrast, EAT was enriched for expression of genes encoding inflammatory pathways, such as Complement and Coagulation Cascades, Focal Adhesion, and Cytokine-Receptor Interaction (Figure 2C).

In patients with CAD 805 genes were differentially expressed between SAT and EAT (Figure 3, Supplemental Table 1). Expression of 427 was increased in EAT and 328 in SAT. GSEA demonstrated that SAT was enriched for expression of genes encoding proteins implicated in the Renin-Angiotensin system, Nicotinate and nicotinamide metabolism, and TGF-beta signaling pathways (Figure 3C). Similar to control patients, EAT was again enriched for expression of genes encoding proteins belonging to Complement and coagulation cascades, cell adhesion molecules, focal adhesion and calcium signaling (Figure 3C).

We then directly compared the gene expression profile of EAT in cases and controls (Figure 4). 326 genes were differentially regulated (1.5 FC, p<0.01). Expression of 312 genes was increased in controls and 14 in cases (Figure 4A, Table 1, Supplemental Table 1). Due to the
small number of genes differentially regulated in the EAT of cases, no KEGG pathway was
significantly enriched in this depot (Figure 4C). However, several pathways were increased in
the EAT of controls, including olfactory transduction, pentose and glucuronate interconversion,
and toll-like receptor transduction (Figure 4C).

We then compared gene expression in SAT between cases and controls (Figure 5).

Expression of 218 genes was significantly different; expression of 82 was increased in the SAT
of controls and 136 in the SAT of cases (Figure 5A). No pathways were significantly enriched in
the SAT of controls; several pathways were increased in the SAT of cases, including those
encoding proteins implicated in circadian rhythms, chemokine signaling, and NOD-receptor
signaling (Figure 5C).

Finally, an advantage of the Human Gene 1.0 array is that it contains probes for microRNAs
(MIRS). Therefore, we utilized this feature to compare microRNA expression in SAT and EAT in
cases and controls (Tables 2 & 3). Expression of MIRS 126 and 1247 was significantly up
regulated in the EAT of controls and cases compared to respective SAT. MIR24-2 expression
was increased in the SAT of cases and controls in comparison to EAT.

Therefore, contrary to our expectations, microarray analysis did not demonstrate greater
inflammatory gene expression in the EAT of cases in comparison to controls. Rather, depot
specific differences predominate, with SAT of both cases and controls enriched in certain
pathways (Renin-angiotensin system, Focal adhesion) and EAT of both cases and controls
enriched in others (Complement and coagulation, calcium signaling)(Figures 2,3).

qRT-PCR and Immunohistochemistry Confirm That There is No Difference in Inflammation
Between EAT of Cases and Controls

We performed qRT-PCR to verify the expression patterns that we observed in the
microarray experiments. First, we confirmed that our samples displayed a depot specific
identity. EAT of both cases and controls had a higher expression of the visceral fat marker
omentin-1 (INTLN1)(Figure 6A). In contrast, the SAT of cases and controls expressed higher
levels of the subcutaneous fat marker neuronatin (NNAT)(Figure 6B). Expression of common inflammatory genes (CCR2, CCL2, IL8, IL6, PAI-1, and TNFα) was not significantly different between groups (Figure 6). Interestingly, expression of three members of the orphan nuclear hormone receptor family was increased in the EAT of control patients in comparison to cases (Figure 6C-E).

We then performed immunohistochemistry for markers of classical (M1) and alternative (M2) macrophage activation. In both cases and controls, EAT had a significantly greater number of classical (CD11c+) and alternative (MRC1+) macrophages than SAT (Figure 7). There was no difference between the number of positively stained cells between the EAT of cases and controls (Figure 7). Furthermore, we did not observe a difference in the ratio of M1/M2 cells per depot, or on a disease specific basis (Figure 7E).

