INDEPENDENT STUDY PROJECT

TITLE: DIFFERENCES IN GENE EXPRESSION BETWEEN YOUNG AND ADULT MICE MAY PLAY A PROTECTIVE ROLE AGAINST INSULIN RESISTANCE IN THE SETTING OF HIGH FAT DIET-INDUCED OBESITY

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KEY SUMMARY POINTS:

- Young mice do not have a significant increase in their adipose tissue mass when fed high-fat diet compared to adult mice.
- Young mice appear protected from developing high-fat diet-induced insulin resistance. Fasting insulin levels in young mice on high-fat diet do not significantly differ from the levels in young mice on normal chow diet. On the other hand, adult mice fed high-fat diet have increased fasting glucose and fasting insulin when fed high-fat diet.
- Visceral fat gene expression differs between adult and young mice in response to high-fat diet. Many of these genes are associated with immune function and inflammation.

ABSTRACT:

As the prevalence of childhood obesity increases in the United States, the prevalence of obesity-related co-morbidities, such as type 2 diabetes mellitus, has increased as well. However, there is currently limited published data on the role of genes and signaling pathways in childhood obesity. Obesity is described as a cause of type 2 diabetes mellitus mediated by chronic inflammation. Our goal is to determine the role of genes in adipose tissue in insulin resistance amongst young pubertal mice by investigating the gene expression profiles of young and adult murine adipose tissue in diet-induced obesity. In our study, we hypothesized that adipose tissue gene profiles differ between young and adult mouse populations. To investigate these differences in adipose tissue between young and adult mice, we utilized genome-wide transcriptional microarray analysis. Results from this study showed that adult mice on high fat diet (HFD) have increased fat mass in comparison to their age-matched controls on normal chow diet (NCD). In contrast, young mice do not significantly increase their fat mass when placed on HFD compared to their age-matched controls on NCD. Furthermore, we found that adult mice exhibit an increase in fasting blood glucose and fasting insulin following HFD. Fasting blood glucose and fasting insulin values in young mice do not differ between NCD and HFD groups. This data suggest that young mice are protected from HFD-induced insulin resistance. In order to illuminate why young mice are protected from HFD-induced insulin resistance, we used microarray analysis and found candidate genes and signaling pathways that could be responsible for mitigating HFD-induced insulin resistance.

INTRODUCTION:

Obesity has been understood as an instigator of many diseases such as type 2 diabetes mellitus, atherosclerosis, cancer, defective immunity, dementia, and other problems. In obesity, the macrophages within adipose tissue are activated to a pro-inflammatory state causing insulin resistance [1]. Historically, salicylates were shown to lower glucose levels in diabetic patients, suggesting a link between inflammation and insulin resistance. To date, many inflammatory cytokines, genes, and pathways have been found to play important roles in obesity and metabolic syndrome [2]. Studies have shown that adipocytes undergo a stress response to activate the JNK and NF-kB pathway, which in turn increases the production of pro-inflammatory cytokines and chemokines such as TNF-a, IL-6, leptin, resistin, and MCP-1.

Childhood obesity is a growing problem with a prevalence rate of 16.9% in all children in the United States as of 2010 [3]. Even in the face of the rapidly growing pediatric obesity epidemic, there has been only limited study of adipose tissue characteristics in children [4]. Several studies have used microarray profiling of adipose tissues from adult mice and humans to identify genes associated with obese states. No studies thus far have addressed the gene profiles of adipose tissue in young mice or children [5].

OBJECTIVE AND HYPOTHESIS:

We hypothesized that adipose tissue characteristics differ between young and adult mice and proposed to investigate whether the gene expression profiles of adipose tissue vary with age in diet-induced obesity. Our preliminary data suggested that young obese mice were protected from high fat diet-induced insulin-resistance, so we secondarily hypothesized that the differences in

gene expression between young and adult mice could play a protective role against insulin resistance.

RESEARCH DESIGN AND METHODS:

In this study, we investigated differences in adipose tissue between young and adult mice using genome-wide transcriptional microarray analysis. This approach has been used successfully to measure differential gene expression in adipose tissues from obese mice and humans [5, 6].

