Effects of Intrinsic Cardiorespiratory Fitness and Caloric Restriction on Metabolites in Plasma and Skeletal Muscle and Skeletal Muscle Mitochondrial DNA

by

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List of abbreviations

4-HNE: 4-hydroxynonenal

8-iso-PGF2α: 8-iso-prostaglandin F2α

8-OHdG: 8-hydroxy-2′-deoxyguanosine

8-oxo-dG: 8-oxo-2'-deoxyguanosine

8dOHG: 8-dihydroxyguanosine

AA: Amino acid

ACADSB: Short/branched chain acyl-CoA dehydrogenase

ACC: Acetyl-CoA carboxylase

ACO: Aconitase

ADP: Adenosine diphosphate

AF: Atrial fibrillation

Akt: Protein kinase B

AMP: Adenosine monophosphate

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPK: 5' adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

AS160: Akt substrate of 160 kDa

ATP: Adenosine triphosphate

ATP10A: Mitochondrial ATPase complex subunit ATP10

AUC: Area under the curve

β-HAD: Beta-hydroxy-acyl-CoA-dehydrogenase

BCAA: Branched-chain amino acid

BCKA: Branched-chain ketoacid

BDNF: Brain-derived neurotrophic factor

BMI: Body mass index

BP: Blood pressure

CHD: Coronary heart disease

COPD: Chronic obstructive pulmonary disease

COX: Cytochrome oxidase

CPS: Carbamoyl phosphate synthetase

CPT: Carnitine palmitoyltransferase

CR: Caloric restriction

CRF: Cardiorespiratory fitness

CRP: C-reactive protein

CS: Citrate synthase

CVD: Cardiovascular disease

DBP: Diastolic blood pressure

DG: Diglyceride

DHEAS: Dehydroepiandrosterone sulfate

DM: Diabetes mellitus

DNMT: DNA methyltransferase

DZ: Dizygotic twins

ECG: Electrocardiogram

eNOS: Endothelial nitric oxide synthase

ETC: Electron transport chain

F6P: Fructose-6-phosphate

FA: Fatty acid

FAD: Flavin adenine dinucleotide

FAO: Fatty acid oxidation

FAT/CD36: Fatty acid translocase

FBP: Fructose 1,6-bisphosphate

FFA: Free fatty acid

FFM: Fat free mass

FPG: Fasting plasma glucose

FoxO: Forkhead box O

G6P: Glucose-6-phosphate

GAP-43: Growth Associated Protein 43

GI: Gastrointestinal

h^2: Heritability

HAHDB: Hydroxyacyl-CoA dehydrogenase

HAT: Histone acetyltransferase

HCR rat: High capacity running rat

HDAC: Histone deacetylase

HDL: High density lipoprotein

HF: Heart failure

HFD: High-fat diet

HILIC: Hydrophilic interaction chromatography

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

HSL: Hormone-sensitive lipase

hTERT: Human telomerase reverse transcriptase

IFG: Impaired fasting glucose

IGF-1: Insulin-like Growth Factor 1

IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKKβ: IκB kinase, beta

IL-6: Interleukin 6

IWMC: Investigational Weight Management Clinic

LCAD: Long-chain acyl-CoA dehydrogenase

LC-MS: Liquid chromatography-mass spectrometry

LCR rat: Low capacity running rat

LDL: Low density lipoprotein

LPL: Lipoprotein lipase

LysoPA: Lysophosphatidic acid

LysoPC: Lysophosphatidylcholine

LysoPE: Lysophosphatidylethanolamine

LysoPG: Lysophosphatidylglycerol

LysoPI: Lysophosphatidylinositol

LysoPS: Lysophosphatidylserine

MANOVA: Multivariate analysis of variance

MDA: Malondialdehyde

MDRTC: Michigan Diabetes Research and Training Center

METs: Metabolic equivalents

MFN: Mitofusin

MMTT: Mixed meal tolerance test

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRM: Multiple reaction monitoring

MS: Mass spectrometry

mtDNA: Mitochondrial DNA

MTE: Mitochondrial thioesterase

MZ: Monozygotic twins

NAD^+: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide hydride

nDNA: Nuclear DNA

NDUFS: NADH-ubiquinone oxireductase Fe-S

NMDA: N-methyl-d-aspartate

Nor: Neuron-derived orphan receptor

NRF: Nuclear respiratory factor

Nur77: Nerve growth factor IB

OXPHOS: Oxidative phosphorylation

$p16^{INK4a}$: Cyclin-dependent kinase inhibitor 2A (inhibitor of CDK4)

PA: Phosphatidic acid

PAD: Peripheral artery disease

PAI-1: Plasminogen activator inhibitor-1

PC: Phosphatidylcholine

PCR: Polymerase chain reaction

PE: Phosphatidylethanolamine

PEG: Polyethylene glycol

PEPCK-C: Phosphoenolpyruvate carboxykinase-C

PFK: Phosphofructokinase

PG: Phosphatidylglycerol

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1, alpha

PKB: Protein kinase B

PKC: Protein kinase C

PI: Phosphatidylinositol

PI3K: Phosphatidylinositide 3-kinase

PPAR: Peroxisome proliferator-activated receptor

PS: Phosphatidylserine

PUFA: Polyunsaturated fatty acid

QC: Quality control

qPCR: Quantitative polymerase chain reaction

QUAD: Quadratic

REE: Resting energy expenditure

RMR: Resting metabolic rate

ROS: Reactive oxygen species

RPLC: Reversed-phase liquid chromatography

RQ: Respiratory quotient

RQE: Respiratory quotient at VO_2max

RQR: Respiratory quotient at rest

RSD: Relative standard deviation

RT: Retention time

RUNX: Runt-related transcription factor

SBP: Systolic blood pressure

SD: Standard deviation

SEM: Standard error of mean

SIM: Selected ion monitoring

SIRT: Sirtuin

SOD: Superoxide dismutase

SPH: Synaptophysin

SREBP-1c: Sterol regulatory element-binding protein-1c

TCA: Tricarboxylic acid

TEM: Transmission electron microscopy

TFAM: Mitochondrial transcription factor A

TG: Triglyceride

TGF-β1: Transforming growth factor-beta 1

TIG: Tazarotene-induced gene

TNF-α: Tumor necrosis factor-alpha

UCP: Uncoupling protein

UDP: Uridine diphosphate

UQCRC: Ubiquinol-cytochrome c reductase core

VLDLR: Very low density lipoprotein receptor

WC: Waist circumference

WT: Wilms tumor

Abstract

Individuals with higher cardiorespiratory fitness (CRF), most often assessed by measuring maximal oxygen consumption while performing incremental exercise, have a decreased risk of a wide variety of metabolic diseases and a reduced mortality rate. Rats selected for increased CRF show improved metabolic status and longevity and a higher mitochondrial capacity for fatty acid (FA) and branched-chain amino acid (BCAA) catabolism as they age. To determine whether these findings are associated with CRF-induced delayed metabolic aging in humans, we assessed CRF reported as VO_2max (ml/min/kg fat free mass), fasting plasma metabolites that are related to FA and amino acids (AA) metabolism, as well as 2 hr-postprandial skeletal muscle metabolites levels in lean (n = 28 plasma, n = 15 muscle) and obese (n = 124 plasma, n = 99 muscle) individuals, the latter before and after 4-6 months of caloric restriction (CR) by 800 kcal/day of high-protein liquid diet. VO_2max fell with age, but was higher at any age in lean compared to obese. Multiple linear regression analysis of plasma metabolites demonstrated that FA-derived acylcarnitines and the ratio of BCAA intermediates to their substrates increased with age, suggesting that aging reduces capacity for fatty acid oxidation (FAO) and BCAA catabolism. Regardless of age, higher CRF was associated with lower levels of FA-derived acylcarnitines. In addition, CRF counteracted the age-associated increase in FA-derived acylcarnitines, suggesting that higher CRF directly mitigates age-associated incomplete FAO. CR also decreased FA-derived acylcarnitine levels and decreased the ratio of BCAA intermediates to their substrates, suggesting that CR decreases metabolic markers of aging. Multiple linear regression analysis of skeletal muscle metabolites showed that most phospholipids decreased with age and polyunsaturated phospholipids increased with age, consistent with the age-induced increase in susceptibility to lipid peroxidative damage of mitochondria. AAs and a ratio of valine intermediate to valine were negatively correlated with VO_2max, but FA-derived acylcarnitines were positively correlated with VO_2max,

suggesting that higher CRF is associated with improved mitochondrial capacity for AA catabolism and FAO. VO_2max was also positively correlated with glutathione (the most abundant endogenous antioxidant), DHEAS and UDP-n-acetyl glucosamine, all of which decrease with age, suggesting that higher CRF is associated with lessened oxidative stress which may play a role in slowing aging. Unlike plasma metabolites, CRF was not statistically associated with age-related changes in skeletal muscle metabolite levels. Rather, CRF was directly associated with changes in skeletal muscle metabolites independent of age and its effect was in part mediated by mitochondrial DNA (mtDNA) count number, suggesting an expansion of mitochondria with CRF. CR decreased isoleucine+leucine-to-their-substrates ratio and increased FA-derived acylcarnitines, suggesting that CR improves mitochondrial capacity for AA catabolism and FAO as well. CR also decreased the levels of polyunsaturated FAs (PUFAs), and increased levels of glycocholic acid, a metabolite inversely related to lifespan in humans, again supporting the notion that CR delays metabolic aging. Because both high CRF and CR can improve substrate utilization of mitochondria and show evidence of delayed metabolic aging, the effects of high CRF and CR are comparable. Both appear to induce metabolite profiles consistent with a 'younger' metabolic state, offering insight into the mechanisms by which they are associated with enhanced metabolic health and longevity.

Chapter 1
Introduction

Living longer, healthier lives has long been a goal. Modern society has brought a tremendous number of advances, including better sanitation, assured food supply and safer working conditions and medical advances have reduced infant mortality and enhanced infectious disease control. Collectively, these developments allow us to live longer than our ancestors; however, we are now at an increased risk of developing multiple chronic diseases, including cardiometabolic diseases, cancer and neurodegeneration due to aging and increasingly, unhealthy diets. Essentially, we live longer but are sicker. Improving conditions such that we remain healthy throughout our lives (healthspan) is an important goal.

It is generally accepted that cardiorespiratory fitness (CRF) is positively associated with improved health and longevity. Though CRF is largely genetically determined, it can be increased by regular aerobic training. To maintain peak CRF, regular aerobic training must continue throughout the lifespan. This is nearly impossible, as older individuals may lack the endurance to train. Additionally, aerobic training can be time-consuming and is often not ideal for people in modern society. Caloric restriction (CR) is another strategy that promotes health and extends lifespan in most animals. However, CR is also difficult to maintain long-term as our brains provide a 'food reward', encouraging continued consumption, particularly high-fat and high-sugar diets prevalent in modern society. Moreover, eating together is a common social interaction, thus the potential anti-social nature CR may decrease adherence.

Because lifelong aerobic training and CR are nearly impossible to achieve, the creation of comparable drug mimetics offers a potentially viable alternative to maximize lifespan healthspan. Before mimetics can be developed, the mechanisms by which aerobic training

and CR benefit health and increase lifespan must be determined. Mitochondria are intricately involved in both metabolism and aging, providing a key nexus that links health and longevity while offering a potential drug target worthy of additional exploration.

1.1 Aims and scope

This thesis involves the intrinsic (genetic, non-trained) component of CRF and how it may relate to caloric restriction. The aim is to better understand how high intrinsic CRF and CR are associated with good health and longevity. I focus on the effects of CRF and CR on mitochondrial metabolism as these organelles are innately involved in metabolism and whose function decreases with age.

My research was conducted with samples and data obtained from obese adults who participated in the University of Michigan Investigational Weight Management Clinic who have undergone a program of clinically-monitored weight loss using caloric restriction and the systematic collection of clinical data and samples. This clinic is directed by Dr. Amy Rothberg and the research arm of the program is directed by Dr. Charles Burant. These data were derived from samples collected at baseline and again after 3-4 months of CR. Non-obese individuals were recruited and serve as controls both for the IWMC cohort. We performed analysis of both plasma, and skeletal muscle, the latter a primary organ that determines CRF and is responsible for nutrient metabolism. For my studies of metabolism, I employed a targeted metabolomic approach to identify metabolites involved in nutrient (amino acids, glucose and fat) metabolism, as well as an untargeted metabolomic approach to identify additional metabolites that are affected by age, CRF or CR. I also assessed mitochondrial DNA (mtDNA) and examined how the levels of mtDNA and mutations may be associated with age-dependent changes in metabolism.

1.2 Study overview

My thesis has 7 Chapters including an introduction. The additional 6 Chapters consist of background knowledge about the effects of CRF and CR on health and lifespan (Chapters 2 and 3), biological research to identify how CRF and CR are associated with health and longevity (Chapters 4 and 5), methodological research to clarify the importance

of metabolomic data normalization (Chapter 6), as well as implications of the current study and future directions (Chapter 7).

In Chapter 2, I review the evidence that intrinsic CRF is genetically determined. In addition, I describe the benefits of high CRF as improved health and decreased mortality, based upon several longitudinal studies in humans. I include in-depth studies from a rat genetic model of aerobic treadmill running capacity that serves as the basis for my clinical studies. I also review additional human studies to identify potential mechanisms by which high CRF is associated with good health and extended lifespan. The review will show that despite an extensive number of studies, how CRF effects nutrient metabolism and modulates aging remain unknown.

In Chapter 3, I review the anti-aging effects of CR, i.e. CR attenuates age-related diseases in animals and humans. In addition, I review the mitochondrial theory of aging, the modulating effects of CR on age-related decline in mitochondrial function, as well as epigenetic regulations linked to the anti-aging effects of CR. Although my review demonstrates that the modulating effects of CR on the age-related decline in mitochondrial function have been extensively documented, the influence of mtDNA on this relationship is uncertain and was therefore included in my project.

Chapter 4 contains the first part of my original research, in which I study the effects of CRF and CR on age-related changes in plasma metabolite levels. In this study, I employed a targeted metabolomic approach to quantitate the levels of amino acids (AAs), branched-chain ketoacids (BCKAs), free fatty acids (FFAs) and acylcarnitines in fasting plasma of non-obese and obese subjects, the latter at both before and after 12-16 weeks of CR. The results demonstrate that aging is associated with incomplete whole body fatty acid oxidation (FAO) and decreased mitochondrial capacity of branched-chain amino acid (BCAA) catabolism. High CRF and CR are directly associated with more complete FAO. High CRF also counteracts age-related incomplete FAO, and CR improves the mitochondrial capacity of BCAA catabolism. Both high CRF and CR delay metabolic aging, consistent with a 'younger' metabolic state.

The results described in Chapter 4 inspired me to extend my work to mtDNA and metabolomic analysis of human skeletal muscle, described in Chapter 5. In this study, I sought to identify mechanisms by which high CRF and CR delay metabolic aging. I chose

skeletal muscle because it is a major organ that is accessible, contributes to CRF and is responsible for nutrient metabolism. I utilized a targeted metabolomic approach to quantitate the levels of AAs, oleic acid, acylcarnitines, glycolysis metabolites, TCA cycle metabolites and nucleotides, as well as an untargeted metabolomic approach to identify additional metabolites, especially lipids. Because mtDNA is affected by aging and differed between high and low CRF rats as well as between ad libitum-fed and CR rats, I also analyzed mtDNA in these samples via real-time PCR. I found that metabolite levels in skeletal muscle change with age. High CRF and CR are associated with better mitochondrial capacity of AA and fatty acid (FA) utilization, resulting in more TCA cycle metabolite production. Unlike plasma metabolites, CRF does not modulate the effect of age-related changes in skeletal muscle metabolite levels. Rather, it is directly associated with changes in metabolite levels in skeletal muscle, and this effect at baseline is partly mediated by mtDNA count number. However, CR diminishes the effect of CRF on mtDNA count number. High CRF is also associated with higher levels of UDP-n-acetyl glucosamine and DHEAS that are observed to decrease with age, suggesting that high CRF delays metabolic aging. CR also delays aging by decreasing the level of glycocholic acid, which is a marker of short lifespan, and by decreasing the level of polyunsaturated FAs (PUFAs), which are associated with susceptibility to lipid peroxidative damage to mitochondria that usually increases with age. These benefits of high CRF and CR are again consistent with a 'younger' metabolic state, and may be associated with improved metabolic health and longevity.

Chapter 6 is methodological research that describes metabolomic data normalization. Because liquid chromatography-mass spectrometry (LC-MS) used for metabolomic research usually drifts over the course of a run and not all internal standard are available to set a quality control for all metabolites, a drift correction method is necessary for data normalization to ensure that data from samples analyzed in many batches over an extended period of time can be compared in an accurate manner. In this study, I demonstrate a quadratic drift correction method among 14 batches of analysis of 418 plasma samples obtained during a mixed meal tolerance test (MMTT) in non-obese and obese subjects, the latter at both before and after 12-16 weeks of CR. Corrected data reveal differential meal tolerance in non-obese and obese subjects, whereas greater

variability in the uncorrected data resulted in the loss of statistical significance among most of the comparisons.

In Chapter 7, I describe the implications of my studies and future directions. In this Chapter, I discuss how results from metabolomic studies in fasting plasma (Chapter 4) and postprandial skeletal muscle (Chapter 5) are consistent with the benefits of high CRF and CR on health and longevity as previously documented by others and reviewed in Chapter 2 and 3. My results fit within the established literature, suggesting they represent potential mechanisms that link high CRF and CR to improved health and longevity. Based upon the results of my research, I recommend that future research focus on the development of effective targeted therapies to improve health and maximize lifespan.