Discussion

There has been much recent interest in the potential role of EAT as a direct link between obesity and CAD(23, 24). Initial studies showed that EAT had greater mRNA and protein expression of inflammatory genes and clusters of inflammatory cells(10, 25). Subsequent analyses have demonstrated that EAT tends to have a more inflammatory gene expression profile than SAT, and this is true even in the absence of CAD(12-14). The majority of the data suggests that EAT is very similar to abdominal VAT in that it tends to be more immunologically active than SAT even in normal conditions. There may be an additional increase in inflammation in the setting of atherosclerosis, but it is the adipose organ itself, not the disease state, that dictates the majority of differences in gene expression(13, 14). This concept was originally proposed by Chatterjee et al. in 2009(26).

Our study confirms many of these findings and makes several novel observations. First, we confirmed that type of adipose tissue, rather than the disease condition, is responsible for most of the differences in gene expression. 693 genes were differentially expressed between SAT and EAT in controls, and 805 in cases. In particular, there is a hallmark panel of genes whose
expression is consistently increased in EAT (INTLN1, SYT4, CFB, ESR, INMT, PRG4, and ALOX15) compared to SAT irrespective of the presence or absence of CAD(13). In contrast, when directly comparing the EAT of cases and controls there are fewer differences in gene expression (326 probes different). However, among these differences, we found that expression of three members of the NR4A subfamily of orphan nuclear hormone receptors was increased in the EAT of patients without CAD. This has not been previously reported, and is very interesting considering that members of this family have been shown to be protective in mouse models of atherosclerosis(17, 18).

The NR4A subfamily of orphan nuclear hormone receptors belong to the same class of transcription factors as peroxisome proliferator activated receptor gamma (PPARγ). Their protein structure is similar to other members in this class, having separate DNA and ligand binding domains. They are called “orphan” receptors because their endogenous ligands have yet to be discovered. NR4A family members are expressed in a broad array of tissues and have been shown to protect against the initiation and progression of atherosclerosis(27, 28). Recent studies have highlighted NR4A1 as a key transcriptional regulator of lipid homeostasis, vascular remodeling, and inflammation(29). NR4A1 has at least three important anti-inflammatory effects relevant to CAD. First, NR4A1 activity is required for the differentiation of Ly6c- monocytes that patrol the endothelium and maintain blood vessel integrity. Nr4a1 null mice lack this anti-inflammatory monocyte population and display an exaggerated inflammatory response(16, 17). Second, within Ly6c+ monocytes, Nr4a1 feeds back to inhibit inflammasome activity and trans-activation of NFkB by IL2. Third, Nr4a1 suppresses induction of macrophage chemotactic protein (MCP-1) expression in response to lipopolysaccharide(30). Although we do not know the specific cell type responsible for NR4A1 expression in EAT, we hypothesize that it is macrophages. Hirata et al. reported have shown that the ratio of M2 (alternatively activated) to M1 (classically activated) macrophages is greater in the EAT of patients without CAD(25).

The inflammatory phase of myocardial infarction (MI) is characterized by influx of Ly6c\textsuperscript{HI}CCR2\textsuperscript{HI}
monocytes(31). The transition to the reparative phase of MI is dependent upon NR4A1 down
regulation of inflammatory cytokine production and transition to Ly6c\textsuperscript{Low}CCR2\textsuperscript{Low} macrophages. Deletion of NR4A in this context leads to excessive inflammation and maladaptive post-MI remodeling(31). Therefore, we hypothesize that within the EAT of patients without CAD there exists a greater population of anti-inflammatory macrophages. Whether or not this is true will require further research.

We showed that epicardial adipocytes are smaller than subcutaneous adipocytes, which is consistent with their visceral lineage. This confirms work by prior authors(32). We did not however see a difference in the size of epicardial adipocytes between cases and controls; this is in contrast to the work of Vianello et al. who demonstrated that epicardial adipocyte size was greater in patients with CAD than in those without(33). We hypothesize that this could be due to differences in the patient population. Vianello et al. included only male patients in their study, and the average BMI of patients in their study was lower(33).