Each study group was placed on respective diets for three weeks prior to tissue collection. There were 8-12 mice per cohort. Primary study cohorts were as follows:

1) "Young" HFD – 60% HFD started at 3 weeks of age (at weaning)
2) "Adult" HFD – 60% HFD started at 6 months of age
3) "Young" NCD – fed NCD through duration of study
4) "Adult" NCD – fed NCD through duration of study

During the study, body weight was measured weekly in HFD and NCD groups. At study week 3, we collected fasting blood samples for glucose and insulin values, and performed DEXA (dual energy x-ray absorptiometry) scans to evaluate body composition. All values are expressed as means ± standard error (SE) unless otherwise noted. We used analysis of variance (ANOVA) to determine differences between groups, and repeated measures ANOVA testing for comparisons over time using a Bonferroni or Tukey correction for multiple comparisons. P values of <0.05 were considered significant. At the end of the study period, we collected the visceral fat for RNA isolation. We used Ambion TRIzol reagent for tissue homogenization, followed by Qiagen RNeasy kit for RNA isolation.

We used the Affymetrix Mouse Gene 1.0 array, which provides coverage of 28,853 genes and expressed sequence tags. Three samples were sent for each of the groups described above, with each sample representing 3-4 mice. The UCSD GeneChip Core performed labeling, hybridization, and scanning of the array. A biostatistician with extensive experience in bioinformatics and microarray analysis performed statistical analysis of these data. The Affymetrix CEL files were analyzed for gene expression signal using the *rma* method of Irizarry et al. [7] and normalized using quantile normalization. Genes were tested for differential expression using the *samr* method of Tusher et al. [8]. Statistical significance of gene expression differences was estimated using p-values obtained from sample permutations while performing the unpaired two-class t-test on log2-transformed signal intensities. We set the s0.perc regularizing parameter in the samr method to 50 in order to avoid spuriously small variances. A false discovery rate (described as the "q-value") was calculated to represent a corrected p-value that accounts for multiple comparisons. For this study, genes with a q-value under 0.2 were considered statistically significant and entered into a program for pathway analysis [9].

RESULTS:

Young mice do not have significantly increased adipose tissue mass on HFD

We generated data on the body composition of our young and adult experimental mouse cohorts. Weekly weights for each study group revealed that adults fed HFD had the most pronounced weight gain over three weeks. There was a much smaller difference in weight gain in young mice fed HFD compared to their age-matched controls fed NCD (Figure 1). DEXA scans revealed that adult mice on HFD have increased fat mass in both percentage and in absolute value. In contrast, there was a trend for increased fat mass in young mice on HFD, but this difference was not

statistically different. The average adipose tissue mass for adult NCD mice was 10.21 ± 0.89 g (32 + 3%) compared to 18.00 ± 0.55 g (45 ± 1%) for adult HFD mice. Young NCD mice average 3.89 ± 0.33 g (19 ± 2%) fat mass while the young HFD mice average 6.07 ± 0.70 g (25 ± 3%) fat mass. The lean mass was similar in both young and adult mice regardless of diet. When comparing adult mice with young mice on NCD, adult mice had more fat mass than young mice (Figure 2). Therefore, adult mice gain more fat mass than young mice on HFD.

Young mice appear protected from developing HFD-induced insulin resistance

In addition to body composition data, we also obtained the fasting blood glucose and insulin levels in the mice. Fasting glucose values were not significantly different between groups (data now shown). There was a trend for higher fasting insulin values in adult mice on HFD when compared to age-matched NCD controls, but this difference was not statistically different. However, fasting insulin values were significantly higher in adult mice than young mice regardless of diet, suggesting that adult mice are more insulin resistant than young mice (Figure 3). In addition, insulin values were similar between young mice on HFD compared to their age-matched controls on NCD. This suggests that the young mice were protected from developing insulin resistance.

Visceral fat gene expression differs between adult and young mice in response to HFD

We sought to find out why young mice were seemingly protected from HFD-induced insulin resistance. We collected the intraabdominal fat tissue, extracted the RNA from these samples, and submitted them to the UCSD GeneChip Core for microarray analysis.

In adult mice, a total of 1360 genes showed a significant fold change between the HFD group and the NCD group with a false-discovery rate <0.2 (Table 1). Using a pathway analysis program called GOrank, we determined that the top 5 pathways with significant differences in gene expression between the HFD group and the NCD group were related to the immune response as well as to lipid metabolism (Table 2). This finding continues to support the theory that insulin resistance is associated with chronic inflammation in obese states.