Chapter 2
Systematic literature review:
Influence of intrinsic cardiorespiratory fitness
on health and longevity in humans

2.1 Introduction

Intrinsic cardiorespiratory fitness (CRF) is defined as the relative ability of the circulatory, respiratory, and muscular systems to supply oxygen to skeletal muscle during sustained physical activity (1). It is usually reported in metabolic equivalents (METs), maximal oxygen uptake (VO_2max) per body weight or VO_2max per fat-free mass (FFM) and measured by specific tests on the treadmill or cycle ergometer (1). One metabolic equivalent is defined as the amount of oxygen consumed while sitting at rest and is equal to 3.5 ml O_2 per kg body weight x min. In testing CRF, in men of 30-40 years, less than 9 METs is considered poor CRF and greater than 15 METs is considered excellent (2)

This chapter contains details about the heritability of CRF, and the benefits of high intrinsic (untrained) CRF as related to health and longevity in humans. These benefits have been demonstrated in longitudinal studies in humans and remain after adjusting for multiple variables such as age, sex, race, study year, obesity, hypertension, diabetes mellitus (DM), hyperlipidemia, cardiovascular diseases (CVD), smoking, alcohol intake, physical activity, fiber intake, body mass index (BMI), waist circumference (WC), percent of body fat, fasting plasma glucose (FPG), systolic blood pressure (SBP), diastolic blood pressure (DBP), lipid profiles, medication, electrocardiogram (ECG) and family history of related diseases. To identify potential mechanisms responsible for the benefits of high CRF, research in the rat genetic model of aerobic treadmill running capacity and humans as related to CRF are also included.

2.2 CRF is highly genetically determined.

Although aerobic training can increase CRF (3-24), there are large interindividual differences in CRF at baseline and in response to similar training (25, 26). Moreover, the health benefits of CRF are independent of physical activity and even more dominant than CRF increases by physical activity. Multiple studies have demonstrated that CRF predicts health and age-adjusted mortality, even when physical activity was included in analysis (27-41), and that the effects of physical activity on health and mortality lost significance when CRF was included in the model (34, 40, 41). This suggests that intrinsic CRF is highly genetically determined. This has been supported by studies using the intraclass correlation coefficient (*r*) of body weight and/or FFM-adjusted VO_2max among monozygotic twins (MZ) (42-48), dizygotic twins (DZ) (42-48), siblings (44, 48-52), and parents-offsprings (49-51). After correction for body weight and FFM, *r* remained significant even after adjustment by age (44, 49-52), sex (44, 47, 50-52), fat mass (52), skinfolds (49, 50), physical activity (50), smoking (50) and socio-economic status (50). Within MZ, DZ, siblings and parent-offspring, *r* values for weight-adjusted VO_2max were 0.62-0.95, 0.04-0.51, 0.18-0.41 and 30.03-0.34, and *r* values for FFM-adjusted VO_2max, were 0.61-0.70, 0.28-0.57, 0.29-0.65 and 0.21-0.29, respectively. The ratio of VO_2max variance between and within twin pairs, sibling pairs and families (F-ratio) was evaluated within two of these studies (44, 52). After correcting for age and sex, F-ratios of MZ, DZ, siblings and parent-offspring for body weight-adjusted VO_2max were 5.79 (44), 3.09 (44), 2.56 (44) and 2.63 (52), and for FFM-adjusted VO_2max, were 4.07 (44), 3.64 (44), 5.11 (44) and 2.72 (52), respectively. These studies demonstrate that VO_2max variance between twin pairs, sibling pairs and families was 2.56-5.79 times higher than VO_2max within each grouping. After correction for age and sex, the F-ratio of VO_2max variance within MZ pairs to variance within DZ pairs was 1.90 for body weight-adjusted VO_2max and 1.21 for FFM-adjusted VO_2max (44). Without age and sex adjustment, this F-ratio ranged between 13.90-16.45 for body weight-adjusted VO_2max (42, 43). The heritability (h^2) of body-weight adjusted VO_2max was estimated to be between 38.0-93.4% (42, 44-47, 52). A meta-analysis found that the h^2 of body-weight adjusted VO_2max was 72% (48).

2.3 The relationship between CRF and health

Research has included both children and adults. In children, low CRF has been associated with obesity, weight gain and increased adiposity. In adults, low CRF has been associated with obesity, weight gain, impaired fasting glucose (IFG), DM, hypertension, metabolic syndrome, cardiovascular disease (CVD) event, cancer and postoperative hospital stay.

2.3.1 High CRF is protective against obesity, weight gain and increased adiposity.

CRF was negatively associated with BMI in boys and girls 7-13 years of age, at baseline and after 12 (53) and 18 months (33) of follow-up. Boys and girls with high (above median of each sex) baseline CRF were 89% and 85% less likely to remain or become overweight, respectively (33). Each 15% increase in baseline VO_2max was associated with 1.38 kg less fat mass gain in Hispanic boys (average baseline age of 11.7 years) 4 years later after adjusting for age, Tanner stage, and lean mass (54). CRF is also inversely associated with obesity and BMI in men and women. A separate study reported that every 1-ml/kg/min (or about 0.28 MET) increase in baseline VO_2max resulted in a 13% decrease in obesity incidence and a 9% decreased incidence of a 10 kg weight gain 20 years later among all subjects (55). This inverse relationship between high VO_2max (ml/kg/min) and rate of weight gain after a shorter period (1 year) of follow-up has also been documented in women (56).

2.3.2 High CRF is protective against IFG.

Higher CRF was found to protect against incidence of IFG. A study in men showed that those in the highest quintile for fitness were 14% less likely to develop IFG than those in the lowest fitness quintile in the 5.1 years since baseline (57). Similarly, a separate study in men documented a 47% lower incidence of IFG in the highest-fitness group after an average follow-up of 6 years (58).

2.3.3 High CRF is protective against DM.

A protective effect of high CRF against DM was documented in men and women after 4-26 years of average or median follow-up (29, 31, 37, 41, 57-66). Within these studies, the

highest-fitness group experienced 33-85% lower incidence of DM when compared to their lowest-fit counterparts (29, 31, 37, 57-61, 63-65). The lower incidence of DM persisted in all strata of BMI (29, 57), WC (57), percent fat (57), baseline fasting glucose (31) and parental DM (63) with a range of 21-75%. A few studies also evaluated the decreased incidence of DM per 1-unit increase in CRF. One reported a 21% and 3% decrease in DM incidence per 1-min increase in treadmill duration in non-obese and obese subjects, respectively (29). A separate study documented an 8% decreased DM incidence per 1-MET increase; this was preserved in all strata of age, sex, race, smoking, obesity, hypertension and hyperlipidemia with a range of 2-13% (37). Another study documented that for each standard deviate (SD = 1.6) increase in maximal METs, and individual was 70% less likely to develop DM (41). A meta-analysis (66) demonstrated that a 1-MET increase was associated with 5% decrease in DM incidence and assumed that this 1-MET increase would prevent 23 cases of DM per 100,000 people per year with a DM incidence of 5 per 1000 person-year.

2.3.4 High CRF is protective against hypertension.

A negative relationship between CRF and incidence of hypertension was found in both men and women after 4-18 years of average or median follow-up (29, 36, 67-71). Compared to the lowest-fitness group, the highest-fitness group had a 27-65% lower incidence of hypertension (29, 36, 67-71). The lower hypertension incidence remained in all strata of age (67), BMI (29, 67) and the presence of pre-hypertension at baseline (67) with a range of 22-76%. The decreased incidence of hypertension per 1-unit increase in CRF has also been previously calculated. A 1-min increase in treadmill duration was associated with 17% and 19% decreased incidence of hypertension in non-obese and obese subjects, respectively (29). A 1-MET increment was associated with 10-32% decreased hypertension incidence (67, 68, 70). Interestingly, one study documented that the positive association between BMI and hypertension incidence was weakened after adding CRF into the model of analysis (68).

2.3.5 High CRF is protective against metabolic syndrome.

Metabolic syndrome is defined as the presence of ≥3 of the following criteria

according to the National Cholesterol Education Program/Adult Treatment Panel III (NCEP/ATP III) (72): abdominal obesity (WC >102 cm in men and >88 cm in women), triglycerides ≥150 mg/dl, HDL <40 mg/dl in men and <50 mg/dl in women, SBP ≥130 mmHg or DBP ≥85 mmHg, and glucose ≥110 mg/dl. It has been shown that high CRF is protective against incidence of metabolic syndrome in both men and women. Research in young adults (18 to 30 years or age) discovered that the highest-fitness group were 47%, 75% and 21% less likely to develop metabolic syndrome among all subjects, non-obese, and obese subjects, respectively, compared to their lowest-fit counterparts after 15 years of follow-up (29). Per every 1-min increase of treadmill duration in non-obese and obese group, metabolic syndrome incidence decreased by 22% and 7%, respectively (29). Another study also found a negative association between CRF and incident metabolic syndrome after the average follow-up of 5.7 years (73). Men and women in the highest-fitness group had 53% and 63% lower metabolic syndrome incidence than those in lowest-fitness group of the same sex, respectively (73). This lower incidence remained in all strata of BMI, age and number of baseline metabolic risk factors (according to the criteria of metabolic syndrome) with a range of 41-85% (73).

2.3.6 High CRF is protective against CVD events.

A protective effect of high CRF against CVD (overall, coronary heart disease (CHD), atrial fibrillation (AF) or stroke) events was observed in both men and women after 3-18.2 years of average or median follow-up (40, 74-90). Participants in the highest-fitness group experienced 22-62% lower overall CVD event incidence as compared to their lowest-fitness counterparts (83, 85-87). The lower incidence persisted when subjects were dichotomized as healthy (54%) and unhealthy (46%) (82) and when only participants with hypertension were evaluated (30% in men and 59% in women) (84). Subjects in the highest-fitness group had 19-67% lower incidence of CHD compared to those in the lowest-fitness group (40, 77-79, 83, 85, 88). The lower CHD incidence persisted when people were dichotomized by race (65% in white and 73% in black) (90), when only participants with hypertension were enrolled (29% in men and 53% in women) (84) and when only people with one or more risk factors (smoking, high cholesterol level and high SBP) were included with a range of 71-85% (78). A meta-analysis found that people in the highest-fitness group

(≥10.9 METs) had 36% lower incidence of overall CVD and/or CHD event than those in the lowest-fitness group (<7.9 METs) (91). When compared with the lowest-fitness group, the highest-fitness group had 56% and 57% lower incident AF among all subjects and subjects with known CVD, respectively (89). Men and women in the highest-fitness category had 29% and 31% lower stroke incidence, respectively, as compared to the lowest-fitness group (85). The decrease in overall CVD, CHD and AF incidence per unit increase of CRF has also been previously calculated. Each 1-MET increase was associated with 10% and 23% decreased incidence of overall CVD event in men and women, respectively (87). A 1-min increment of treadmill duration resulted in 13% decrease in incidence of overall CVD events (80). Additionally, each 20-W increase of workload during the exercise test was associated with a 9% decreased incidence of overall CVD events (86). The decreased incidence of overall CVD event per each MET increase was preserved in all strata of smoking, BMI, hypertension and LDL cholesterol level with a range of 17-29% (82). Each 1-MET increase was also associated with a 20% decrease in CHD incidence (88). Similarly, a separate study found 6% and 3% decreased incident CHD per each MET increment in men and women, respectively (81). The decreased incidence of CHD per 1-MET increment was preserved in all strata of Framingham risk score (http://cvdrisk.nhlbi.nih.gov) with a range of 3-13% (81). A meta-analysis showed that a 1-MET increase was associated with a 15% decrease in incident overall CVD/CHD events, and the decreased incidence persisted in all strata of age, sex, smoking, number of risk factors, exercise ECG, follow-up duration, ergometer used for testing, and maximal/submaximal workload testing with a range of 10-23% (91). The decrease in AF incidence per 1-MET increment of CRF was also documented (7% among all subjects and 6% among subjects with known CVD), and the decreased incidence per each MET increase remained in all strata of age, sex, race, smoking, obesity, hypertension, DM and hyperlipidemia with a range of 8-16% (89).

2.3.7 High CRF is protective against cancer.

A study in men found that the highest-fitness group had 27% lower cancer incidence (overall, lung and gastrointestinal (GI) tract) than their lowest-group counterparts after an average follow-up of 16.7 years, and this was preserved when subjects were dichotomized as physically active (26%) and physically inactive (33%) (92). The authors also noted a

20% and 12% decreased incidence of lung and GI tract cancer, respectively, per 1-MET increase (92).

2.3.8 High CRF is protective against postoperative hospital stay.

CRF and postoperative (hepatobiliary surgery) hospital stay duration were negatively associated (93). Furthermore, patients in the high CRF group (VO_2max >10 ml/kg/min) spent shorter total period in a hospital and in a critical care unit than those whose VO_2max <10 ml/kg/min, and the results were consistent when patients were dichotomized into those younger or older than 75 years of age (93). Interestingly, high CRF patients spent similar durations of both total and critical care unit stay regardless of age group (93).

2.4 The relationship between CRF and mortality

The association between CRF and all-cause mortality, CVD mortality, stroke mortality, dementia mortality, cancer mortality and postoperative mortality to date has only been documented in adults.

2.4.1 High CRF is protective against all-cause mortality.

High CRF was found to be protective against all-cause mortality in both men and women after 6.2-28.7 years of average or median follow-up (27, 28, 30, 34, 35, 38, 39, 82, 83, 86, 87, 90, 94-131). Compared to subjects in the lowest-fitness group, those in the highest-fitness group demonstrated a 24-74% lower all-cause mortality (27, 28, 34, 39, 83, 86, 87, 94-96, 98-100, 102, 105, 107, 108, 110, 114, 118, 120, 121, 123, 125-127, 129). A meta-analysis found that people in the highest-fitness group (≥10.9 METs) had 41% lower all-cause mortality than those in the lowest-fitness group (<7.9 METs) (91). The lower all-cause mortality was preserved when only people with diseases were enrolled. As compared with their lowest-fitness counterparts, subjects with peripheral artery diseases (PAD) (128), hypertension (30), DM (104, 111) and documented or suspected CHD (35) in the highest-fitness group had 41%, 57%, 52-78% and 40% lower all-cause mortality, respectively. The lower all-cause mortality also persisted in all strata of race (90, 113, 120), BMI (35, 38, 103, 106, 107, 111, 121, 123, 125), WC (35, 38, 101, 123), fat mass (101), FFM

(101), percent fat (35, 101, 111, 123), waist-to-hip ratio (123), DM or glucose (97, 106, 122), hypertension or BP (98, 106), cholesterol level (98, 106), metabolic syndrome (112), CVD (106, 119, 120, 122), symptom of CHD (115), chronic obstructive pulmonary disease (COPD) (106), smoking (28, 98, 101, 106), alcohol intake (126), dietary intake (124), physical activity (34), leisure time (39), health status (82, 98) and number of risk factors (82, 122) with a range of 6-83%. Interestingly, the lower mortality persisted after sub-stratifying subjects in each lowest-fitness (130) and highest-fitness group (131) into 3 groups. In the lowest-fitness group, the highest-lowest group at all age ranges had 40-47% lower all-cause mortality than the lowest-lowest group counterparts (130). Among those in the highest-fitness group, the highest-highest group had 39% lower all-cause mortality than those in the lowest-highest group (131). The results from sub-stratification suggest that there is no threshold of CRF as a predictor of all-cause mortality.

The decrease in all-cause mortality per unit increment of CRF was previously calculated in several studies. A 1-MET increase was associated with a 7-19% decrease in all-cause mortality (106, 108-110, 114, 116, 117, 120, 125, 127). Additionally, each 20-W workload increase during the exercise test was associated with a 14% decreased all-cause mortality (86). A meta-analysis showed that each 1-MET increase was associated with a 13% decrease in all-cause mortality (91). The decrease in all-cause mortality per 1-MET increase was also observed when only subjects with PAD were enrolled (18%) (128) and when subjects were stratified by race (120) and CVD (120) with a range of 9-24%. The aforementioned meta-analysis that reported decreased all-cause mortality in the fittest individuals also documented a decrease in all-cause mortality per 1-MET increment in all strata of age, sex, smoking, number of risk factors, exercise ECG, average of follow-up duration, ergometer used for testing, and maximal/submaximal workload testing with a range of 9-16% (91). Interestingly, significant associations of metabolic syndrome (112, 116), WC (118), BMI (105, 116, 121), physical activity (34) and leisure time (39) vs. all-cause mortality disappeared after adding CRF into the model. Moreover, fit people with risk factors such as obesity (30, 35), high WC (30, 35, 123), high percent of fat (30, 35, 123) and high waist-to-hip ratio (123) had no higher all-cause mortality as compared with their fit/normal counterparts even they were hypertensive (30). A few studies found that CRF was a more dominant predictor of all-cause mortality than several other risk factors. Even

in the presence of risk factors, including overweight or obesity (82, 111, 125), high WC (123), high percent of fat (123), high waist-to-hip ratio (123), DM (122), CVD (122), high BP or hypertension (82, 98), high cholesterol (98), smoking (28, 82), poorer dietary intake (124) and less physically active (34, 39), fit people demonstrated lower all-cause mortality than unfit people without any risk factors.