A novel finding in our study is that EAT appears to have a greater number of blood vessels than SAT. This has not been previously shown. This was unexpected, as visceral adipose depots have previously been demonstrated to have a lower blood vessel density than SAT(34). However, it may be that EAT is different than abdominal VAT in this regard. Furthermore, EAT is known to have a blood supply from the underlying coronary arteries, therefore it follows that there may be a higher vascularity in this this adipose depot which is in close proximity to the heart(8).

Interestingly, we found that the expression level of miR126 was higher in the EAT of both cases and controls compared to SAT. miR126 is one of the most abundant microRNAs in endothelial cells(35). miR126 promotes regeneration of endothelial cells and regulates endothelial cell turnover(36). The increased expression of this miR in EAT is consistent with the increased number of endothelial cells in this depot.
Our study has several limitations. First, we have a modest number of patients. Second, we could not obtain samples of visceral fat to compare the three depots. Third, samples of EAT were only obtained proximal to the right coronary artery, because this is easiest area for the surgeon to take the biopsy from. These limitations aside, we feel that we have made some novel observations regarding the characteristics of EAT that add to the present literature.

**Conclusions**

We conclude that the adipose depot, rather than disease status, is the predominant determinant of gene expression in EAT. EAT has greater inflammatory gene expression than SAT even in the absence of coronary disease; this is consistent with its visceral origin. In regards to disease specific gene expression, patients without coronary disease had higher expression of all three members of the orphan nuclear hormone receptor family in EAT. This is consistent with mouse studies demonstrating a protective and anti-inflammatory effect of these nuclear receptors in atherosclerosis. We hypothesize that these receptors may play an important role in limiting adipose tissue inflammation in cardio-metabolic disorders.

**Disclosures**

**Acknowledgements**

This manuscript is dedicated to the memory of Dr. John N. Fain. The authors would like to thank the DERC morphology core and the UMASS Genomics Core for assistance with sample preparation.
References


circulating plasma levels of Omentin after myocardial ischemia. *Cardiovasc Diabetol.* 2017;16(1):87.


Figure 1. EAT Contains Smaller Adipocytes and Greater Number of Blood Vessels. A) Adipocyte size was determined using Adiposoft software on H & E stain sections of SAT and EAT in both cases and controls (top panel right). The mean adipocyte size in SAT was larger than those in EAT (Top panel left). This was true for both cases and controls. (**p<0.01 SAT vs. EAT, two-tailed Student’s t-test, n=13 per group. In each column, individual subjects are plotted, and error bars show the mean and standard deviation per group). B) Sections of SAT and EAT were stained with vWF and the number of blood vessels per high power field was quantified (bottom panel right). EAT had more blood vessels per field than SAT. (*p<0.05 EAT vs. SAT, two-tailed Student’s t-test, n=13 per group. In each column, individual subjects are plotted, and error bars show the mean and standard deviation per group).
Figure 2. Comparison of Gene Expression Between SAT & EAT in Controls. A) Volcano plot of 693 probes that were differentially expressed at a level of 1.5 FC (ANOVA p<0.05). 383 were up regulated in EAT (red) and 310 in SAT (green). B) Hierarchical clustering of differentially expressed genes in control SAT and EAT. C) KEGG Pathway analysis of differentially expressed genes in SAT and EAT.
Figure 3. Comparison of Gene Expression Between SAT & EAT in Cases. A) Volcano plot of 805 genes that were differentially expressed at a level of 1.5 FC (ANOVA p<0.05). 427 were up regulated in EAT (red) and 378 in SAT (green). B) Hierarchical clustering of differentially expressed genes in SAT and EAT of cases. C) KEGG Pathway analysis of differentially expressed genes in SAT and EAT of cases.
Figure 4. Comparison of Gene Expression in EAT Between Cases and Controls.  A) Volcano plot of 326 genes that were differentially expressed at a level of 1.5 FC (ANOVA p<0.05).  312 were up regulated in the EAT of control patients (red) and 14 in the EAT of cases (green).  B) Hierarchical clustering of differentially expressed genes in EAT of cases and controls of.  C) KEGG Pathway analysis of differentially expressed genes in EAT of controls. No pathways were significantly enriched in the EAT of cases.
Figure 5. Comparison of Gene Expression in SAT Between Cases and Controls.  