In young mice, we did not see any significant gene expression changes between the HFD group and the NCD group (Table 1). We suspect that this is reflective of the apparent protection that young mice on HFD have from developing insulin resistance.

A large number of genes (4667) significantly differed in expression between normal chow-fed adult and young mice (Table 1). Upon closer examination of these genes, most of them belong in pathways pertaining to reproduction. It is possible that the visceral fat samples for the adults in this study were contaminated with testicular tissue, thus confounding the picture.

In the group comparing adult and young mice on HFD diet, there were 611 genes with significant expression change (Table 1). The top 5 significant pathways that encompass the genes that differed between the adult and young mice are associated with immune response and inflammation (Table 2). This finding suggests that adult mice on HFD have a marked immune and inflammatory response compared to young mice on HFD, and could also suggest that the chronic inflammation secondary to obesity is the cause of insulin resistance in adult mice.

The effect of age was apparent in the comparison adult versus young mice on HFD. Immune-related pathways and inflammation-related pathways had the most profound number of pathways with significant gene expression changes (Figure 4). This data again suggest that HFD does not exacerbate immune inflammatory responses in young mice as compared to adult mice, which in turn may play a role in their protection against insulin resistance. We can also see an effect of diet (HFD versus NCD) in adult mice on pathways pertaining to immune function, inflammation, insulin regulation, and lipid metabolism (Figure 4).

In addition to the increased expression of immune and inflammatory genes in adults with HFD, there is also downregulation of genes related to insulin action in adults on HFD (Figure 5). The estrogen receptor 1 (ESR1) gene was downregulated in adult mice on HFD. While it is known that insulin-mediated metabolism is impaired with high-fat feeding, some studies demonstrated that restoration of estrogen to physiological concentrations in humans and rodents can protect against HFD-induced insulin resistance [11]. Estrogen has also been found to protect pancreatic beta cell function in type 2 diabetes [12].

DISCUSSION

Obesity has been known to cause chronic inflammation, and in turn lead to insulin resistance. To date, our findings suggest that young mice and adult mice respond differently when fed a high-fat diet. Both groups show no change in lean mass after high-fat feeding, but young mice accrue less adipose tissue than adult mice in response to HFD. Moreover, our studies demonstrate that young mice appear to be protected from insulin resistance, whereas adult mice are not. Microarray studies revealed no significant gene expression changes between young mice on

NCD and young mice on HFD. On the other hand, adult mice on HFD have significant changes in the expression of genes pertaining to immune response and inflammation as compared to young mice on HFD. Moreover, there is also downregulation of metabolism-associated genes in adults on HFD, including ESR1, which serves important functions in insulin-mediated metabolism.

However, multiple other non-genetic factors may play a role in the different responses between adult and young mice to HFD. There is literature showing that young mice have higher basal metabolic rates, which in turn could be the reason that young mice do not significantly increase their adipose mass and appear to be protected from obesity-induced insulin resistance [13]. Although we did not investigate the role of brown adipose tissue (BAT) in this study, there may be more BAT in young mice. Future studies may uncover whether BAT mass contributes to the metabolic rate in young mice and mitigates their response to high-fat diet.

Despite our findings, our studies are limited by several factors. The adult normal chow sample had a remarkable upregulation in reproduction-associated genes, suggesting possible contamination of epididymal fat with testicular tissue. We lack reliable tissue from the adult cohort fed NCD that is necessary for determining the effect of HFD versus NCD on adults. Therefore, this study should be repeated to ensure no contamination of tissues has occurred to confound our data.

Even with a robust microarray study, gene expression does not necessarily reflect protein function. Future studies will be necessary to confirm the observed changes in gene expression by conducting RT-PCR followed by the examination of associated protein expression by Western

blot. Once protein levels are confirmed, we can perform functional studies by using cell lines or generating targeted knockout or transgenic mice to characterize the role of putative targets on metabolic endpoints such as adipogenesis and glucose transport. To date we have not identified specific gene candidates for PCR and Western blot analysis. Future work in identifying interesting candidates would require finding a gene signature for adult and young mice so that we can find specific genes that differ between those two populations for study [14].