2.4.2 High CRF is protective against CVD mortality.

A protective effect of high CRF against CVD (overall, CHD and heart failure (HF)) mortality was discovered in both men and women after 7-26 years of average or median follow-up (27, 28, 30, 35, 82, 86, 87, 94-96, 98, 101, 103, 107, 109, 112-117, 126-128, 132-136). As compared with the lowest-fitness group, subjects in the highest-fitness group had 11-82% lower overall CVD mortality (27, 28, 86, 87, 96, 98, 107, 114, 126, 132). This lower overall CVD mortality was preserved when only people with diseases were enrolled, including hypertension (70%) (30), documented or suspected CHD (49%) (35) and PAD (60%) (128). The lower overall CVD mortality also persisted in all strata of race (107), BMI (35, 103, 107, 127, 132), percent fat (35, 101), fat mass (101), FFM (101), WC (35), hypertension or BP (96, 98, 127, 132), DM or glucose (127, 132), metabolic syndrome (112), heart disease (96), symptom of CHD (115), stroke (96), health status (82, 98, 132), cholesterol level (98, 132), alcohol intake (126) and smoking (28, 98, 127, 132) with a range of 2-83%. For CHD and HF mortality, subjects in the highest-fitness group had 17-84% lower CHD mortality (95, 134, 135) and 75% lower HF mortality (136) than their lowest-fitness counterparts. The lower HF mortality persisted in all strata of BMI and number of risk factors (obesity, hypertension, DM, smoking and family history of CVD) with a rage of 27-75% (136). For overall CVD mortality, each 1-MET increase was associated with 5-26% decreased mortality (87, 109, 114, 116, 117). A separate study documented a 12% decrease in overall CVD mortality per 20-W workload increase during an exercise test (86). The decrease in overall CVD mortality per unit increase of CRF was also observed in all strata of BMI, BP, LDL cholesterol level, smoking and health status with a range of 15-51% (82). For CHD mortality, 1-SD increase of cumulative work per body weight (0.57 kJ/kg) during the exercise test was associated with 15% decreased mortality (133). The decrease in CHD mortality per 1-MET increment was also found when subjects were

dichotomized as healthy (18%) and unhealthy (28%) (82). Interestingly, the effects of obesity (116) and metabolic syndrome (112, 116) on overall CVD mortality were weakened after adding CRF into the model of analysis. In addition, fit subjects who had high BMI, high WC or high percent of fat had no higher overall CVD mortality than their fit/normal counterparts (30, 35) even if they had hypertension (30). A few studies found that CRF was a more dominant predictor of overall CVD mortality than several other risk factors. Fit smokers (28) as well as those with any combination of smoking, high cholesterol, and high BP (98) had lower overall CVD mortality compared to unfit people with no risk. Another study also documented that fit people with high BMI, high BP, high glucose, high cholesterol, or who smoked had lower overall CVD mortality than unfit subjects with no risk (132). Interestingly, after including CRF as a parameter, classification of the risk of overall CVD mortality in asymptomatic individuals without known CVD at both 10 and 25 years was improved (137), suggesting that CRF is a critical factor for prediction of overall CVD mortality.

2.4.3 High CRF is protective against stroke mortality.

A study in men documented an inverse relationship between CRF and stroke mortality (138). Men in the highest-fitness group experienced 68% lower stroke mortality than their lowest-fitness group counterparts after an average follow-up of 10 years.

2.4.4 High CRF is protective against dementia mortality.

A negative relationship between CRF and dementia mortality in both sexes was identified after an average follow-up of 17 years (139). Compared to the lowest-fitness group, subjects in the highest-fitness group had 34%, 69% and 51% lower mortality from Alzheimer's disease, vascular dementia and overall dementia (Alzheimer's disease plus vascular dementia), respectively (139). Each MET increment was associated with 13%, 18% and 14% decreased mortality from Alzheimer's disease, vascular dementia and overall dementia, respectively, and the decrease in mortality from overall dementia per 1-MET increment was preserved in all strata of age, sex, BMI and chronic medical conditions with a range of 7-34% (139).

2.4.5 High CRF is protective against cancer mortality.

A negative association between CRF and cancer mortality was observed in both men and women after 7-24.9 years of average or median follow-up (32, 92, 94, 96, 99, 140-147). As compared with the lowest-fitness group, people in the highest-fitness group demonstrated 37-64% lower overall-cancer mortality (92, 96, 99, 140-143, 147). A meta-analysis found 45% lower overall-cancer mortality in the highest-fitness group as compared with the lowest-fitness group with or without adjustment for adiposity (148). The lower cancer mortality in the highest-fitness group compared to their lowest-group counterparts was also found in specific cancer mortality, including smoking-related cancer (66%) (140), nonsmoking-related cancer (46%) (140), lung cancer (57%) (32, 144), overall GI tract cancer (44-45%) (144, 146), colon cancer (39%) (146), colorectal cancer (42-47%) (144, 146), liver cancer (72-78%) (144, 146) and breast cancer (45%) (145) regardless of pre-DM and DM (144). The lower overall and specific cancer mortalities were preserved in all strata of BMI (143, 147), percent body fat (143, 147), WC (143), DM (144) and smoking (32, 141) with a range of 16-67%. Moreover, people with high CRF who had high BMI, high WC or high percent body fat had lower overall cancer mortality than those with low CRF who had none of these risk factors (144). Interestingly, positive correlations of BMI, percent of fat and WC with overall cancer mortality were weakened after adding CRF into the model of analysis, suggesting that CRF attenuates obesity-induced cancer mortality (143).

2.4.6 High CRF is protective against postoperative mortality.

Patients in the high-fitness category (≥5 METs) had lower immediate postoperative (Coronary Artery Bypass Graft) and 30-day postoperative mortalities as compared with those in the low-fitness group (<5 METs) (149). Another study in men and women also investigated the relationship between preoperative CRF and postoperative (hepatobiliary surgery) mortality and found that patients in the high CRF group (VO_2max >10 ml/kg/min) had 48% lower postoperative mortality than those whose VO_2max <10 ml/kg/min (93). When patients were dichotomized as <75 years and >75 years of age, those in the high CRF group had a mortality rate of less than 5% regardless of age group, but those in the low CRF group who were younger than 75 years had an 11% mortality rate and those who were

older than 75 years had a 22% mortality rates (93). In summary, high CRF is associated with lower postoperative mortality in all strata of age, and interestingly, high CRF attenuates age-related postoperative mortality.

2.5 The rat genetic model of aerobic treadmill running capacity

In-depth studies that identify how CRF influences health and longevity are necessary. However, completing such studies in humans is difficult. Therefore, a rat genetic model of aerobic treadmill running capacity was established. The model was first developed (150) by running 18 male and 24 female outbred Sprague-Dawley rats on the treadmill once a day for 5 days. The treadmill was set at a constant 15° inclination and the initial speed of 10 m/min was increased by 1 m/min every 2 min. The total distance run on the single best day was taken as the measure of endurance performance. The 2 lowest-performing pairs and 2 highest-performing pairs were selectively bred through 3 generations. After 3 generations of selection, performance of the high-selected line averaged 659±36 m, whereas those of the low-selected line averaged 388±28 m. After that, a larger-scale selection was completed in 96 male and 96 female non-sibling N:NIH rats (151) by Steve Britton and Lauran Koch in 1996 (152, 153). Rats ran on the treadmill as previously described. Based on maximal running capacity, the top 13 males and females were bred together to initiate the high capacity running (HCR) line, and the bottom 13 males and females were bred together to initiate the low capacity running (LCR) line. In this breeding scheme, each of the 13 families contributed 1 male and 1 female to the next generation, which were in-turn rotationally bred with another pre-arranged family. By 2011, they had bred for 28 generations and the HCR and LCR rats differed in maximal running capacity about 7-fold (154). In additional to higher running capacity, HCR rats also demonstrate better health, including lower body weight (153, 155-178) despite higher food intake per body weight (167, 176, 178), higher resting metabolic rate (RMR) per body weight (165, 176, 178, 179), higher energy expenditure of activity (179), lower adiposity (152, 158, 161, 164, 166, 168, 171, 172, 176, 180, 181), higher insulin sensitivity (152, 158, 159, 161, 163-166, 168, 169, 172, 178, 180-183), more favorable plasma lipid profiles (152, 158, 159, 161, 163-165, 168, 178, 180, 181), less hepatic lipid accumulation (164, 176), less age-related hepatic fibrosis and apoptosis (164), lower BP (152, 161, 164, 180, 181), less

cardiac arrhythmia (184), better cardiac function (152, 155, 161, 180, 181, 185, 186), greater endothelial-mediated vasodilatation (161, 181), better pulmonary function (160), higher tissue O_2 diffusive capacity (152, 156, 161), more O_2 uptake by skeletal muscle (155, 161, 180), higher capillary density in muscle (152, 157), less sleep problem (187), better coping with stress (188) and more resistance to high-fat diet (HFD), including less weight gain (154, 158, 159, 161, 176, 179, 180), less or no increase in fat mass (154, 158, 159, 161, 166, 176, 179, 180), no reduced insulin sensitivity (154, 158, 159, 161, 166, 180, 182), less increase in intramuscular lipid density (159), no increased hepatic lipid accumulation (176) and more reduction in respiratory quotient (RQ) when compared to their LCR counterparts (176). Moreover, female HCR rats are protected from ovariectomy-associated increases in adiposity and insulin resistance (178).

Survivability evaluation at generations 14, 15 and 17 found that median lifespan of HCR was 28-45% longer than LCR rats (154, 185). Genetic analysis identified increased variation between HCR and LCR lines but decreased difference within lines over breeding generation with h^2 for the running capacity phenotype of 47 ± 2 and $43\pm2\%$ for HCR and LCR lines, respectively, over 28 generations (174). Interestingly, microarray-based gene analysis on skeletal muscle of HCR and LCR rats found the largest differentiation between lines in old-rest rats and smallest differentiation in young-rest rats (189). The results suggest that the differentiation in gene expression is age-dependent. Up-regulation of genes related to mitochondrial pathways in skeletal muscle of HCR rats was documented, with an overall difference of 2,838 genes between HCR and LCR lines (189). The data also suggested that the changes in gene expression that was related to aging was smaller in HCR than LCR, suggesting a slowing of aging in the muscle. A difference in cardiac gene expression between HCR and LCR rats was also revealed (190). 1,540 out of 28,000 screened cardiac genes differed between lines (190). Because the rat model exhibits the difference in health, longevity and genetic phenotype between lines as well as high h^2 within lines, the model is comparable with previous human data, and is therefore valuable for further in-depth studies of CRF.

2.6 In-depth studies to identify mechanisms by which high CRF is associated with good health and longevity

To understand how CRF determines health and longevity, the rat genetic model of aerobic treadmill running capacity was initially used to identify differences between HCR and LCR lines. A few studies in humans have also been completed. The differences between high and low CRF include mitochondrial content, mitochondrial biogenesis, oxidative phosphorylation (OXPHOS) and mitochondrial respiration, reactive oxygen species (ROS) production, oxidative stress, anti-oxidative stress, indicators of insulin sensitivity, branched-chain amino acid (BCAA) catabolism and lipid metabolism, as well as physical activity and leanness.

2.6.1 High CRF is associated with higher mitochondrial content and related markers.

Mitochondrial content quantified by transmission electron microscopy (TEM) (169) and by estimation from the yield of mitochondria from isolation procedure (171) was found to be higher in skeletal muscle of HCR rats. Citrate synthase (CS) activity, a marker of mitochondrial content, was higher in HCR rat skeletal muscle compared to their LCR counterparts (152, 157-159, 161, 169-172). Liver CS activity was also higher in HCR than LCR rats (164). CPS1, another marker of hepatic mitochondrial content, was higher in HCR rats as well (164).

2.6.2 High CRF is associated with more indicators of mitochondrial biogenesis.

PGC-1α plays a major role in controlling mitochondrial biogenesis and was higher in skeletal muscle of HCR compared to LCR rats (152, 161, 172, 178, 181). MtDNA count number, an indicator of mitochondrial biogenesis (191), was higher in HCR skeletal muscle as well (172). Following HFD, a decrease in mRNA expression of PGC-1α in skeletal muscle was observed only in LCR rats (166). A positive correlation between VO_2max and mtDNA count number has also been documented in human skeletal muscle (192).

2.6.3 High CRF is associated with more indicators of OXPHOS and higher mitochondrial respiration.

Bioinformatic analysis of skeletal muscle gene expression data revealed that gene

sets involving OXPHOS were more highly expressed in HCR vs. LCR rats (165). Several enzymes involving mitochondrial OXPHOS were higher in skeletal muscle of HCR rats, including Complex I (172, 178), Complex II (172, 178), Complex III (172), UQCRC2 (a part of Complex III) (152, 158, 161, 181), COXI (a part of Complex IV) (152, 158, 159, 161, 171, 181), COXII (a part of Complex IV) (172), COXIV (a part of Complex IV) (172), Complex V (152, 158, 161, 172, 178, 181), UCP2 (152, 158, 161, 181), UCP3 (154, 159, 163, 172) and PGC-1α (152, 158, 161, 172, 178, 181). Following HFD, skeletal muscle mRNA expression of PGC-1α decreased in LCR but not HCR rats (166), and COXI and UCP3 skeletal muscle mRNA expression increased more in HCR vs. LCR rats (159, 161). Tweedie and colleagues (170) discovered that the proportion of electron transport capacity that was attributable to leak, an index of mitochondrial uncoupling that drives OXPHOS (193), was greater in skeletal muscle of HCR rats. Mitochondrial protein deacetylation via SIRT3 activation, another indicator of OXPHOS (194-201), was reduced in HCR skeletal muscle at rest, and was rapid during exercise, suggesting that HCR rats have a greater ability to continuously produce ATP through OXPHOS during exercise (202). Mitochondrial respiration in skeletal muscle was also directly measured, and a higher respiration was found in HCR rats, indicating a greater capacity of complex IV in the mitochondria (170-172). In an exercise mimicking condition, HCR rats also demonstrated higher mitochondrial respiration in skeletal muscle, which possibly contributes to greater running capacity (203). HCR liver mitochondria also demonstrated higher respiration regardless of diet (176).

2.6.4 High CRF is associated with lower markers of oxidative stress and oxidative DNA damage likely due to a greater capacity for anti-oxidative stress.

Surprisingly, skeletal muscle of HCR rats revealed higher mitochondrial ROS production, as evidenced by since greater H_2O_2 emission (170, 171). However, markers of mitochondrial oxidative stress (4-HNE) (171) and mitochondrial oxidative DNA damage (8dOHG) (170) were lower in skeletal muscle of HCRs. This can be explained by greater ROS buffering capacity because markers of anti-oxidative stress were higher in skeletal muscle of HCR rats, including total SOD activity (170) and SOD2 expression (171). Following HFD, SOD activity in skeletal muscle were significantly reduced in LCRs, but non-significantly increased in HCR rats (166). Less oxidative stress was also revealed in liver

mitochondria of HCR rats, as 4-HNE level was lower when compared to LCR rats (164). In human plasma, higher CRF was associated with lower markers of oxidative stress (MDA and 8-iso-PGF2α) as well as markers of oxidative DNA damage (8-OHdG), but was associated with higher activity of an antioxidant enzyme glutathione peroxidase (204).

2.6.5 High CRF is associated with increased insulin sensitivity.

Real-time PCR analysis of skeletal muscle found that Nur77, a gene associated with insulin sensitivity, was up-regulated in HCR rats (163). Additionally, a BCAA catabolism associated gene set was more highly expressed in HCR vs. LCR rats (165). This is important because BCAAs are markers of insulin resistance (205-207). Many proteins associated with insulin sensitivity have been documented to be higher in the skeletal muscle of HCR vs. LCR rats, including IκBα (182), beta-2 adrenergic receptor (154, 168), AMPKγ3 (163), Nur77 (154, 163, 168, 172), PPAR-γ (152, 158, 161, 181), Akt1 (169), Akt2 (169) and Nor1 (172). In addition, phosphorylation of Akt on threonine T308 and serine S473, mechanisms that drive insulin signaling, were higher in HCR rats as was phosphorylation of AS160, a downstream substrate of Akt (169). Skeletal muscle glucose uptake and oxidation (169) as well as insulin-stimulated glucose transport and insulin signal transduction were higher in skeletal muscle of HCRs (154, 168, 172). The increased insulin sensitivity in HCR rats was associated with a greater response to beta-adrenergic stimulation in skeletal muscle, as indicated by increased isoproterenol-induced lipolysis (glycerol release), which was associated with higher basal triacylglycerol lipase activity, ser660 phosphorylation of HSL and beta-2 adrenergic receptor protein in skeletal muscle (163). Following HFD, phosphorylation of IRS-1 on serine S407, a mediator of IKKβ-induced insulin resistance, was higher only in skeletal muscle of LCR rats (182). In metabolomic analysis, mitochondria of HCR skeletal muscle demonstrated a greater capacity for BCAA catabolism (202). Further supporting this finding, mitochondrial protein deacetylation via SIRT3 activation, a regulator of BCAA catabolism (208), was reduced in HCR at rest, and was rapid during exercise (202). A study in human skeletal muscle also showed that high CRF is associated with increased indicators of insulin signaling, including protein expression of PI3K and PKB Ser473 phosphorylation (209). A separate human study demonstrated that

high CRF is associated with a higher insulin secretion and a higher shift in substrate utilization toward primarily carbohydrate oxidation during exercise (210).