A) Volcano plot of 218 genes that were differentially expressed at a level of 1.5 FC (ANOVA p<0.05). 82 were upregulated in the SAT of control patients (red) and 136 in the SAT of cases (green). 

B) Hierarchical clustering of differentially expressed genes in SAT of cases and controls of. 

C) KEGG Pathway analysis of differentially expressed genes in SAT of cases. No pathways were significantly enriched in the EAT of cases.
Figure 6. qRT-PCR Verifies Down-Regulation of NR4A1, NR4A2, and NR4A3 in EAT of Cases. A) INTLN1 expression was greater in EAT than SAT in both cases and controls (n=11-12 per group, ***p<0.001 vs. SAT, 2 Way ANOVA and Tukey’s Multiple Comparison Test). B)
NNAT expression was greater in SAT than EAT in both cases and controls (n=11-12 per group, ***p<0.001 vs. EAT, 2 Way ANOVA and Tukey’s Multiple Comparison Test). C) NR4A1 mRNA expression was significantly decreased in EAT of cases compared to controls (n=11-12 per group, *p<0.05 vs. control EAT, 2 Way ANOVA and Tukey’s Multiple Comparison Test). D) NR4A2 expression was reduced in EAT of cases, in comparison to all other groups (n=11-12 per group, **p<0.01 vs. SAT con, ## p<0.01 vs. EAT con, ^^ p<0.01 vs. SAT case, 2 Way ANOVA and Tukey’s Multiple Comparison Test). E) NR4A3 expression was reduced in EAT of cases in comparison to SAT of cases and EAT of controls (n=11-12 per group, ## p<0.01 vs. SAT cases, ** p<0.01 vs. EAT controls). In each column, individual subjects are plotted, and error bars show the mean and standard deviation per group.
Figure 7. Staining of EAT for Classical and Alternative Macrophage Markers Shows No Difference Between Cases and Controls. A) Staining of EAT for the classical macrophage marker CD11c showed no difference between cases and controls. EAT of both cases and controls had more CD11c+ cells than SAT. *p<0.05 and ***p<0.001 EAT for indicated comparisons using the Kruskal-Wallis Test with Dunn’s Multiple Comparison Test. In each
column, individual subjects are plotted, and error bars show the mean and standard deviation per group. B) Representative CD11c staining in Cases and Controls. C) Staining of EAT for the alternative macrophage marker MRC1 showed no difference between cases and controls. EAT of both cases and controls had a greater number of MRC1 positive cells than SAT.

***p<0.001 EAT for indicated comparisons using the Kruskal-Wallis Test with Dunn’s Multiple Comparison Test. In each column, individual subjects are plotted, and error bars show the mean and standard deviation per group. D) Representative MRC1 staining in Cases and Controls. E) The average values CD11c and MRC1 positive cells per subject and tissue were divided to create a ratio. There was no difference in the ratio per tissue or group using the Kruskal-Wallis Test.
### Table 1. Clinical Characteristics of Study Participants