In conclusion, we have shown that age affects the metabolic response to high-fat diet. Our data support the conclusion that HFD-induced insulin resistance is associated with a chronic inflammatory state in adult mice. Additionally, we have begun to evaluate differences in gene expression between adult and young mice that could account for the apparent protection that young mice have from developing HFD-induced insulin resistance. Epigenetic modifications may explain differences in gene expression between these genetically identical groups, as studies have attributed the effect of diet and other environmental factors in epigenetic changes that lead to insulin resistance [15]. This work serves as a starting point for these future studies.

FIGURE LEGENDs:

Figure 1. Adult mice fed HFD gain more weight. Error bars represent standard error (SE). HF = high fat diet. NC = normal chow diet. Please note the difference in the y-axis scale between graphs. *p <0.05, **p<0.01, ***p<0.001

Figure 2. Young mice gain less fat mass than adult mice when fed HFD. White bars represent percent body fat of lean mass. Grey bars represent percent body fat of fat mass. HFD= high fat diet. NCD= normal chow diet. NS = not significant. Error bars represent standard error (SE). P-values were generated using ANOVA with Bonferroni correction for multiple comparisons. *p < 0.05.

Figure 3. Young mice do not develop insulin resistance when fed HFD. The Y-axis represents insulin levels in ng/ml. Adult mice have significantly greater insulin levels in response to HFD than young mice on HFD. Error bars represent standard error (SE). P-values were generated using ANOVA with Tukey correction. **p < 0.01.

Figure 4. Pathways with genes that had statistically significant differences. Each vertical bar represents one pathway, and the height of the bar signifies number of genes in the pathways. The horizontal axis is grouped by functional ontology of the gene pathways. The graph on top represents the comparison group of HFD versus NCD in adults. The graph on the bottom represents the comparison group of Adult versus Young mice on HFD.

Figure 5. **Heatmap of microarray data**. Each row represents one gene (as indicated on the right). Each column represents one sample (each sample consists of RNA from 3-4 mice). Genes are clustered by function (top genes are related to lipid metabolism, bottom genes are related to immune response and inflammation). Color legend represented at the bottom of the figure.

Table 1. Number of statistically significant genes that had expression changes between the comparison groups.

Table 2. Top 5 pathways determined by GOrank for each comparison group that contains genes that showed statistically significant expression changes.

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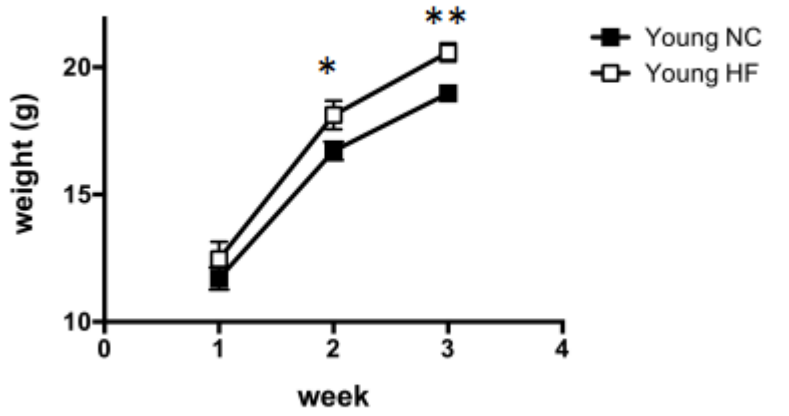
**
*
Young NC
Young HF
weight (g)
20
15
10
0
1
2
3
4
week