2.6.6 High CRF is associated with ***increased lipid metabolism as well as indicators of lipid metabolism.***

A study in skeletal muscle of HCR and LCR rats using real-time PCR found that Nur77 and UCP3, two genes associated with fatty acid metabolism, were up-regulated in HCR rats (163). Bioinformatic analysis of gene expression revealed that a gene set involved in fatty acid oxidation (FAO) was more highly expressed in HCR vs. LCR skeletal muscle (165). Real-time PCR analysis indicated up-regulation of several FAO related genes in skeletal muscle of HCR vs. LCR rats, including LPL, CPT1A, CPT1B, CPT2, HAHDB, UCP2, UCP3, ACO1 and ACO2 (165). Additionally, several proteins responsible for lipid metabolism were also higher in HCR skeletal muscle, including Nur77 (154, 163, 168, 172), β-HAD (152, 157, 161, 172), UCP2 (152, 158, 161, 181), UCP3 (154, 161, 163, 168, 172), FAT/CD36 (154, 163, 168, 172) and AMPKγ3 (163). Following HFD, skeletal muscle UCP3 significantly increased in HCR but not LCR rats (159). Skeletal muscle of HCR rats demonstrated higher rates of FAO (166, 169, 172) and palmitate oxidation (154, 168) compared to their LCR counterparts.

Metabolomic analysis indicated that skeletal muscle mitochondria of HCR rats have a greater capacity for FAO (202). Mitochondrial protein deacetylation via SIRT3 activation is a regulator of FAO (211), and in HCR rats was found to be reduced at rest and rapid during exercise (202). In liver, HCRs had lower SREBP-1c, a regulator of fatty acid synthase genes, and lower activity of ACC, an inhibitor of FAO, suggesting a less hepatic lipogenic phenotype (164). Conversely, activity of β-HAD, the rate-limiting enzyme for FAO, was higher in HCR vs. LCR liver at rest (164). Isolated liver mitochondria of HCR rats demonstrated higher complete fatty oxidation (176) and higher complete palmitate oxidation (164) than their LCR counterparts. In addition, HCR liver displayed lower peroxisomal enzyme activity for both acyl CoA oxidase and catalase, suggesting a compensatory increase in peroxisomal activity to mitigate the reduced liver mitochondrial oxidative capacity in LCRs (164). Following HFD, liver palmitate oxidation remained unchanged in HCR but decreased significantly in LCR rats (159). The increase in whole

body FAO after HFD exposure was also significantly greater in HCRs as demonstrated by a greater reduction in respiratory quotient (RQ) following HFD (176). It has also found that genes involved in lipid metabolism (ACADSB, VLDLR, CPT1A and MTE1) were more highly expressed in the left ventricle of HCR vs. LCR rats (190). In humans, high CRF is associated with higher fat oxidation during exercise (209, 212, 213).

2.6.7 High CRF is associated with increased markers of physical activity and leanness.

Orexin-induced activity and PEPCK-C, markers of high physical activity and leanness, were higher in skeletal muscle of HCR vs. LCR rats (179). In addition, NAD-dependent deacetylase SIRT1, which is associated with high energy expenditure, was higher HCR skeletal muscle (179). Furthermore, HCR rats were less sensitive to fatigue and demonstrated faster metabolic recovery after maximal muscle contraction, both of which possibly contribute to greater running capacity (173). Analysis of brain and plasma revealed higher striatal dopaminergic activity, a neurotransmitter responsible for physical performance, and lower plasma corticosterone level in HCR rats as compared with their LCR counterparts after 8 weeks of running trial (162). The lower plasma corticosterone level suggests that HCR rats are less stressed by training (162).

2.7 Summary

- CRF is defined as the ability of the circulatory, respiratory, and muscular systems to supply oxygen during sustained physical activity. It is reported in METs, VO_2max per body weight or VO_2max per FFM and measured by exercise tests on equipment such as a treadmill or cycle ergometer.
- There are large interindividual differences of CRF in the absence of training and in response to similar aerobic training. Moreover, the effects of CRF on health and longevity are independent of physical activity and even more dominant than physical activity. Heritability studies in twins and families demonstrate that CRF is highly genetically determined .
- High CRF is protective against several diseases and pathologies, including obesity, DM, hypertension, metabolic syndrome, coronary heart disease, cardiac arrhythmia, stroke,

and cancer, as well as postoperative hospital stay and is independent of any other risk factors.

- High CRF is also associated with lower all-cause mortality and specific mortality such as CVD mortality, stroke mortality, dementia mortality and cancer mortality, as well as postoperative mortality regardless of any other risk factors.
- The rat genetic model of aerobic treadmill running capacity is useful for in-depth studies to identify mechanisms by which high CRF is associated with good health and longevity.
- In-depth studies in the rat genetic model of aerobic treadmill running capacity and humans demonstrated that high CRF is associated with higher mitochondrial content, higher mitochondrial biogenesis, higher OXPHOS and mitochondrial respiration, greater ROS buffering capacity, higher indicators of insulin sensitivity, higher capacity for BCAA and lipid catabolism, as well as higher markers of activity and leanness.

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Chapter 3

Systematic literature review: Anti-aging effects of caloric restriction

3.1 Introduction

Caloric restriction (CR) is defined as a reduction in caloric intake, typically by 20–40% of ad libitum consumption, whereas protein and micronutrients intake are adequate to avoid malnutrition (1, 2). CR without malnutrition is the most acceptable non-genetic paradigm to increase lifespan (2-4). The effect of CR on longevity was first reported in a rat study in 1935 (5). Because of this study, the anti-aging effects of CR were widely investigated and CR was shown to extend lifespan in various non-primate animals, including rats, mice, yeast, *Drosophila*, rotifers, bowl and doily spiders, and *C. elegans* (6-14), by 20-100% (15-27). Several studies in these animals found that the effect of CR on longevity was associated with attenuation of age-related pathologies, including cardiometabolic diseases (28-37), cognitive decline (10, 38-47), cancer (31, 48-103) and immunodeficiency (104-109). To determine whether those benefits could be applied to humans, studies in non-human primates were initiated in 1987 and 1989 (110, 111). Further studies in non-human primates demonstrated that 30% CR decreased age-related mortality by 65-67% (112, 113), decreased cardiovascular disease (CVD) incidence by 50% (112), decreased incidence of cancer (112, 114) by 50% (112), and that CR attenuated age-related cardiometabolic diseases (112, 114-133), cognitive decline (112, 134, 135), and immunodeficiency (109, 136). In humans, an observational study established in 1978 reported lower mortality rates related to heart diseases (-59%), cerebrovascular diseases (-59%) and cancer (-69%) in Okinawans who consumed less energy than the Japanese average (137). Following that hallmark study, several additional observational and experimental studies in humans reported an attenuating effect of CR on age-related cardiometabolic diseases (2, 138-168), cognitive decline (169-173) and cancer (150, 174-177). This review focuses on the beneficial effects of CR on cardiometabolic health, the

nervous system, cancer prevention and tumor regression, as well as the immune system. In addition, the modulating effects of CR and its molecular mechanisms on the age-related decline in mitochondrial function, as well as epigenetic regulations linking CR to anti-aging are discussed.

3.2 CR and cardiometabolic health

The benefits of CR on age-related cardiometabolic diseases have primarily been studied as related to the attenuation of atherosclerosis risk factors because atherosclerosis is one of the most common causes of death and disability throughout the world (178). Atherosclerosis is an inflammatory condition of the endothelium, leading to endothelial injury and dysfunction (179, 180), and thus narrowing the lumen of arteries and obstructing the blood supply (181). Classic risk factors for atherosclerotic diseases (cardiovascular, cerebrovascular, and peripheral vascular diseases) include insulin resistance and associated increases in blood pressure (BP) and dyslipidemia (high LDL cholesterol, low HDL cholesterol and high triglyceride) (182-185). In addition, elevation in inflammatory markers, including CRP, cytokines, hemostasis factors (such as factor VII, fibrinogen, and PAI-1) and homocysteine, are predictive of atherosclerosis events (186-188).

3.2.1 CR improves insulin sensitivity.

Elevated plasma insulin and small elevations of glucose are characteristics of insulin resistance (189). Aging is associated with increased incidence of insulin resistance (190-192) and is associated with the increases in visceral adiposity and proinflammatory cytokines that interferes with insulin signaling (193). Reciprocally, insulin resistance leads to aging as well (194, 195). High plasma glucose contributes to aging because of the associated hyperglycemia-induced oxidative stress (194). Excessive glucose increases reactive oxygen species (ROS) production, as caused by overload of mitochondrial phosphorylation (OXPHOS) and shunting of physiological glycolysis to other pathways, including enolization and α-ketoaldehyde formation, protein kinase C (PKC) activation, dicarbonyl formation and glycation, and sorbitol and hexosamine metabolisms (194). High plasma insulin also contributes to aging via its mitogenic action (195). Excessive insulin

expedites the appearance of later generations of cells, which are physiologically present at a later age of life, causing early onset of age-related diseases (195).

Several studies in rodents (29, 31, 33-37), non-human primates (112, 114-118, 120, 122, 126-133) and humans (2, 142, 143, 146, 148, 149, 151-154, 156, 163, 165, 168, 196) have demonstrated that CR in lean and obese subjects improved insulin sensitivity. Several mechanisms by which CR improves insulin sensitivity have been proposed. First, CR decreases circulating fatty acids (180), which are substances that inhibit 1) insulin-stimulated glucose uptake at the level of glucose transport and/or phosphorylation, 2) insulin-stimulated glycogen synthesis and 3) insulin-stimulated glucose oxidation (197). Second, CR limits accumulation of intramyocellular triacylglycerols (180), lipids that inhibit glucose uptake to skeletal muscle (198) and therefore induce insulin resistance (198-200). CR also reduces hepatic diacylglycerols (34), lipids that activate PKC which inhibits insulin-stimulated insulin receptor kinase phosphorylation, and therefore impairs activation of downstream insulin signaling (201-203). In addition, CR decreases levels of proinflammatory cytokines (204, 205), substances that inhibit insulin receptor signaling and glucose transport in insulin-sensitive tissues (206, 207). Moreover, CR increases Akt2 activation, and therefore enhances insulin-stimulated glucose uptake to skeletal muscle (37).

3.2.2 CR decreases BP.

Arterial stiffness increased with age (208-212), leading to age-related elevated BP (208, 210-213) in humans. It has been documented that CR decreases BP in rodents (28, 30, 32) and non-human primates (124). CR decreases systolic (SBP) and diastolic (DBP) blood pressures in both non-obese and obese humans as well (138, 140, 143, 144, 151, 158, 159, 161, 163, 164, 166). SBP and DPB were lower in non-obese and obese people who completed CR compared to non-CR controls (139, 145, 146, 152, 155, 162). Potential mechanisms by which CR decreases BP may involve factors that mitigate elevated BP. CR decreases plasma norepinephrine concentration and excretion of catecholamines (214-216), decreases sympathetic nervous activity (145, 215, 216), ameliorates aging-associated decrease in diastolic function (155), reduces arterial stiffness (166), modulates the sympathetic-renin-angiotensin axis (185) and stimulates the adiponectin/AMPK/eNOS

signaling axis (32).

3.2.3 CR improves lipid profile.

Total cholesterol, LDL cholesterol and triglycerides are atherogenic stimuli, whereas HDL cholesterol is protective against atherosclerosis (217). Total cholesterol, LDL cholesterol and triglycerides in humans increase with age until middle-age and then gradually decrease (218-223), but there is no specific pattern of age-related change in HDL cholesterol (218-222, 224, 225). CR reduces plasma total cholesterol and triglycerides in mice (29). In monkeys, CR is associated with lower levels of total cholesterol (114, 124), LDL cholesterol (128) and triglycerides (114, 121, 124). In addition, CR increases HDL cholesterol in monkeys (121). Like rodents and monkeys, CR improves lipid profiles in humans. CR decreases total cholesterol and triglycerides (142-144, 147, 151, 152, 156-158, 160-163), reduces LDL cholesterol (144, 147, 151, 152, 156-158, 160-162), and increases HDL cholesterol (141, 142, 144-146, 151, 152, 156, 160-163). A potential mechanism responsible for CR-induced favorable lipid profiles is the improvement of lipoprotein metabolism caused by a reduction in circulating fatty acids after CR (226).

3.2.4 CR reduces inflammation.

Chronic inflammation increases with aging (227, 228). The production of proinflammatory cytokines by mononuclear leukocytes in response to stimulation increases with aging as well (227). Several human studies have found that CR decreases various markers of inflammation in plasma, including CRP (152, 159, 161, 162, 229), IL-6 (152, 158, 159, 161, 230), TNF-α (152, 158, 159, 161), TGF-β1 (159), and PAI-1 (231, 232).

3.3 CR attenuates the age-related decline in cognitive function.

Several studies in rodents found that CR decelerated age-related changes in cognitive function, including learning (38-43, 47), memory (40), motor strength (10) and motor coordination (10, 39, 42, 46), as well as behavior alteration (10). In non-human primates, higher locomotor activity (134, 233) and increased memory (233) were observed after CR. In addition, CR reduced age-related brain atrophy in rhesus monkeys after 20 years of follow-up (112). In humans, 30% CR was associated with 20% increase in verbal

memory score (173). The benefits of CR on cognitive function are primarily associated with synaptic plasticity, neurogenesis and protective effects against neurodegenerative diseases (234, 235).

3.3.1 CR promotes synaptic plasticity.

The term "synaptic plasticity" refers to changes in the numbers, structure, and functional status of synapses as adaptive responses to a range of environmental challenges (236). Synapses are highly specialized sites of interneuronal signal transduction that dictate the immediate functions and long-term plasticity of the brain, and they are highly prone to degeneration during aging (234). Several previous studies have suggested that CR enhances synaptic function and increases synaptic resistance to aging-induced degeneration in rodents. In rats, CR prevents age-related deficits in long-term potentiation (237, 238), a synaptic activity that produces a long-lasting increase in signal transmission between two neurons (239) and is believed to correlate with learning and memory (235). Additionally, CR stabilizes the levels of presynaptic proteins (GAP-43, SPH and alpha-Synuclein) in aged rats (240). The synaptic terminals of rats maintained on CR, theirs are more resistant to oxidative impairment of glucose and glutamate transportation across the cell membrane and exhibit enhanced mitochondrial function (241). Beneficial effects of CR on neurotransmitters released from synapses and neurotransmitter receptors located on synapses have also been documented. CR prevents age-related changes in serotonin and dopamine levels in the cerebral cortex of rats (242), enhances evoked dopamine accumulation in the striatum of aged rats (243), and prevents the age-related decline in NR1 and GluR1 subunits of NMDA and AMPA receptors in rats, respectively (244). Brain-derived neurotrophic factor (BDNF), a protein that helps protect neurons against excitotoxic, oxidative and metabolic insults (234, 245, 246), also regulates synaptic plasticity and might therefore facilitate learning and memory (247). A few previous studies also demonstrated that rodents maintained on CR had increased BDNF levels in hippocampal and cortical neurons (245, 246, 248-250).

3.3.2 CR induces neurogenesis.

The capacity of the brain for neurogenesis decreases with aging (251). CR has been

shown to increase the number of newly generated neural cells in the brain of rodents (248-250). CR promotes survival of newly generated neurons (248, 250). Lee and colleagues (249) demonstrated that neurogenesis requires BDNF. As previously mentioned, rodents maintained on CR had increased BDNF levels in hippocampal and cortical neurons (245, 246, 248-250).

3.3.3 CR is protective against age-related neurodegenerative diseases.

Initial research documenting the neuroprotective effect of CR found that CR attenuated the age-related loss of spiral ganglion neurons in C57BL/6 mice (252). After that, rodent studies evaluated the effects of CR on common human age-related neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, and demonstrated that CR protected against these diseases (234, 235). In Alzheimer's disease rodent models, CR ameliorated age-related behavioral deficits in the triple-transgenic mouse model (44) and double knockout of presenilin-1 and presenilin-2 (cDKO) mouse model (45). Wu and colleagues (45) also showed that CR attenuated ventricle enlargement, caspase-3 activation and astrogliosis, and reduced the induction of tau hyperphosphorylation in the cDKO mice. In addition, hippocampal neurons of presenilin-1 mutant mice maintained on CR showed increased resistance to excitotoxic injury compared with ad libitum-fed mice (253), and this effect of CR was similar in rats that contracted excitotoxin-induced Alzheimer's desease (254). Zhu and colleagues (253) also showed that levels of oxidative stress in the hippocampus of presenilin-1 mutant mice maintained on CR were lower than their ad libitum counterparts, suggesting that suppression of oxidative stress is a mechanism responsible for the neuroprotective effects of CR on Alzheimer's disease incidence. Another potential mechanism by which CR protects neurons from Alzheimer's disease is that CR attenuates β-amyloid neuropathology, which has been observed in mice (255) and monkeys (135).

In humans, epidemiologic studies revealed a positive correlation between caloric intake and Alzheimer's disease (169, 171). Moreover, a study in humans found that for every 1-kg/m^2 increase in BMI at age 70 years, Alzheimer's disease risk increases by 36% (172). In a Parkinson's disease rodent model, the nigro-striatal dopaminergic neurons of mice maintained on CR were less vulnerable to MPTP toxicity, as since there were more

survived dopaminergic neuron survival was higher and motor deficits lower after exposure to MPTP when compared to non-CR animals (256). Similarly, a case-control study in humans observed higher caloric intake in patients with Parkinson's disease (170).