<table>
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<tr>
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<th>Controls (n=13)</th>
<th>Cases (n=13)</th>
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<tr>
<td><strong>Age</strong></td>
<td>67.1 ± 9.9</td>
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<tr>
<td><strong>Female</strong></td>
<td>6 (46%)</td>
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<td><strong>Body Mass Index (kg/m2)</strong></td>
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<td>29.5 ± 6.3</td>
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<tr>
<td><strong>Hypertension</strong></td>
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<td>9 (69.2%)</td>
<td>0.99</td>
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<tr>
<td><strong>CHF</strong></td>
<td>6 (46.1%)</td>
<td>4 (30.7%)</td>
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<td><strong>Diabetes Mellitus</strong></td>
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<td>4 (30.7%)</td>
<td>0.99</td>
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<td>2 (15.4%)</td>
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<td>10 (76.9%)</td>
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<td><strong>Obese</strong></td>
<td>7 (53.8%)</td>
<td>4 (30.8%)</td>
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</tr>
<tr>
<td><strong>Gensini Score</strong></td>
<td>0.7 ± 1.3</td>
<td>53.7 ± 27.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Statins</strong></td>
<td>6 (46%)</td>
<td>7 (53%)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Ejection fraction (%)</strong></td>
<td>54.8 ± 18.1</td>
<td>53.22 ± 16.3</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>HbA1c%</strong></td>
<td>6.0 ± 0.8</td>
<td>6.3 ± 0.9</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>TC (mg/dL)</strong></td>
<td>179.3 ± 54.6</td>
<td>153.4 ± 47.6</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>LDL (mg/dL)</strong></td>
<td>108.0 ± 41.9</td>
<td>88.8 ± 33.3</td>
<td>0.28</td>
</tr>
<tr>
<td><strong>HDL (mg/dL)</strong></td>
<td>45.7 ± 15.0</td>
<td>41.6 ± 13.5</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>TG (mg/dL)</strong></td>
<td>119.6 ± 42.3</td>
<td>115.1 ± 6.0</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Abbreviations: CHF= congestive heart failure, COPD=chronic obstructive pulmonary disease, HbA1c%=hemoglobin A1c, TC=total cholesterol, LDL=low density lipoprotein cholesterol, HDL=high density lipoprotein cholesterol, TG=triglycerides
Table 2. Differential miRNA Expression in SAT and EAT of Controls

<table>
<thead>
<tr>
<th>ID</th>
<th>Control EAT Avg (log2)</th>
<th>Control SAT Avg (log2)</th>
<th>FC</th>
<th>ANOVA P-val</th>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>8159371</td>
<td>5.19</td>
<td>4.41</td>
<td>1.72</td>
<td>0.0005</td>
<td>MIR126</td>
<td>microRNA 126</td>
</tr>
<tr>
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<td>5.81E-10</td>
<td>MIR1247</td>
<td>microRNA 1247</td>
</tr>
<tr>
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<td>6.68</td>
<td>-1.53</td>
<td>0.0239</td>
<td>MIR24-2</td>
<td>microRNA 24-2</td>
</tr>
</tbody>
</table>

Abbreviations: FC=fold change, SAT=subcutaneous adipose tissue, EAT=epicardial adipose tissue, MIR=microRNA
## Table 3. Differential miRNA Expression in SAT and EAT of Cases

<table>
<thead>
<tr>
<th>ID</th>
<th>Case EAT Avg (log2)</th>
<th>Case SAT Avg (log2)</th>
<th>FC</th>
<th>ANOVA P-val</th>
<th>Gene Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>7981326</td>
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<td>microRNA 1247</td>
</tr>
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<td>1.52</td>
<td>0.0072</td>
<td>MIR126</td>
<td>microRNA 126</td>
</tr>
<tr>
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<td>1.52</td>
<td>0.0011</td>
<td>MIRLET7C</td>
<td>microRNA let-7c</td>
</tr>
<tr>
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<td>8.44</td>
<td>9.02</td>
<td>-1.5</td>
<td>9.73E-08</td>
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<td>microRNA 22</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>microRNA 24-2</td>
</tr>
</tbody>
</table>

Abbreviations: FC=fold change, SAT=subcutaneous adipose tissue, EAT=epicardial adipose tissue, MIR=microRNA