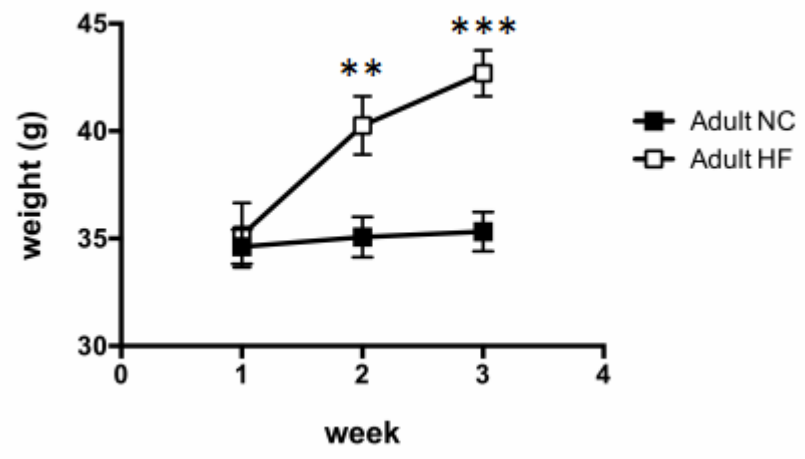
***
**
Adult NC
Adult HF
weight (g)
45
40
35
30
0
1
2
3
4
week

Figure 1

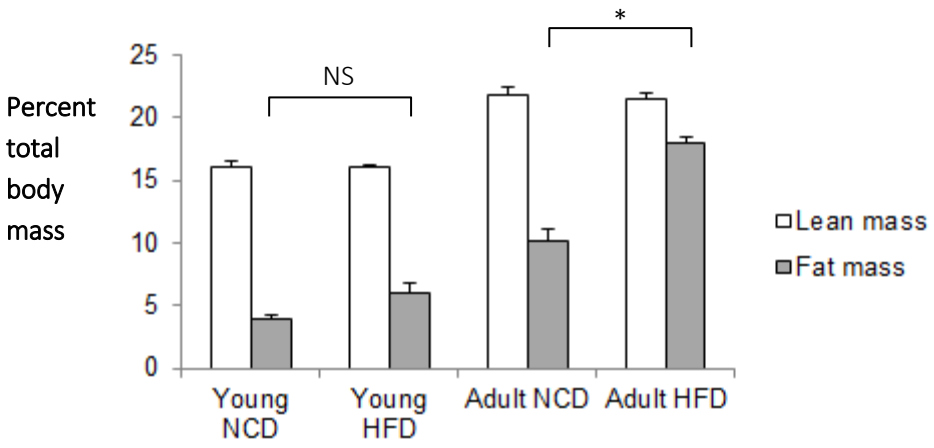
Percent total body mass
25
20
15
10
5
0
NS
*
Young NCD
Young HFD
Adult NCD
Adult HFD
Lean mass
Fat mass

Figure 2

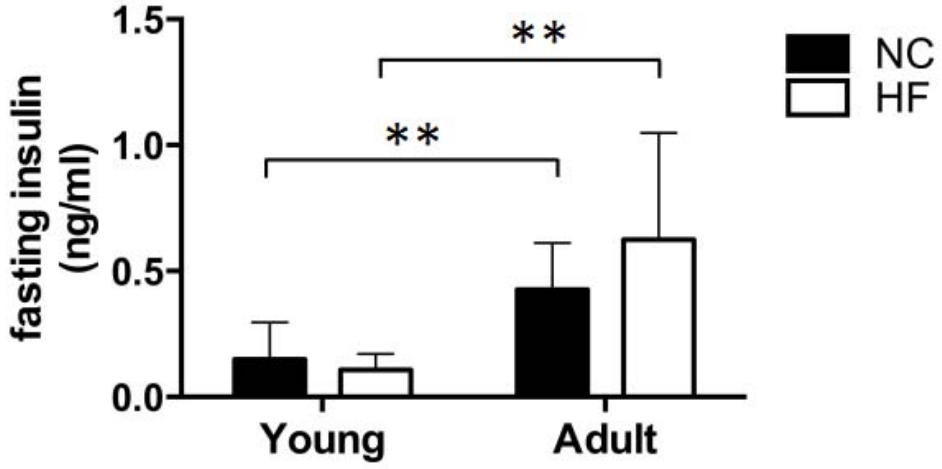

Figure3

Comparison group 1	Comparison group 2	No. of significantly different genes (q value <0.2)
Adult (NCD)	Young (NCD)	4667
Adult (HFD)	Young (HFD)	611
Adult (NCD)	Adult (HFD)	1360
Young (NCD)	Young (HFD)	0