3.4 CR has anti-cancer effects.

Aging is associated with increased cancer incidence (257, 258), especially cancer of the breast, lung, prostate, and colon (258). The age-associated increased cancer incidence is caused by increased DNA damage (259), decreased DNA repair (258), decline in immune function (258), decreased antitumor action (258), tumor-suppressor gene defects (258) and aberrant DNA methylation (260, 261) that increases expression of oncogenes (4). Numerous rodent studies have demonstrated that CR can prevent cancer and promote tumor regression (102). These effects have been documented in both solid and lymphoid cancer. Rodents maintained on CR experienced 26-94% lower incidence of mammary cancer (53, 60, 62, 65, 71, 75, 77, 78, 82, 99, 100), prostate cancer (87), liver cancer (55, 58), skin cancer (73, 92) and lymphoid cancer (52, 56). In tumor-induced models of rodents, those maintained on CR demonstrated lesser progression of mammary tumor (57, 83, 88, 91, 93, 101, 103), prostate tumor (67, 72, 74, 80, 81, 95), brain tumor (69, 79, 84, 90, 97), pancreatic tumor (89, 98), hepatic tumor (54, 66, 85), intestinal tumor (51, 68, 94, 96) and lymphoid tumor (52, 63). A meta-analysis evaluating 58 rodent and 1 chicken study demonstrated that CR was associated with 80% lower cancer incidence (102). The anti-cancerous effect of CR has also been observed in longitudinal studies of non-human primates. Rhesus monkeys maintained on 30% CR had 50% (112) lower risk of cancer incidence after more than 20 years of follow-up (112, 114). In humans, an observational study reported 69% lower cancer mortality in Okinawans who consumed less energy than the Japanese average (137). Another study also found that the Okinawans experienced 68-83% lower hormone-dependent cancer mortality as compared with people from USA, Sweden, Greece and Italy (150). A study in women with a history of anorexia nervosa and low BMI demonstrated 50% reduction in the incidence of breast cancer, suggesting that CR is protective against breast cancer in humans (174). Positive correlations of BMI with incident cancers of uterus, cervix, gall bladder, kidney, thyroid and bone marrow were also observed in humans (177). Moreover, the protective effects of CR against breast cancer

related-mortality (175), breast cancer recurrence (175), as well as prostate tumor recurrence (176) have been documented. Several mechanisms by which CR protects against cancer have been discovered (262), including 1) a reduction in IGF-1 level (59), resulting in increased cell apoptosis (263), 2) an activation of AMPK (264) that can inhibit cell growth, cell proliferation and tumorigenesis (265), 3) reversal of the aging-induced aberrant DNA methylation (260) that can silence the expression of oncogenes (4) and 4) reduced accumulation of oxidized DNA and protein (266-268) that cause genomic instability (269), a characteristic of most cancer cells (269, 270).

3.5 CR decelerates age-induced immunodeficiency.

Prior studies in rodents and non-human primates demonstrated the beneficial effects of CR on the delay of immunosenescence (109). In rodents, it has been show that CR maintains naive CD4 and CD8 T cells, which represent the body's reserve to combat new pathogens, in peripheral blood and spleen (107). A potential mechanism responsible for their maintenance by CR is increased thymic production (107). In addition, CR maintains the proliferative capacity of T cells in rodents (104-106, 108), which is most likely caused by increased apoptosis of old cells in the aged CR animals (105, 106, 108) and increased resistance of T cells to oxidative stress (271). In non-human primates, CR also preserves naive T cells and T cell proliferation in aged animals (109, 136), and therefore preserves T cell receptor diversity (136). Furthermore, CR improves T cell function and reduces the production of proinflammatory cytokines from memory T cells (136).

3.6 The mitochondrial theory of aging and the modulating effect of CR on the age-related decline in mitochondrial function

Because mitochondria act as a central regulator of nutrient utilization (272), mechanisms by which CR delays aging are likely associated with modulation of mitochondrial function (273). Several modulating effects of CR on the age-related decrease in mitochondrial function have been documented, including antioxidant effects, increased resistance to age-related damage of mitochondrial DNA (mtDNA), improvement of mitochondrial respiratory function and coupling efficiency of mitochondrial OXPHOS, as well as attenuation of the age-induced reduction in mitochondrial turnover (274, 275).

3.6.1 The mitochondrial theory of aging

Cellular aging is associated with mitochondrial damage induced by ROS production (276-285). The age-related increase in the rate of ROS production leads to oxidative damage, somatic mutations and decreased transcription of mtDNA (286-291), causing a progressive decline in the bioenergetic function of cells with aging (292, 293). It has also been demonstrated that the somatic mutations and decreased transcription of mtDNA due to ROS-induced oxidative damage in aged tissues impair OXPHOS and mitochondrial respiration (294-296) through electron leakage from Complex I and Complex III of the respiratory chain (297, 298), resulting in further overproduction of ROS, and further increasing oxidative damage, i.e. aging-induced decline in mitochondrial function is a vicious cycle (289, 299-301). Moreover, the rate of mitochondrial turnover declines with aging, resulting in an age-associated accumulation of mitochondria with aberrant function (302-304).

3.6.2 Antioxidant effects of CR

A reduction in ROS production by CR has been documented in mitochondria of various organs of rodents, including rat liver (305, 306), rat skeletal muscle (307), mouse liver (308), and rat brain (309). Complex I of the mitochondrial electron transport system is a specific site where CR reduces ROS production (306, 310-312). In addition, CR attenuates the increase in ROS normally seen with aging (313, 314). A mechanism responsible for CR-induced decreased ROS production is the reduction in mitochondrial proton leakage (306, 313, 315, 316). A study in mice suggested that the reduction in mitochondrial proton leakage was due to a reduction in polyunsaturated fatty acid (PUFA) in the mitochondrial membrane by CR (317).

3.6.3 CR may be protective against age-related damage of mtDNA.

Gredilla and colleagues (310) showed that CR was associated with lower 8-oxo-dG, an indicator of mtDNA oxidative damage, in rat heart. This effect of CR has also been documented in skeletal muscle. Skeletal muscle 8-oxo-dG was lower in aged CR vs. ad libitum fed mice due to the protective effect of CR against the aging-associated rise in oxidant production (314).

3.6.4 CR improves mitochondrial respiratory function and the coupling efficiency of mitochondrial OXPHOS.

It has been demonstrated that CR induces more efficient mitochondrial respiratory function as skeletal muscle (314, 318, 319) and brain tissue (320) of CR animals exhibit a greater mitochondrial respiratory capacity. This beneficial effect is mediated by a greater affinity of complex IV for O_2 that is induced by CR (318, 321) and an increase in expression of electron transport chain (ETC) complexes (319). CR also improves the coupling efficiency of mitochondrial OXPHOS. Lanza and colleagues (314) demonstrated that aged mice maintained on CR possessed a greater mitochondrial coupling efficiency of mitochondrial OXPHOS in skeletal muscle than their ad libitum fed counterparts.

3.6.5 CR attenuates age-related decline in mitochondrial turnover.

Mitochondrial turnover depends on a balance between mitochondrial biogenesis and autophagy (322). A previous study in mice demonstrated that CR increases mRNA markers of mitochondrial biogenesis, including PGC-1α, NRF1, TFAM, MFN1 and MFN2 (323). A study in human skeletal muscle also demonstrated that CR increases expression of genes encoding proteins involved in mitochondrial biogenesis, including PGC-1α and TFAM (324). A study in rats found that life-long CR reversed aging-induced decrease in mtDNA count number, an indicator of mitochondrial biogenesis (324, 325), in liver and skeletal muscle of rats and increased mtDNA count number in rat brain (326). Another study in rats which assessed autophagic vacuoles and expression of autophagy related proteins documented that CR prevented the age-related decline in autophagy in rat hepatocytes and cardiomyocytes (274). However, there has been some data that questions the effect of CR on mitochondrial biogenesis, at least in rats (327).

3.6.6 Mechanisms by which CR improves mitochondrial function

CR improves mitochondrial function through multiple pathways. For example, CR activates mitochondrial SIRT3, resulting in the deacetylation of several targets, including 1) isocitrate dehydrogenase 2 (328), which is essential for cellular metabolism in lipid synthesis, cellular defense against oxidative stress, oxidative respiration, and oxygen-sensing signal transduction (329), 2) cyclophilin D, resulting in increased resistance to

mitochondrial permeability transition, and consequently reduced activation of mitochondrial-mediated pathways of apoptosis (330), 3) SOD2 which helps reduce oxidative stress (331), 4) LCAD (332), a key enzyme in beta-oxidation (333), 5) respiratory complex II that is responsible for OXPHOS (334) and 6) two subunits of the mitochondrial electron transport complexes: NDUFS1 and the cytochrome bc 1 complex Rieske subunit (335). This effect is mediated by an elevated NAD^+-to-NADH ratio secondary to the energetic stress imposed by CR (336, 337). In addition, previous studies have demonstrated that SIRT1 is required to achieve the beneficial effects of CR (338, 339). CR increased AMPK-induced phosphorylation at Thr^{177} and Ser^{538} residues (340) and subsequent deacetylation by SIRT1 (341, 342), which facilitates PGC-1α translocation to the nucleus (275), resulting in increased mitochondrial biogenesis (320, 323, 343-346). However, this mechanism is still controversial as a few studies found no increase in PGC-1α activity (347, 348) and no increase in mitochondrial biogenesis (319, 327, 349) with CR. Modulation of age-related changes in lipid membrane composition is another mechanism related to the beneficial effect of CR on mitochondrial function (275). CR increases essential fatty acids while attenuating highly unsaturated fatty acids in rat liver mitochondria (350) and heart (351), therefore reducing lipid peroxidizability.

3.7 Epigenetic regulations linking to anti-aging effects of CR

Epigenetic regulations have been recognized as major contributors to CR-related longevity (4). These regulations include DNA methylation and histone modification, resulting in regulation of age-related gene expression (352, 353).

3.7.1 CR and DNA methylation

DNA methylation plays an important role in the regulation of gene expression and maintenance of DNA integrity and stability in various biological processes (4, 354-356). Two major changes in DNA methylation occur during aging (4). These changes involve globally decreased (357-362) but locally increased (362, 363) DNA methylation status of age-related genes, such as RUNX3 and TIG1, leading to a reduced capability for homeostasis and loss of chromatin integrity, predominantly due to aberrant gene expression (364). CR reverses aberrant DNA methylation during aging (260, 261) by increasing DNMT1 (365)

and DNMT3b (366) activity and is associated with changes in specific gene loci rather than globally patterned changes (261). The reverse effect results in hypermethylation and consequently silences the expression of oncogenes such as H-Ras (367), as well as metabolism-related genes such as TNF-α (368), WT1 (369) and ATP10A (370). The silencing of expression leads to cancer prevention and good metabolic health (4).

3.7.2 CR and histone modification

Histone is a protein found in eukaryotic cell nuclei that packages and orders the DNA into nucleosomes (371, 372), the basic structure of the chromatin unit (373). Histone modifications are associated with both gene activation and gene repression (4) and affect nucleosomes by directly changing nucleosome configuration (373). Histone acetylation and deacetylation are the most prevalent mechanisms of histone modifications (353). In addition to histone acetylation and deacetylation, histone methylation is another important modification that regulates gene expression (374).

3.7.2.1 Histone acetylation and deacetylation

Histone acetylation and deacetylation processes are catalyzed by the specific enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively (374, 375). Because it has been widely accepted that HDAC activity is increased during CR, global deacetylation has been suggested as a protective mechanism of CR against metabolic alterations and aging (365). At least four classes of the HDAC family have been identified: class I HDAC (HDAC1, HDAC2, HDAC3 and HDAC8), class II HDAC (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10), class III HDAC (SIRT1, 2, 3, 4, 5, 6 and 7) and class IV HDAC (HDAC11) (4). Among all HDACs, activation of SIRT1, which is a class III NAD^+-dependent HDAC, is found in diverse CR models of animals, humans and in vitro system (12, 19, 341, 365, 376-382). Two potential mechanisms by which SIRT1 mediates CR-induced metabolic alterations and subsequent delay aging have been identified (4). First, SIRT1 activation negatively regulates proapoptotic factors such as p53, Ku70 and FoxO, resulting in increased stress resistance (383-389). Second, SIRT1 activation regulates key metabolism-associated genes such as PGC-1α, leading to inhibition of adipogenesis and increased insulin secretion from pancreatic β-cells, which facilitates stress resistance and longevity

(390, 391). Potential mechanisms are not limited to SIRT1, as previous studies also demonstrated that altered binding enrichment of HDAC1 on the promoter region of age-related genes such as $p16^{INK4a}$ and hTERT leads to gene expression changes and contributes to longevity under CR condition (365, 392, 393).

3.7.2.2 Histone methylation

CR-induced histone methylation can regulate expression changes in age-related genes, including $p16^{INK4a}$ and hTERT, contributing to longevity (365, 382, 394-396). Unlike histone acetylation and deacetylation, which are always associated with open chromatin status and subsequent gene activation, differentially methylated forms of histones show unique association patterns with specific proteins that recognize these markers, and therefore lead to gene silencing or activating effects (353, 365, 397). Specific histone methylation can also act as a transcription modulator by interacting with different transcription factors and may regulate the CR-induced aging delay (394-396).

3.8 Summary

- CR is defined as a reduction in caloric intake, typically by 20–40% of ad libitum consumption, whereas protein and micronutrients intake are sufficient to avoid malnutrition.
- CR without malnutrition is a widely accepted non-genetic paradigm to increase lifespan.
- It has been suggested that CR extends lifespan by attenuating age-related diseases and pathologies such as insulin resistance, hypertension, dyslipidemia, chronic inflammation, cognitive decline, cancer and immunodeficiency.
- Mechanisms by which CR delays aging are likely associated with modulation of mitochondrial function. CR has antioxidant effects, attenuates age-related oxidative damage to mtDNA, improves mitochondrial respiratory function and the coupling efficiency of mitochondrial OXPHOS, and prevents the age-related decline in mitochondrial turnover.
- Several mechanisms by which CR alters mitochondrial function were reviewed, including activation of mitochondrial SIRT3, which results in the deacetylation of

several targets, and modulation of age-related changes in lipid membrane composition. However, the mechanism by which CR increases PGC-1 α activity and therefore mitochondrial biogenesis through phosphorylation by AMPK and subsequent deacetylation by SIRT1 is still controversial.

- Epigenetic regulations have been recognized as major contributors to CR-related longevity. Regulations include DNA methylation and histone modification, resulting in alteration of age-related gene expression. Histone acetylation and deacetylation are the most prevalent mechanisms of histone modifications. In addition to histone acetylation, histone methylation is another important modification.

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Chapter 4
Effects of intrinsic cardiorespiratory fitness and caloric restriction on metabolites in human plasma

4.1 Introduction

Intrinsic cardiorespiratory fitness (VO_2max) is highly positively related to human lifespan and negatively related to all-cause mortality and cardiovascular disease (CVD) regardless of age, sex, race, and other risk factors as reviewed in chapter 2. Approximately 50% of the variation in the intrinsic (untrained) VO_2max in the untrained state, after adjusting for age, sex, body mass, and body composition is thought to be genetic (1). Rats bred for high or low VO_2max (high capacity running (HCR) and low capacity running (LCR) rats) show divergence in skeletal muscle oxidative capacity and multiple aspects of metabolic health, including more insulin sensitivity, lower plasma triglyceride level, lower blood pressure (BP), lower visceral adiposity, as well as lower body weight (2-5). Paralleling the observations in humans with high VO_2max, HCR rats have a 23-40% longer median and absolute lifespan (6) and show evidence of slowed aging with reduced changes in skeletal muscle gene expression with age (7).

Mitochondria are central to intermediary metabolism in all cells and play a role in the normal aging process. Mitochondrial dysfunction in aging is thought to be the result in decreased oxidative phosphorylation (OXPHOS) efficiency, increase in the production of reactive oxygen species (ROS), decrease in anti-oxidant effect, as well as a reduction in mitochondrial turnover due to decreased mitochondrial biogenesis and mitophagy (8, 9), resulting in a shortened lifespan (9-11). Over-nutrition, as seen in obesity, can lead to 'accelerated aging' (12). Recent metabolite profiling studies have shown a relationship between obesity and changes in multiple metabolites in the blood, most notably the essential branched-chain amino acids (BCAAs) and aromatic amino acids, fatty acids (FAs)

and their downstream metabolites acylcarnitines, all formed as part of mitochondrial catabolism (13-21). The alterations in these metabolite, and others, likely reflect changes in the balance of cellular metabolite uptake and their ultimate metabolism. The oxidation of both FAs and BCAAs are dependent upon the availability of oxidized NAD^+ and FAD in the mitochondria to accept electrons and donate them to the electron transport chain for ATP synthesis (22). With a high cellular energy state as seen in obesity, ADP levels are limited and OXPHOS slows, with the resultant generation of ROS and a decline in mitochondrial mass, function and turnover (22). Experimental high fat feeding in mice reduced both healthspan and lifespan (12). Conversely, caloric restriction (CR) can markedly improve cellular energetics and mitochondrial function as reviewed in chapter 3.

While high VO_2max and CR both are associated with increased longevity and alterations in mitochondrial function, it is unclear if there is a link between the two phenomena. To address the possible interconnectedness in humans, we asked whether we could detect a modulation by increased VO_2max in the observed age-related changes in blood metabolites (23). Further, we asked whether CR alters metabolism in a similar matter. To do this we investigated the relationships between age, VO_2max and a set of plasma metabolites that have been associated with aging, insulin resistance and/or risk of type 2 diabetes mellitus (DM) and CVD. The levels of amino acids (AAs), free fatty acids (FFAs) and their metabolites generated by mitochondrial metabolism were measured by targeted metabolomics profiling in plasma in non-obese individuals and obese people before and after aggressive CR to induce weight loss. In an attempt to lessen diet influences on the metabolite levels, subjects consumed a standardized isocaloric diet for 3 days prior to sampling. The results suggest that age related changes in the metabolite levels are slowed with higher VO_2max. The data also suggests that CR improves apparent 'metabolic age'.

4.2 Materials and methods

4.2.1 Participants

Participants in this study were enrolled as a part of "Investigational Weight Management Clinic (IWMC)"

Inclusion criteria

1. Males or females whose age were 18-85 years old.
    a. Subjects whose BMI was 18.5-28.0 kg/m^2 were categorized as "non-obese" group. Subjects in this group must be non-diabetic.
    b. Subjects whose BMI was equal or greater than 30.0 kg/m^2 were categorized as "obese" group.
2. Able and willing to provide written informed consent for the trial.

Exclusion criteria

1. Evidence of inherited disorders of lipid metabolism.
2. History of cancer within the last 5 years
3. Human immunodeficiency virus (HIV) antibody positive.
4. Patients with solid organ transplants.
5. Participation in any other clinical trial within 90 days of entry into this trial.
6. Pregnant or lactating females.
7. Uncontrolled thyroid disease
8. Unstable angina or New York Heart Association class II failure or above
9. Gastrointestinal disease specifically GI motility disorders
10. Unstable neuropsychiatric disease including major depression/anxiety, eating disorder such as bulimia or anorexia
11. End stage renal or hepatic disease
12. Autoimmune disorders
13. Body weight fluctuation of more than 5 kg in the previous 3 months
14. Prior bariatric surgery
15. A history or current alcohol/substance abuse and change in smoking habits or cessation in the past 6 months.
16. Women of childbearing age must use a reliable form of contraception.
17. Any medical condition, which in the opinion of the investigator would make the patient unsuitable for recruitment, or could interfere with the patient participating in or completing the protocol.
18. Unwilling or unable to consent for the study.

CR was applied only for the obese group. Indeed, non-obese subjects were studied

only once as the control group. All studies were approved by the Institutional Review Board of the University of Michigan and all subjects gave informed consent.

4.2.2 CR protocol

The protocol lasted 100 weeks with 1 assessment visit (visit 0) and 26 program visits (visit 1-26) and was divided into 2 sections.

1. Rapid weight loss section

   Participants received 800 kcal/day (5 packs) of high-protein liquid shake (HMR® 800) approximately containing 16 g of protein, 21 g of carbohydrate and 2 g of fat per pack. They were also encouraged to have at least 150 min/week of exercise at intervals at their convenience. The goal is at least 15% of weight loss, which usually takes 12 to 16 weeks.

2. Weight maintenance section

   After achieving the goal, participants received 1,200-1,800 kcal/day of regular diet plus partial meal replacement with high-protein liquid shake (HMR® 800) and maintained the same exercise. The study was ended at week 100.

Our assessment was focused only on the time point at baseline before CR (week 0) and at the end of rapid weight loss section (week 12 to 16). The assessment included demographic data, body mass index (BMI), body composition, VO_2max, resting energy expenditure (REE), respiratory quotient at rest (RQR), respiratory quotient at VO_2max (RQE), fasting plasma glucose and insulin, HOMA-IR ((fasting plasma glucose x fasting plasma insulin)/405), as well as fasting plasma metabolites, including AAs, branched-chain ketoacids (BCKAs), FFAs and acylcarnitines.

4.2.3 Body composition

Body composition was measured by dual energy x-ray absorptiometry (Lunar Prodigy ADVANCE Plus, GE Healthcare, Chicago, IL).

4.2.4 Exercise testing for VO_2max, REE, RQR and RQE

The equipment for the exercise test included 3 complete metabolic carts (Viasys Healthcare Vmax Encore VS 29n, ParvoMedics TrueOne 2400 and Physiodyne Max II), 2

treadmills (Trackmaster), 2 portable/wearable metabolic mask systems (COSMED K4B2 and COSMED Fitmate), 12 lead ECG (Quentin), POLAR T2 technology system (heart rate monitor and capture) and SenseWear® monitors (Bodymedia Inc., Pittsburgh, PA, USA). VO_2max was assessed using modified Bruce protocol described elsewhere (24). Ventilation volume and respiratory gas exchange were continuously monitored as well to calculate RQR and RQE.

4.2.5 Mixed Meal Tolerance Test (MMTT)

Fasting plasma for all analyses were obtained during MMTT. To minimize interpersonal variation during the test, all subjects were provided an isocaloric diet menu containing a macronutrient mix at the 50^{th} percentile for Americans that was consumed for 3 days prior to testing. Following a 12-hour overnight fast, blood was collected from a peripheral venous catheter placed in either in the arm (antecubital) or hand (0 min). Each participant then consumed a liquid mixed meal (237 ml of Ensure® Original Therapeutic Nutrition containing 22% of fat, 64% of carbohydrate and 14% of protein) within 10 min. In addition to the initial fasting sample (0 min), venous blood was obtained 15, 30, 60, 90, 120, 150 and 180 min after mixed meal consumption.

4.2.6 Plasma glucose and insulin

Plasma glucose and insulin were measured by the Michigan Diabetes Research and Training Center (MDRTC). Plasma glucose analysis was performed on a Cobas Mira Chemistry Analyzer (Roche Diagnostics Corporation, Indianapolis, IN, USA). Reagents and calibrators used for the analysis were from Sekisui Diagnostic LLC (Lexington, MA). Plasma insulin was analyzed by a double-antibody radioimmunoassay using an ^{125}I-Human insulin tracer (Linco Research, St. Charles, MO, USA), a guinea pig anti-porcine insulin first antibody (MDRTC, 68.5% cross-reaction to human proinsulin) and a goat anti-guinea pig gamma globulin-PEG second antibody (MDRTC) and standardized against the Human Insulin International Reference Preparation (National Institute for Biological Standards and Control).

4.2.7 Plasma metabolomics

Targeted metabolomic approach was used to study metabolites listed in Table A4.1 of appendices. The study was quality controlled by reference human plasma samples obtained from the American Red Cross. Seventy µl of plasma was extracted by adding 280 µl of extraction solvent (1:1:1 methanol: acetonitrile: acetone) containing internal standard mixture (see Table A4.2 of appendices), vortexing for 10 sec, allowing to rest on ice for 5 min, and then centrifuging at 4 °C for 10 min. The supernatant was divided into 2 aliquots (140 µl each) and dried by vacuum centrifuge at 45 °C. One aliquot was reconstituted using 140 µl of 8:2 methanol:water for AA analysis. Another was reconstituted using 140 µl of 8:2 water:methanol for BCKA, FFA and acylcarnitine analysis.

All metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 LC connected to an Agilent 6410 tandem quadrupole MS. MS parameters were as follows: capillary voltage 4000 V, gas temperature 325 °C, gas flow 10 l/min and nebulizer pressure 40 psi. Mobile phase A was 0.1% of formic acid in water and mobile phase B was 0.1% of formic acid in acetonitrile.

For AA analysis, hydrophilic interaction chromatography (HILIC) was performed using a VWR Cogent Diamond Hydride column, 4 µm particle size, 150 mm x 2.1 mm inner diameter (i.d.) (Radnor, PA). The flow rate was 0.25 ml/min and the gradient consisted of an 11-min linear ramp from 95 to 25% B, 2 min at 10% B and 7 min of re-equilibration at 95% B. Detection was performed using multiple reaction monitoring (MRM) in positive ion mode (see details in Table A4.3 of appendices).

For BCKA, FFA and acylcarnitine analysis, reversed-phase liquid chromatography (RPLC) was performed using a Waters Acquity HSS T3 column, 1.8 µm particle size, 2.1 x 100 mm i.d. (Milford, MA), with a flow rate of 0.25 ml/min. For BCKA and FFA, the gradient consisted of a 10-min linear ramp from 0 to 100% B, 5 min at 100% B and 8 min of re-equilibration at 0% B. Detection was performed using selected ion monitoring (SIM) in negative ion mode (see details in Table A4.4 of appendices). For acylcarnitine analysis, the same column was used with the following gradient: 7-min linear ramp from 0 to 80% B, 3 min at 100% B and 5 min of re-equilibration at 0% B. MRM in positive ion mode was used with precursor/product ion transitions specified elsewhere (25).

Acylcarnitine species present in the internal standard mix (NSK-B) were reported as absolute concentration as determined by isotope dilution MS (peak area of unlabeled compound-to-peak area of internal standard x concentration of internal standard). Other acylcarnitine species were reported as relative concentration (peak area of biological compound-to-peak area of internal standard with closest matching RT x concentration of internal standard). Metabolites other than acylcarnitines with exact-matching stable isotope internal standards were quantitated by calibration curves using peak area ratios to their internal standards. Other metabolites without exact-matching stable isotope internal standards were quantitated using the ratio of their peak area to that of the internal standard with closest matching retention time (RT), as indicated in Table A4.5 of appendices. Metabolite concentrations used for calibration curves are listed in Table A4.5 of appendices. Metabolite peak areas were determined using Agilent Masshunter Quantitative Analysis software for triple quadrupole MS version B.07.00.

4.2.8 Statistical analyses

All figures were depicted using Prism 7 Version 7.0a. P-value <0.050 was considered statistically significant. Comparison of the assessments between the non-obese and the obese groups were analyzed using unpaired-two-tailed student's t tests. For the comparison between obese subjects at baseline and after CR, we used paired-two-tailed student's t tests. All metabolomic data were reported as average$\pm$standard error of mean (SEM).

Correlations of metabolites vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline] were analyzed by Multivariate Analysis of Variance (MANOVA) and Analysis of Variance (ANOVA). We employed MANOVA to compare means of metabolites grouped based on their correlations. It uses the covariance between the metabolites considered in a group to test the statistical significance of the mean differences across different experimental conditions. MANOVA is a procedure for comparing multivariate sample means. MANOVA is simply an ANOVA with several dependent variables. It uses the covariance between outcome variables in testing the statistical significance of the mean differences. The groups of metabolites considered in our analysis were identified by calculating correlations amongst them, then thresholding them by testing whether they are

different than zero by employing Fisher's z-transform test and controlling the False Discovery Rate at the 10% level, followed by applying agglomerative clustering. In carrying out the MANOVA testing, we used the so called Type II tests that test for an effect after testing for another one. To clarify this point, we provided a simple example. Suppose that we are interested in testing the effect of covariate for a particular group of metabolites, as a function of sex, race, age, diabetes status, VO_2max and BMI. Then, the p-value obtained for the BMI covariate shows the significance controlling for the presence of the remaining variables in the model. Finally, once groups of metabolites were identified as significantly differential, we further examined the impact of covariates on each metabolite in the group, by employing the standard t-test in a regression test (ANOVA) for the null hypothesis of whether its coefficient is zero or not.

4.3 Results

4.3.1 Participant characteristics

Participant characteristics are detailed in Table 4.1. Twenty-eight non-obese (14 males and 14 females) and 124 obese (56 males and 68 females) individuals participated in this study. Most were Caucasian (92.8% of non-obese and 93.6% of obese). Obese individuals were heavier (39.7 ± 0.43 vs. 22.5 ± 0.39 kg/m^2) and older (50 ± 0.79 vs. $43\pm$ 2.04 years of age). Among 124 obese subjects, 47 of them had type 2 DM and 32 of them received 1 or more anti-diabetic drugs prior to the enrollment. An additional 6 obese subjects began anti-diabetic drug(s) after type 2 DM was diagnosed during testing. Body weight decreased by 17.8 ± 0.56% with CR (range 4.1-35.7%).

4.3.2 Non-metabolite parameters

Non-metabolite parameters are detailed in Table 4.1. The obese group had higher lean and fat mass and fat percentage. The obese group also had higher fasting plasma glucose and insulin, and HOMA-IR and these values continued to be higher after removal of diabetic individuals (p-value <0.001 in both male and female). These parameters significantly improved after CR, but continued to be significantly different than non-obese. Total REE and REE corrected for lean+bone (fat-free) mass (FFM) was higher in obese than

non-obese males, but was not different after correcting for lean mass in the females (Table 4.1). Average REE and corrected REE fell significantly following weight loss, indicating an increased oxidation of FAs, despite an isocaloric diet for 3 days prior to testing. VO_2max corrected for FFM was lower in obese individuals than the non-obese. Because VO_2max per FFM negatively correlated with age ($r = -0.42$; p-value = 0.023 for non-obese group, $r = -0.47$; p-value <0.001 for obese group at baseline and $r = -0.53$; p-value <0.001 for obese group after CR) and the average age of our obese subjects were higher, we examined VO_2max per FFM between non-obese and obese subjects in sub-group of our participants with ages >35 years (n = 54 obese males, n = 63 obese females, n = 8 non-obese males and n = 11 non-obese females). VO_2max per FFM was still significantly lower in the obese group (Table A4.6 of appendices). After CR in the obese, VO_2max per FFM was increased slightly, but significantly compared to baseline. The correlation of VO_2max per FFM between at baseline and after CR was significantly positive ($r = 0.689$; p-value <0.001). We detected no difference in the RQE between non-obese and obese or in obese after CR.

4.3.3 Metabolites and their difference between groups

Concentrations of all AA-related metabolites are detailed in Table 4.2. Consistent with insulin resistance profiles (glucose, insulin and HOMA-IR), previously proved insulin resistance-related AAs, including BCAAs (isoleucine+leucine and valine), aromatic AAs (phenylalanine and tyrosine), alanine and glutamic acid (13-21), were significantly higher in obese subjects. These AAs decreased significantly after CR. Methionine, which is also correlated with insulin resistance (26), was not significantly different between groups, but CR decreased the level. Previously proved insulin sensitivity-related AAs include asparagine, glutamine-to-glutamic acid ratio, glutamine, glycine and serine (17, 27, 28). Asparagine, glutamine-to-glutamic acid ratio, glutamine and serine levels were significantly lower in the obese group. Glycine was lower in obese male than non-obese male participants, but there was no significant difference between groups in females. CR increased the levels of all these 5 markers, yet glutamine-to-glutamic acid ratio was still significantly lower than what we found in non-obese participants. Ketovaline was the only BCKA that was significantly higher in obese subjects and this was found only in male participants. CR decreased ketoisoleucine+ketoleucine and ketovaline levels. C3 carnitine

and C5 carnitine are intermediate metabolites of valine and isoleucine+leucine catabolism, respectively. Like their substrates, C3 carnitine and C5 carnitine are known as markers of insulin resistance (20, 21). C3 carnitine and C5 carnitine were significantly higher in obese participants. Their levels decreased after CR. To evaluate mitochondrial capacity of BCAA catabolism, we inferred fluxes by calculating ratios of C3 carnitine and C5 carnitine to their substrates and ketoacids of the substrates. Higher ratio means lower mitochondrial capacity of BCAA catabolism since there were more accumulations of intermediate metabolites. We found that C3 carnitine-to-valine ratio, C3 carnitine-to-ketovaline ratio and C5 carnitine-to-ketoisoleucine+ketoleucine ratio were significantly higher in obese participants. C5 carnitine-to-isoleucine+leucine ratio showed a higher trend (p-value = 0.051) in the obese group.

Concentrations of all FA-related metabolites are detailed in Table 4.2. Oleic acid levels were not different between groups. Obese male subjects had significantly higher palmitic acid level than the non-obese males, but the difference was not found between groups in females. After CR, oleic acid remained unchanged whereas palmitic acid showed a trend of reduction in female subjects (p-value = 0.051). Long-chain acylcarnitine levels reflect coming of FAs into the mitochondria via carnitine shuttle (29). C18:0 carnitine, C20:0 carnitine and C20:1 carnitine were significantly lower in obese subjects. Other long-chain acylcarnitines were not significantly different between groups. CR increased C18:1 carnitine, C18:2 carnitine, C20:0 carnitine, C20:1 carnitine, C20:2 carnitine, C20:3 carnitine and C20:4 carnitine levels whereas other long-chain acylcarnitines remained unaltered. We also inferred fluxes by calculating ratios of C16:0 carnitine and C18:1 carnitine, which reflect coming-in of palmitic acid (16:0 FA) and oleic acid (18:1 FA) respectively, to the levels of palmitic acid and oleic acid. Straightforwardly, higher ratio represents higher coming of FAs into the mitochondria. We found that C16:0 carnitine-to-palmitic acid ratio was significantly lower in obese participants. C18:1 carnitine-to-oleic acid ratio was also lower in obese subjects but did not reach statistically significance (p-value = 0.125). Both C16:0 carnitine-to-palmitic acid ratio and C18:1 carnitine-to-oleic acid ratio were significantly increased after CR. Medium-chain (C6-C14) acylcarnitine levels reflect fatty acid oxidation (FAO) in the mitochondria (29). It is already known that FAO consists of several steps, but which steps are rate-limiting is still unclear. Here we identified rate-

limiting steps by the average concentration of all medium-chain acylcarnitines in non-obese, obese at baseline and after CR. We found that C10:0 carnitine had the highest concentration in every group (Figure A4.1 of appendices). Therefore, we determined the shortening of C10:0 carnitine to get C8:0 carnitine as a starting point of the rate-limiting step of FAO. After identifying the rate-limiting step, we again inferred a flux using C8:0 carnitine-to-C10:0 carnitine ratio to evaluate mitochondrial capacity of FAO. Higher ratio means lower mitochondrial capacity of FAO, i.e. incomplete FAO since there was more accumulation of the first product of the rate-limiting step. We found that C8:0 carnitine-to-C10:0 carnitine ratio was significantly higher in the obese group and CR decreased the level. C8:1 carnitine was significantly higher in obese subjects. C6:0 carnitine was significantly higher in obese males, but not in obese females. C10:0 carnitine, C12:0 carnitine and C14:2 carnitine were significantly lower in the obese group. The levels of C8:0 carnitine, C10:1 carnitine, C12:1 carnitine, C12-OH carnitine, C14:0 carnitine, C14:1 carnitine, C14-OH carnitine, C16:1 carnitine and C16-OH carnitine were not significantly different between groups. After CR, C6:0 carnitine, C8:1 carnitine, C12:0 carnitine, C12:1 carnitine and C14:0 carnitine were significantly decreased whereas C8:0 carnitine, C10:0 carnitine, C10:1 carnitine, C12-OH carnitine, C14:1 carnitine, C14:2 carnitine, C14-OH carnitine, C16:1 carnitine and C16-OH carnitine remained unchanged.