Table 1

Comparison Groups	No. of sig genes (q value <0.2)	Top 5 pathways (determined by GOrank)
Adult versus Young (HFD)	611	1. immune response 2. cell activation 3. leukocyte activation 4. regulation of immune system process 5. defense response
HFD versus NCD (adults)	1360	1. immune response 2. defense response 3. cellular lipid metabolic process 4. lipid metabolic process 5. innate immune response

Table 2

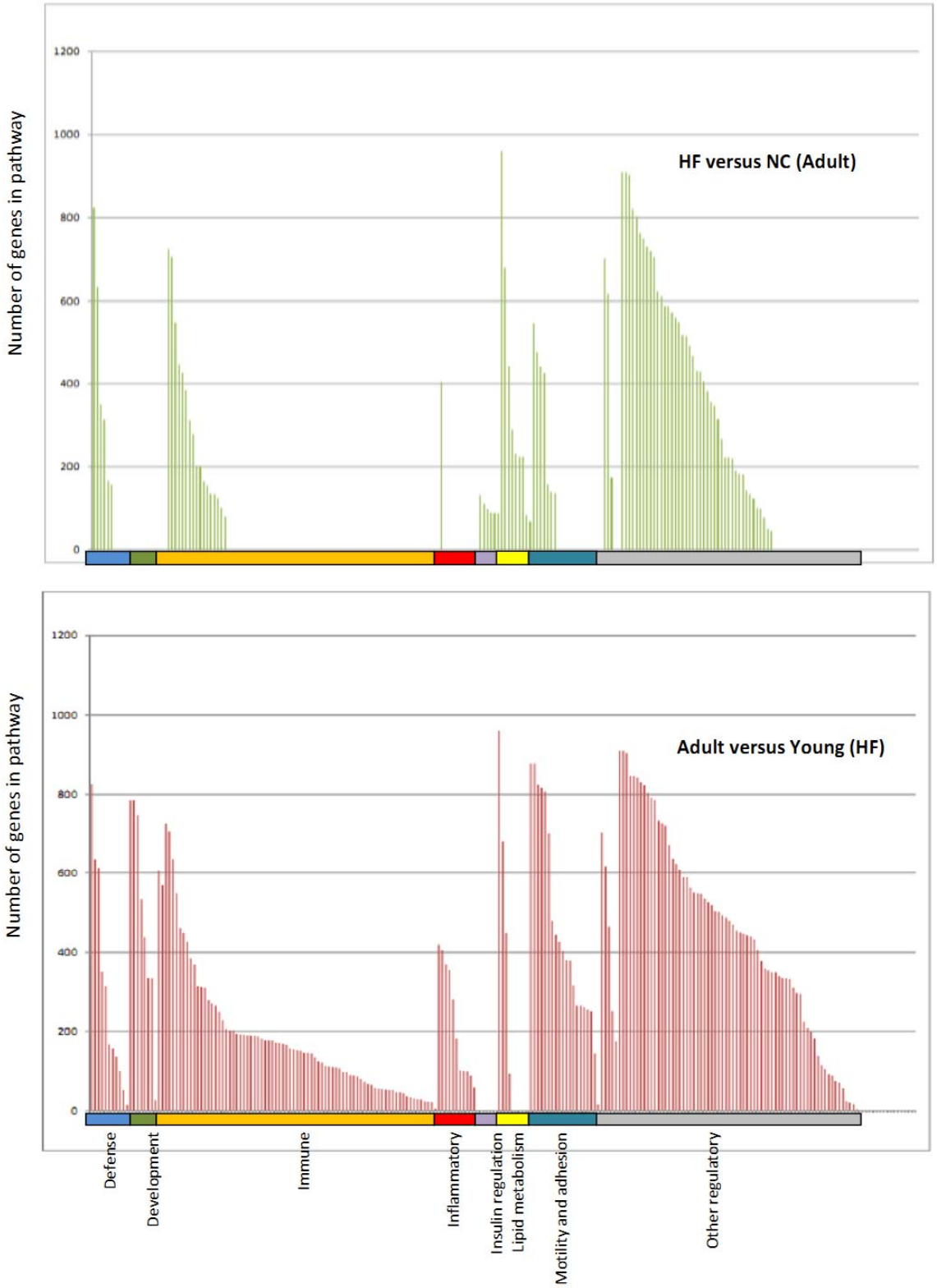
Number of genes in pathway
1200
1000
800
600
400
200
0
HF versus NC (Adult)
Number of genes in pathway
1200
1000
800
600
400
200
0
Adult versus Young (HF)
Defense
Development
Immune
Inflammatory
Insulin regulation
Lipid metabolism
Motility and adhesion
Other regulatory

Figure 4

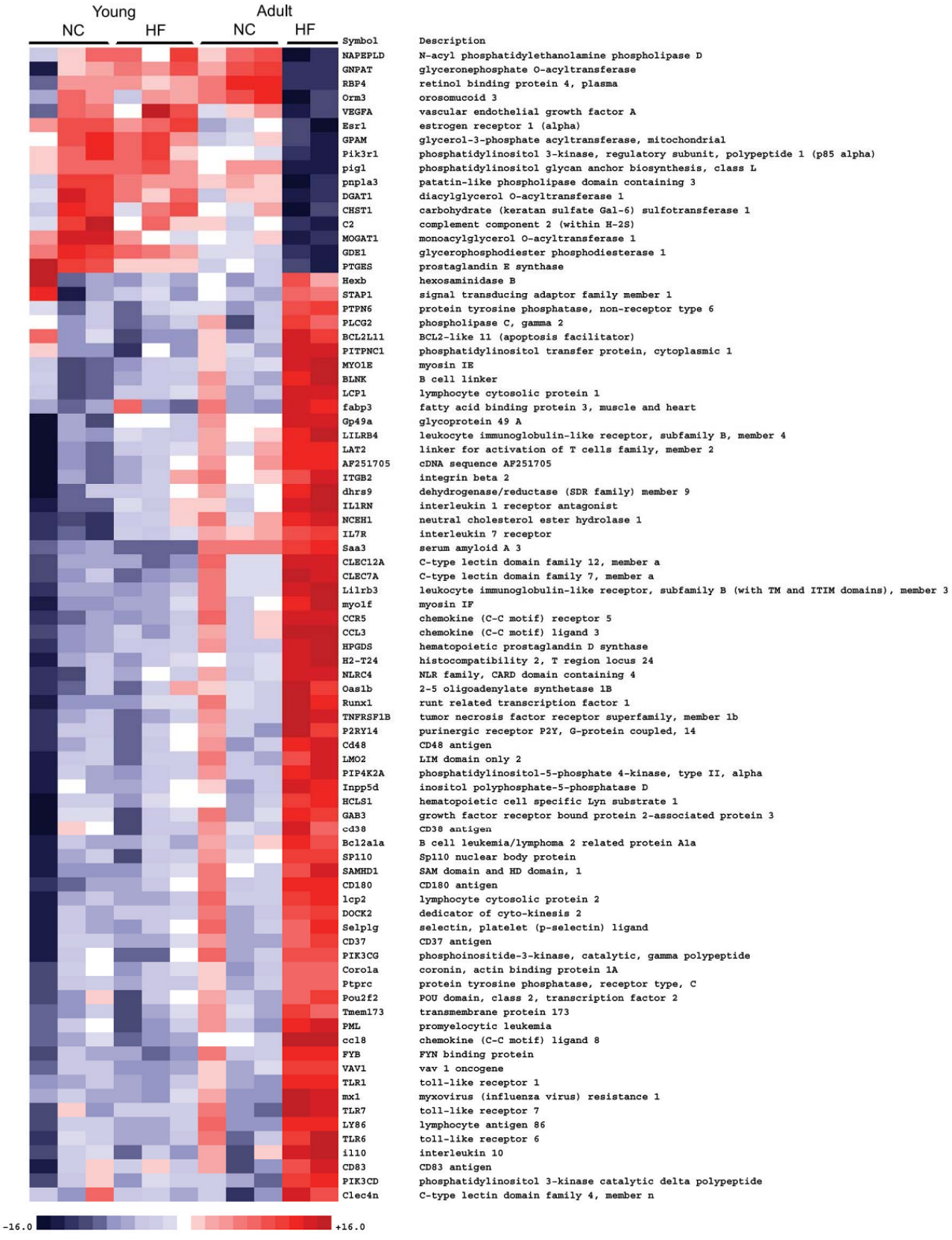
Young
Adult
NC
HF
NC
HF
Symbol Description
NAPEPLD N-acyl phosphatidylethanolamine phospholipase D
GNPAT glyceronephosphate O-acyltransferase
RBP4 retinol binding protein 4, plasma
Orm3 orosomucoid 3
VEGFA vascular endothelial growth factor A
Esr1 estrogen receptor 1 (alpha)
GPAM glycerol-3-phosphate acyltransferase, mitochondrial
Pik3r1 phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
pigl phosphatidylinositol glycan anchor biosynthesis, class L
pnpla3 patatin-like phospholipase domain containing 3
DGAT1 diacylglycerol O-acyltransferase 1
CHST1 carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
C2 complement component 2 (within H-2S)
MOGAT1 monoacylglycerol O-acyltransferase 1
GDE1 glycerophosphodiester phosphodiesterase 1
PTGES prostaglandin E synthase
Hexb hexosaminidase B
STAP1 signal transducing adaptor family member 1
PTPN6 protein tyrosine phosphatase, non-receptor type 6
PLCG2 phospholipase C, gamma 2
BCL2L11 BCL2-like 11 (apoptosis facilitator)
PITPNC1 phosphatidylinositol transfer protein, cytoplasmic 1
MYO1E myosin IE
BLNK B cell linker
LCP1 lymphocyte cytosolic protein 1
fabp3 fatty acid binding protein 3, muscle and heart
Gp49a glycoprotein 49 A
LILRB4 leukocyte immunoglobulin-like receptor, subfamily B, member 4
LAT2 linker for activation of T cells family, member 2
AF251705 cDNA sequence AF251705
ITGB2 integrin beta 2
dhrs9 dehydrogenase/reductase (SDR family) member 9
IL1RN interleukin 1 receptor antagonist
NCEH1 neutral cholesterol ester hydrolase 1
IL7R interleukin 7 receptor
Saa3 serum amyloid A 3
CLEC12A C-type lectin domain family 12, member a
CLEC7A C-type lectin domain family 7, member a