4.3.4 Effects of age and VO_2max on metabolite levels

We noted that groups of the metabolites had significant covariance in the plasma, which was not surprising given the chemical similarities and the convergence of the metabolic pathways used in the oxidation of FAs and AAs in the mitochondria. To enhance our signal for specific metabolic pathways that are affected by age and VO_2max, we used MANOVA, grouping the metabolites by clustering their covariance and then assessing how the metabolites in each group were affected by age and/or VO_2max and the interactions of the terms. All metabolites and insulin resistance profiles at baseline from the non-obese and obese groups formed 4 groups after hierarchal clustering (Figure 4.1 and Table 4.3). Group I mainly consisted of basic AAs, long-chain saturated acylcarnitines, as well as ratios of C16:0 carnitine and C18:1 carnitine to their FA substrates. Group II mainly consisted of BCAAs, aromatic AAs, BCKAs and insulin resistance profiles. Group III mostly consisted of

acylcarnitine intermediates of BCAAs and ratios to their substrates. Group IV consisted of FFAs, long-chain and medium-chain acylcarnitines, as well as C2 carnitine. We then calculated the effects of age and/or VO_2max on metabolite levels, after correction for clinical variables including sex, race, group of subjects, diabetes status and BMI. Results were considered significant when correlations of age and/or VO_2max vs. metabolite cluster were significant using MANOVA. In each cluster of metabolites that were significantly affected by age and/or VO_2max from MANOVA, ANOVA was then used to identify which metabolites drove the significance of the group. Metabolites were considered as drivers of the group if their correlations vs. age and/or VO_2max were significant from ANOVA. Since VO_2max is a function of FFM (30, 31) and fat mass does not have any effect on VO_2max (31), VO_2max adjusted by FFM (VO_2max per FFM) was used for the analysis.

4.3.4.1 Age determines metabolite levels.

From MANOVA, we found that correlations between age and all 4 metabolite groups at baseline were significant (Figure 4.2 and Table A4.7 of appendices). Using ANOVA, Group I displayed significantly positive correlations of age vs. glycine, C18:0 carnitine, C20:0 carnitine and C18:1 carnitine-to-oleic acid ratio, whereas significantly negative correlations of age vs. histidine and threonine were found. In Group II, there was a significantly positive correlation between age and C8:0 carnitine-to-C10:0 carnitine ratio, but significantly negative correlations of age vs. isoleucine+leucine and ketoisoleucine+ketoleucine were observed. The correlations between age and all metabolites in Group III were positive and the significances were found in L-carnitine and ratios of C5 carnitine to its substrates. As with Group III, the correlations between age and metabolites in Group IV were all positive and the significances were found in C6 carnitine, C8:0 carnitine, C10:0 carnitine, C10:1 carnitine, C16:1 carnitine, C18:1 carnitine, C18:2 carnitine, C18:2-OH carnitine, C20:1 carnitine, C20:2 carnitine, C20:3 carnitine and C20:4 carnitine (Figure 4.2 and Table A4.7 of appendices).

4.3.4.2 Regardless of age, VO_2max also determines metabolite levels.

VO_2max falls with age (32-34) and thus in modeling changes in metabolite levels with VO_2max were adjusted for age. From MANOVA, significant correlations of VO_2max per

FFM at baseline vs. Group II and IV at baseline were found (Figure 4.2 and Table A4.7 of appendices). Using ANOVA, Group II demonstrated significantly positive correlation between VO_2max per FFM at baseline and tryptophan but significantly negative correlation with proline. In Group IV, there were significantly negative correlations between VO_2max per FFM at baseline and C2 carnitine as well as several medium-chain acylcarnitines including C6 carnitine, C12:0 carnitine, C14:0 carnitine and C16-OH carnitine (Figure 4.2 and Table A4.7 of appendices).

4.3.4.3 VO_2max counteracts age-induced increase in FA-related metabolites.

To identify how VO_2max alters effects of age on metabolites levels, we assessed the correlation between [age x VO_2max per FFM at baseline] and metabolite levels. From MANOVA, significantly correlations between [age x VO_2max per FFM at baseline] and Group IV at baseline were identified (Figure 4.2 and Table A4.7 of appendices). Using ANOVA, correlations were all negative and significance was found in oleic acid, several medium-chain acylcarnitines including C6 carnitine, C10:1 carnitine, C12:0 carnitine, C12:1 carnitine, C12-OH carnitine, C14:0 carnitine, C14:1 carnitine, C14:2 carnitine, C14-OH carnitine, C16:1 carnitine and C16-OH carnitine, as well as two long-chain acylcarnitines: C18:1 carnitine and C18:2-OH carnitine (Figure 4.2 and Table A4.7 of appendices), suggesting that not only VO_2max determines FA-related metabolite levels, but it also counteracts age-induced increase in FA-related metabolites since the correlation between age and all metabolites in Group IV were positive (as shown earlier). In other words, high VO_2max delays metabolic aging. The counteracting effect was dominant in medium-chain acylcarnitines because ANOVA showed that significant correlations of [age x VO_2max per FFM at baseline] were mostly found in those metabolites.

4.3.5 Effects of CR on age- and VO_2max-related changes in metabolite levels

To assess how CR alters the effects of age and VO_2max on metabolite levels, we identified the effects of age, VO_2max per FFM and [age x VO_2max per FFM] on metabolite levels only in obese subjects at baseline and compared with those effects in obese subjects after CR. Because one of our objectives was to study effects of intrinsic VO_2max, we always used VO_2max per FFM at baseline for the analysis even after CR.

All metabolites and insulin resistance profiles after CR could be hierarchal clustered into 4 groups according to their correlations with each other, and metabolites in each group were identical as those of baseline condition (Figure 4.1 and Table 4.3). As described earlier, the effects of age and/or VO_2max were considered significant using MANOVA, and driving metabolites of the group were identified using ANOVA.

4.3.5.1 Effects of age on metabolite levels persist after CR.

From MANOVA, we found that correlations between age and all 4 metabolite groups after CR were significant as in baseline condition (Figure 4.4 and Table A4.9 of appendices). Using ANOVA, Group I displayed significantly positive correlations of age vs. glutamine, glycine and C20:0 carnitine. In Group II, there was a significantly positive correlation between age and glucose, but significantly negative correlations of age vs. isoleucine+leucine and ketoisoleucine+ketoleucine were observed. The correlations between age and all metabolites in Group III were all positive and the significances were found in C3 carnitine, C4 carnitine as well as ratios of C3 carnitine and C5 carnitine to their substrates. As with Group III, the correlations between age and metabolites in Group IV were all positive and the significances were found in C2 carnitine, C6 carnitine, C8:0 carnitine, C10:0 carnitine, C10:1 carnitine, C12:0 carnitine, C12:1 carnitine, C14:0 carnitine, C14:1 carnitine, C14:2 carnitine, C16:1 carnitine, C18:1 carnitine, C18:2 carnitine, C18:2-OH carnitine, C20:1 carnitine, C20:2 carnitine, C20:3 carnitine and C20:4 carnitine (Figure 4.4 and Table A4.9 of appendices).

4.3.5.2 CR overwhelms effects of VO_2max on FA-related metabolite levels.

From MANOVA, there was a significant correlation between VO_2max per FFM at baseline and group IV that mainly consists of FA-related metabolites in obese subjects at baseline (Figure 4.3 and Table A4.8 of appendices). Using ANOVA, there were significantly negative correlations between VO_2max per FFM at baseline and C2 carnitine as well as several medium-chain acylcarnitines including C6 carnitine, C12:0 carnitine, C12-OH carnitine, C14:0 carnitine, C14-OH carnitine and C16-OH carnitine. However, the significance was not found in obese subjects after CR (Figure 4.4 and Table A4.9 of

appendices), suggesting that CR weakened the effects of VO_2max on FA-related metabolite levels.

4.3.5.3 CR also weakens the counteracting effects of VO_2max on age-induced increase in FA-related metabolites.

From MANOVA, there was a trend of correlation (p-value = 0.116) between [age x VO_2max per FFM at baseline] and Group IV that mainly consists of FA-related metabolites in obese subjects at baseline (Figure 4.3 and Table A4.8 of appendices). Using ANOVA, there were significantly negative correlations of several medium-chain acylcarnitines including C6 carnitine, C8:0 carnitine, C10:0 carnitine, C10:1 carnitine, C12:0 carnitine, C12:1 carnitine, C12-OH carnitine, C14:0 carnitine, C14:1 carnitine, C14:2 carnitine, C16:1 carnitine and C16-OH carnitine, as well as 2 long-chain acylcarnitines: C18:2-OH carnitine and C20:4 carnitine (Figure 4.3 and Table A4.8 of appendices). This counteracting effect was dominant in medium-chain acylcarnitines because ANOVA showed that significant correlations of [age x VO_2max per FFM at baseline] were mostly found in those metabolites. However, the trend of correlation was not found (p-value = 0.494) in obese subjects after CR (Figure 4.4 and Table A4.9 of appendices), suggesting that CR weakened the counteracting effects of VO_2max on age-induced increase in FA-related metabolites.

4.3.6 Not only high VO_2max, but CR also delays age-induced increase in medium-chain acylcarnitines.

As shown earlier, high VO_2max delays metabolic aging, especially age-induced increase in medium-chain acylcarnitines. Indeed, at any age, people with higher VO_2max have lower levels of medium-chain acylcarnitines. Because several medium-chain acylcarnitine levels were significantly lower after CR including C6:0 carnitine, C12:0 carnitine, C12:1 carnitine, and C14:0 carnitine (Figure 4.5), it is suggestive that CR acts like high VO_2max in delay age-induced increase in medium-chain acylcarnitines, i.e. delay metabolic aging.

4.4 Discussion

Using quantitative targeted metabolomics, we identified expected changes in AA and FA metabolism due to obesity and CR, as well as assessed the effects of age and VO_2max on metabolites and how these factors interacted. To reduce metabolite variation between subjects and before and after CR, we provided every subject with a personalized diet plan to reduced diet- and energy-related changes in metabolite levels to increase our ability to see changes related to age and VO_2max. All studies were done on fasting plasma. In the fasting state, more FAs and many AAs are entering the blood, increasing delivery to the tissues for oxidation. This state provides an ideal state to probe whole body mitochondrial oxidative capacity.

We found that VO_2max per FFM was lower in the obese group, consistent with several previous cross-sectional and longitudinal studies which found that higher VO_2max is associated with lower BMI in both lean and obese humans (35-40). CR slightly, but significantly increased VO_2max per FFM in our obese subjects as previously found (41, 42) and likely was due to an increase in pulmonary (43-46) and cardiac (43, 47-49) function following to weight loss. However, the VO_2max after CR was highly correlated to pre-weight loss levels, consistent with a genetically driven component to VO_2max (1, 50-59).

Changes in several AAs and BCAAs intermediates due to obesity and CR were demonstrated in this study. Consistent with insulin resistance profiles, insulin resistance-related AAs and their intermediates were significantly higher in obese subjects, but CR decreased their levels. It was not surprising that our results were consistent with previous human studies (21, 60, 61). Oppositely, insulin sensitivity-related AAs (asparagine, glutamine-to-glutamic acid ratio, glutamine, glycine and serine) were significantly lower in obese subjects, yet CR increased their levels. Our results were again consistent with a previous study in humans (21). To our knowledge, this is the first study that infers fluxes to assess mitochondrial capacity of BCAA catabolism in humans. The inferred fluxes showed that obesity was associated with decrease in mitochondrial capacity of BCAA catabolism, but this was improved after CR. This effect of CR is possibly not only due to improved insulin sensitivity, but also increased mitochondrial protein deacetylation since mitochondrial protein deacetylation was found to be associated with increased mitochondrial capacity of BCAA catabolism (62) and because CR can increase

mitochondrial protein deacetylation via SIRT1 (63) and SIRT3 (64) activation.

Unlike other previous studies in humans (21, 65), we did not see elevated fasting plasma FFAs in obese participants and no significant changes in those levels after CR. This may be due to the isocaloric diet prescription that the participants were provided. Despite similar levels of fasting FFAs between non-obese and obese subjects, we found lower long-chain acylcarnitine levels as well as long-chain acylcarnitine-to-its FFA substrate ratios in the obese group, increasing after CR. The apparent whole body reduction in FA influx into mitochondria may be due to decreases in skeletal muscle uptake of FAs (66), increases in skeletal muscle malonyl-CoA (67, 68), as well as reduction in AMPK (69-71) and CPT-1 activity in skeletal muscle of obese individuals (69-72), with improved influx after CR. As an indicator of mitochondrial FA oxidative capacity, we found C8:0 carnitine-to-C10:0 carnitine ratio was significantly higher in obese subjects compared to non-obese, with significant reduction in the ratio after CR. This suggests that despite higher influx of FAs into the mitochondria, CR improves the capacity of the mitochondria to oxidize the FAs. Reduction in several medium-chain acylcarnitine levels despite higher levels of long-chain acylcarnitines after CR also reflects improvement of incomplete FAO by CR. Whether the relative decrease in FAO reflects a 'defect' in mitochondrial function in obesity (73), or simply reflect the mismatch of fuel delivery to mitochondria to need for ATP generation is somewhat controversial (74), the improvement of FAO in CR would suggest any intrinsic defect is amplified by nutrient overload and improved with CR. Reduction in several medium-chain acylcarnitine levels despite higher levels of long-chain acylcarnitines after CR also reflects improvement of incomplete FAO by CR. As with the effect of CR on BCAA catabolism, the effect of CR on FAO is possibly associated with increased mitochondrial protein deacetylation since mitochondrial protein deacetylation was found to be associated with increased mitochondrial capacity of FAO (62) and because CR can increase mitochondrial protein deacetylation via SIRT1 (63) and SIRT3 (64) activation. Although medium-chain acylcarnitine levels decreased after CR, we found higher levels of those metabolites in non-obese participants. This may be due to much higher coming-in of FA substrates into the mitochondria as compared with those of obese subjects at both baseline and after CR, leading to higher FA intermediates despite more complete FAO (29), but the significantly lower plasma C8:0 carnitine-to-C10:0 carnitine ratio in non-obese than either

the obese or CR plasma indicates a better capacity for FAO. Further fluxomic study will be helpful to prove this explanation.

Our primary goal in this study was to ascertain evidence that VO_2max could alter the age-related changes in mitochondrial metabolism. A previous study in human also revealed that people with high cardiorespiratory fitness (classified using metabolic equivalent (METs)) had a delayed development of dyslipidemia (75) and hypertension (76) compared with their lower counterparts and a separate studies found that people with higher VO_2max had slower increase in age-related arterial stiffness (77) after 2 years of follow-up. Our models suggest that higher VO_2max has an effect to delay metabolic aging. Using MANOVA to leverage the correlations of plasma metabolites, we found that each of the 4 groups of identified metabolites varied with age and 2 of 4 groups varied with VO_2max after adjusting for clinical variables and age when assessed over both non-obese and obese groups (Figure 4.2). These groups contained AAs that previously proved associated with insulin resistance (BCAA and aromatic AAs (Group II)) as well as acylcarnitines (Group IV). This result indicates that age affects metabolism, independent of VO_2max and further, VO_2max has an important effect on metabolite levels, independent of age, BMI, sex, race or diabetes status. The effect of higher VO_2max to modulate age-dependent changes in metabolite levels was most robustly seen in FA-related metabolites levels in which rose with age and were reduced in the face of VO_2max. CR reversed the alterations in metabolites that were observed in obesity, with the exception of medium-chain acylcarnitine levels (Figure 4.5). However, FA-related metabolites were not significantly affected by VO_2max after CR (Figure 4.4), suggesting that the effect of VO_2max on metabolite levels are through a separate pathway from that seen in CR. Indeed, this is consistent with the observation that CR is associated with improvements in insulin sensitivity but is not associated with mitochondrial function (78).

How does higher VO_2max alter age-related changes in metabolite levels and are these changes indicative of the apparent longevity effect of higher VO_2max? These studies were prompted by our findings in the rat genetic model of aerobic treadmill running capacity (79). The HCR rats have a greater capacity for FAO during exercise (62) and have an increase in gene expression (7) and proteomic profiles (62) that are associated with upregulation of genes for FAO and BCAA catabolism. Importantly, the increased longevity

in HCR rats (6) is associated with a delay in the age-dependent changes in gene expression (7). In humans, studies have shown that higher VO_2max is associated with increased ability to oxidize FAs (80) and an increased expression of genes associated with OXPHOS and FAO is found in human skeletal muscle of individuals with higher VO_2max (81). One possibility is that changes in fuel selection towards FAs is associated with reduced generation of ROS and it has been suggested that this occurs through an increased proportion of electron flux through complex II, bypassing ROS production associated complex I (82).