Lilrb3 leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3
myo1f myosin IF
CCR5 chemokine (C-C motif) receptor 5
CCL3 chemokine (C-C motif) ligand 3
HPGDS hematopoietic prostaglandin D synthase
H2-T24 histocompatibility 2, T region locus 24
NLRC4 NLR family, CARD domain containing 4
Oas1b 2-5 oligoadenylate synthetase 1B
Runx1 runt related transcription factor 1
TNFRSF1B tumor necrosis factor receptor superfamily, member 1b
P2RY14 purinergic receptor P2Y, G-protein coupled, 14
Cd48 CD48 antigen
LMO2 LIM domain only 2
PIP4K2A phosphatidylinositol-5-phosphate 4-kinase, type II, alpha
Inpp5d inositol polyphosphate-5-phosphatase D
HCLS1 hematopoietic cell specific Lyn substrate 1
GAB3 growth factor receptor bound protein 2-associated protein 3
cd38 CD38 antigen
Bcl2a1a B cell leukemia/lymphoma 2 related protein A1a
SP110 Sp110 nuclear body protein
SAMHD1 SAM domain and HD domain, 1
CD180 CD180 antigen
lcp2 lymphocyte cytosolic protein 2
DOCK2 dedicator of cyto-kinesis 2
Selplg selectin, platelet (p-selectin) ligand
CD37 CD37 antigen
PIK3CG phosphoinositide-3-kinase, catalytic, gamma polypeptide
Coro1a coronin, actin binding protein 1A
Ptprc protein tyrosine phosphatase, receptor type, C
Pou2f2 POU domain, class 2, transcription factor 2
Tmem173 transmembrane protein 173
PML promyelocytic leukemia
ccl8 chemokine (C-C motif) ligand 8
FYB FYN binding protein
VAV1 vav 1 oncogene
TLR1 toll-like receptor 1
mx1 myxovirus (influenza virus) resistance 1
TLR7 toll-like receptor 7
LY86 lymphocyte antigen 86
TLR6 toll-like receptor 6
il10 interleukin 10
CD83 CD83 antigen
PIK3CD phosphatidylinositol 3-kinase catalytic delta polypeptide
Clec4n C-type lectin domain family 4, member n
-16.0
+16.0

Figure 5