4.5 Summary

In summary, we present the first evidence that in people, a higher VO_2max is associated with delayed metabolic aging and this effect may be separate from the effect of CR to alter aging. The primary effect is seems to be on mitochondrial capacity for BCAA catabolism and FAO. CR also improves metabolism, and seems to improve metabolic age. Thus, high VO_2max and CR can delay metabolic aging, consistent with a 'younger metabolic state', with its attendant decrease in risk for metabolic disease and age-adjusted mortality.

Table 4.1 Participant characteristics and non-metabolite parameters. Data were reported as average (minimum-maximum). $ = p-value <0.050; NA = not applicable

Character-istics	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
Number of subjects	28	124		NA	NA	NA
Male/female	14/14	56/68		NA	NA	NA
Race (n)						
Caucasian	26	116		NA	NA	NA
African American	1	7		NA	NA	NA
Hawaiian	0	1		NA	NA	NA
Asian	1	0		NA	NA	NA
Age (years)						
Male	42 (24-60)	51 (25-65)		0.002$	0.002$	NA
Female	44 (32-59)	49 (28-67)		0.046$	0.046$	NA
All	43 (24-60)	50 (25-67)		<0.001$	<0.001$	NA
BMI (kg/m^2)						
Male	22.9 (19.2-27.0)	39.4 (30.5-54.9)	31.9 (26.6-47.6)	<0.001$	<0.001$	<0.001$
Female	22.1 (19.7-24.4)	40.0 (30.8-57.2)	31.1 (23.2-50.3)	<0.001$	<0.001$	<0.001$
All	22.5 (19.2-27.0)	39.7 (30.5-57.2)	32.6 (23.2-50.3)	<0.001$	<0.001$	<0.001$
% weight loss after CR						
Male	NA	17.1 (4.1-30.7)		NA	NA	NA
Female	NA	17.8 (4.1-35.7)		NA	NA	NA
All	NA	18.6 (5.6-35.7)		NA	NA	NA
Numbers of type 2 DM						
Male	0	21		NA	NA	NA
Female	0	26		NA	NA	NA
All	0	47		NA	NA	NA
Subjects who received one or more anti-diabetic drugs						
Male	0	17	18	NA	NA	NA
Female	0	15	20	NA	NA	NA
All	0	32	38	NA	NA	NA
Lean mass (kg)						
Male	54.1 (43.2-63.3)	69.6 (52.2-97.2)	65.5 (49.7-86.3)	<0.001$	<0.001$	<0.001$
Female	40.1 (30.9-48.1)	50.9 (39.8-70.5)	47.2 (35.5-62.2)	<0.001$	<0.001$	<0.001$
All	47.1 (30.9-63.3)	59.3 (39.8-97.2)	55.5 (35.5-86.3)	<0.001$	<0.001$	<0.001$
Lean+bone (fat-free) mass (kg)						
Male	57.1 (46.6-67.6)	73.2 (55.4-101.1)	69.2 (52.7-90.1)	<0.001$	<0.001$	<0.001$
Female	42.5 (33.1-50.8)	53.8 (42.0-72.8)	50.2 (38.9-65.7)	<0.001$	<0.001$	<0.001$

All	49.8 (33.1-67.6)	62.6 (42.0-101.1)	58.8 (38.9-90.1)	<0.001$	<0.001$	<0.001$
Fat mass (kg)						
Male	13.7 (5.4-23.6)	49.2 (25.6-73.6)	33.4 (18.5-58.8)	<0.001$	<0.001$	<0.001$
Female	17.0 (8.1-25.6)	53.9 (34.5-92.8)	40.3 (20.8-73.2)	<0.001$	<0.001$	<0.001$
All	15.4 (5.4-25.6)	51.8 (25.6-92.8)	37.2 (18.5-73.2)	<0.001$	<0.001$	<0.001$
% Total body fat						
Male	18.9 (9.8-26.9)	39.9 (25.0-50.7)	32.2 (21.2-47.1)	<0.001$	<0.001$	<0.001$
Female	28.4 (14.6-39.6)	49.7 (41.3-57.7)	43.9 (30.5-56.8)	<0.001$	<0.001$	<0.001$
All	23.6 (9.8-39.6)	45.3 (25-57.7)	38.6 (21.2-56.8)	<0.001$	<0.001$	<0.001$
Fasting plasma glucose (mg/dl)						
Male	91 (75-106)	114 (73-233)	103 (69-195)	0.002$	0.022$	0.002$
Female	90 (78-99)	109 (87-176)	97 (56-131)	<0.001$	0.046$	<0.001$
All	90 (75-106)	111 (73-233)	100 (56-195)	<0.001$	0.003$	<0.001$
Fasting plasma insulin (mIU/l)						
Male	9.7 (6.1-13.3)	27.4 (11.0-79.8)	15.0 (7.1-27.5)	<0.001$	<0.001$	<0.001$
Female	9.9 (5.6-14.5)	23.7 (9.2-145.3)	14.3 (6.3-32.2)	0.004$	0.006$	<0.001$
All	9.8 (5.6-14.5)	25.3 (9.2-145.3)	14.6 (6.3-32.2)	<0.001$	<0.001$	<0.001$
HOMA-IR ((fasting plasma glucose x fasting plasma insulin) /405)						
Male	2.2 (1.2-2.9)	8.1 (2.1-33.4)	3.9 (1.7-9.2)	<0.001$	<0.001$	<0.001$
Female	2.2 (1.0-3.4)	6.4 (2.3-36.6)	3.4 (1.3-9.4)	<0.001$	0.005$	<0.001$
All	2.2 (1.0-3.4)	7.2 (2.1-36.6)	3.6 (1.3-9.4)	<0.001$	<0.001$	<0.001$
Resting energy expenditure (REE) (kCal)						
Male	1623 (1212-2101)	2231 (1501-3455)	1946 (1123-2473)	<0.001$	<0.001$	<0.001$
Female	1345 (967-1866)	1784 (1304-2521)	1595 (916-2227)	<0.001$	0.001$	<0.001$
All	1479 (967-2101)	1986 (1304-3455)	1752 (916-2473)	<0.001$	<0.001$	<0.001$
REE per fat-free mass (kCal/kg)						
Male	28.5 (21.9-33.2)	30.4 (21.0-45.5)	28.2 (20.9-37.1)	0.097	0.769	<0.001$
Female	31.8 (22.5-40.8)	33.2 (27.2-39.1)	31.8 (20.5-41.5)	0.162	0.977	0.006
All	30.2 (21.9-40.8)	32.0 (21.0-45.5)	30.1 (20.5-41.5)	0.033$	0.992	<0.001$
Respiratory quotient at rest (RQR)						

Male	0.81 (0.72-0.90)	0.79 (0.67-0.93)	0.77 (0.67-1.06)	0.380	0.070	0.052
Female	0.80 (0.67-0.91)	0.79 (0.66-0.94)	0.78 (0.68-0.97)	0.528	0.109	0.060
All	0.81 (0.67-0.91)	0.79 (0.66-0.94)	0.77 (0.67-1.06)	0.285	0.014[$]	0.006[$]
Respiratory quotient at VO_2max (RQE)						
Male	1.19 (1.04-1.36)	1.16 (0.98-1.40)	1.18 (0.91-1.37)	0.435	0.944	0.152
Female	1.20 (1.03-1.40)	1.14 (0.84-1.35)	1.13 (0.73-1.41)	0.065	0.045[$]	0.352
All	1.19 (1.03-1.40)	1.15 (0.84-1.40)	1.15 (0.73-1.41)	0.051	0.110	0.927
VO_2max (L/min)						
Male	3.09 (1.90-4.30)	3.08 (1.59-5.09)	3.16 (1.62-4.86)	0.965	0.762	0.267
Female	2.17 (1.57-2.90)	2.12 (1.05-3.00)	2.16 (1.19-3.64)	0.698	0.937	0.565
All	2.63 (1.57-4.30)	2.55 (1.05-5.09)	2.61 (1.19-4.86)	0.622	0.929	0.210
VO_2max per fat-free mass (ml/(kg x min))						
Male	53.95 (38.62-68.64)	42.04 (26.44-68.07)	45.59 (26.24-73.73)	<0.001[$]	0.003[$]	<0.001[$]
Female	51.14 (42.19-68.28)	39.59 (14.42-54.62)	42.94 (26.77-73.02)	<0.001[$]	<0.001[$]	<0.001[$]
All	52.55 (38.62-68.64)	40.70 (14.42-68.07)	44.16 (26.77-73.73)	<0.001[$]	<0.001[$]	<0.001[$]

Table 4.2 Concentration of metabolites. Data were reported as average±SEM. $ = p-value <0.050

AAs	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
Alanine (μM)						
Male	322.28±20.86	383.85±10.35	344.26±10.51	0.010$	0.352	<0.001$
Female	322.31±13.40	371.69±9.19	336.47±9.76	0.022$	0.529	<0.001$
All	322.30±12.17	377.18±6.87	339.99±7.13	<0.001$	0.273	<0.001$
Arginine (μM)						
Male	85.82±3.05	81.51±2.63	77.73±2.20	0.436	0.088	0.089
Female	90.93±4.81	79.54±1.94	75.51±2.27	0.020$	0.006$	0.170
All	88.38±2.84	80.43±1.59	76.51±1.59	0.030$	0.001$	0.020$
Asparagine (μM)						
Male	26.29±1.46	22.20±0.63	23.45±0.72	0.007$	0.084	0.051
Female	29.46±1.56	20.16±0.47	21.37±0.58	<0.001$	<0.001$	0.025$
All	27.87±1.09	21.08±0.39	22.31±0.46	<0.001$	<0.001$	0.003$
Aspartic acid (μM)						
Male	3.05±0.24	4.44±0.15	3.69±0.17	<0.001$	0.075	<0.001$
Female	3.38±0.41	4.76±0.24	4.16±0.18	0.015$	0.083	0.015$
All	3.22±0.23	4.61±0.15	3.95±0.13	<0.001$	0.012$	<0.001$
Glutamic acid (μM)						
Male	29.35±3.81	80.63±6.44	59.16±6.51	0.001$	0.027$	0.001$
Female	22.62±3.61	65.06±6.28	45.21±3.24	0.003$	0.003$	<0.001$
All	25.99±2.65	72.09±4.55	51.51±3.48	<0.001$	<0.001$	<0.001$
Glutamine (μM)						
Male	429.16±13.73	387.94±8.67	410.01±6.46	0.031$	0.194	0.002$
Female	402.99±13.21	363.33±7.34	384.67±7.41	0.024$	0.296	0.002$
All	416.07±9.68	374.44±5.70	396.11±5.11	0.001$	0.090	<0.001$
Glutamine-to-glutamic acid ratio						
Male	18.27±2.58	5.92±0.34	10.27±1.04	<0.001$	0.002$	<0.001$
Female	23.54±3.36	8.26±0.81	11.95±1.03	<0.001$	<0.001$	<0.001$
All	20.90±2.14	7.20±0.48	11.19±0.74	<0.001$	<0.001$	<0.001$
Glycine (μM)						
Male	328.22±21.25	263.56±10.41	325.52±11.78	0.007$	0.917	<0.001$
Female	322.24±17.94	311.73±27.60	378.03±36.47	0.865	0.494	0.011$
All	325.23±13.66	289.98±15.94	354.32±20.76	0.305	0.512	<0.001$
Histidine (μM)						
Male	83.28±2.90	81.15±1.51	79.24±1.70	0.525	0.278	0.201
Female	89.11±3.53	76.73±1.26	73.75±1.25	<0.001$	<0.001$	0.017$
All	86.20±2.31	78.73±0.99	76.23±1.05	0.002$	<0.001$	0.009$
Isoleucine+ leucine (μM)						
Male	202.98±6.46	230.62±4.74	209.96±4.62	0.008$	0.480	<0.001$
Female	155.92±4.86	183.60±4.38	171.22±3.82	0.019$	0.199	0.005$
All	181.46±5.74	204.82±3.84	188.70±3.42	0.007$	0.347	<0.001$
Lysine (μM)						
Male	183.61±5.56	216.28±3.82	206.76±4.52	<0.001$	0.017$	0.025$
Female	188.60±6.96	211.23±3.71	202.43±4.37	0.012$	0.177	0.023$

All	186.10±4.40	213.51±2.67	204.39±3.14	<0.001[$]	0.009[$]	0.001[$]
Methionine (μM)						
Male	23.71±1.11	23.46±0.47	21.71±0.54	0.816	0.106	0.002[$]
Female	21.64±0.65	20.43±0.33	18.92±0.44	0.126	0.008[$]	0.001[$]
All	22.68±0.66	21.80±0.31	20.18±0.36	0.224	0.003[$]	<0.001[$]
Phenylalanine (μM)						
Male	57.13±1.54	66.43±1.20	59.34±1.64	<0.001[$]	0.516	<0.001[$]
Female	52.35±1.26	60.15±1.04	54.64±1.06	0.001[$]	0.346	<0.001[$]
All	54.74±1.08	62.99±0.83	56.76±0.96	<0.001[$]	0.335	<0.001[$]
Proline (μM)						
Male	189.36±15.89	196.36±6.78	194.39±6.48	0.657	0.741	0.765
Female	145.22±8.55	178.28±5.18	167.05±5.04	0.008[$]	0.068	0.039[$]
All	167.26±9.82	186.45±4.24	179.39±4.19	0.059	0.227	0.092
Serine (μM)						
Male	103.98±3.95	92.23±1.72	107.36±2.53	0.004[$]	0.537	<0.001[$]
Female	109.37±6.89	89.67±2.15	102.88±2.56	<0.001[$]	0.316	<0.001[$]
All	106.68±3.93	90.83±1.41	104.91±1.81	<0.001[$]	0.677	<0.001[$]
Threonine (μM)						
Male	123.76±5.67	121.40±2.95	125.70±3.22	0.720	0.783	0.192
Female	142.08±8.37	122.08±3.20	123.55±3.40	0.012[$]	0.030[$]	0.685
All	132.92±5.26	121.77±2.19	124.52±2.36	0.035[$]	0.133	0.266
Tryptophan (μM)						
Male	42.10±2.03	40.06±1.03	37.23±1.07	0.377	0.043[$]	0.013[$]
Female	35.17±1.80	35.37±0.69	33.44±0.71	0.736	0.329	0.003[$]
All	38.63±1.49	37.70±0.63	35.15±0.64	0.531	0.023[$]	<0.001[$]
Tyrosine (μM)						
Male	58.25±2.64	71.90±1.70	60.21±1.49	<0.001[$]	0.552	<0.001[$]
Female	52.74±2.53	68.71±1.53	58.10±1.80	<0.001[$]	0.200	<0.001[$]
All	55.50±1.87	70.15±1.14	59.05±1.19	<0.001[$]	0.185	<0.001[$]
Valine (μM)						
Male	257.37±9.32	286.30±5.37	270.99±5.18	0.016[$]	0.235	0.015[$]
Female	212.30±8.37	249.59±4.54	233.96±5.03	<0.001[$]	0.068	0.005[$]
All	234.84±7.52	266.17±3.83	250.68±3.97	<0.001[$]	0.083	<0.001[$]
BCKAs	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
Ketoisoleucine+ketoleucine (μM)						
Male	57.84±3.42	60.86±1.80	54.82±1.62	0.450	0.414	<0.001[$]
Female	48.30±1.80	46.08±1.42	43.18±1.00	0.497	0.032[$]	0.026[$]
All	53.06±2.10	52.76±1.30	48.44±1.04	0.916	0.058	<0.001[$]
Ketovaline (μM)						
Male	13.16±0.67	14.76±0.35	13.83±0.29	0.044[$]	0.322	0.007[$]
Female	12.68±0.39	13.25±0.28	12.36±0.24	0.387	0.565	0.002[$]
All	12.92±0.39	13.93±0.23	13.03±0.20	0.054	0.819	<0.001[$]
FFAs	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese	p-value of non-obese	p-value of obese at baseline vs. obese

				vs. obese at baseline	vs. obese after CR	after CR
Oleic acid (μM)						
Male	238.12±29.98	263.63±9.33	273.75±15.60	0.288	0.307	0.499
Female	302.92±33.35	302.85±9.72	298.67±11.60	0.998	0.886	0.722
All	270.52±22.87	285.14±6.99	287.42±9.52	0.425	0.459	0.806
Palmitic acid (μM)						
Male	152.78±19.44	193.76±7.28	192.86±11.87	0.022$	0.128	0.930
Female	221.73±32.99	231.52±9.70	209.79±9.77	0.708	0.649	0.051
All	187.26±19.93	214.47±6.46	202.15±7.59	0.102	0.423	0.107
Acylcarnitines	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
L-carnitine (μM)						
Male	44.54±3.01	50.69±1.39	47.39±1.38	0.056	0.368	0.018$
Female	41.81±2.49	44.73±1.10	40.88±1.05	0.280	0.718	<0.001$
All	43.18±1.94	47.42±0.91	43.82±0.89	0.047$	0.759	<0.001$
C2 carnitine (μM)						
Male	11.83 ±1.22	12.66±0.48	12.39±0.51	0.472	0.634	0.593
Female	12.77±1.16	11.67±0.39	11.68±0.45	0.279	0.333	0.987
All	12.30±0.83	12.12±0.71	12.00±0.34	0.804	0.709	0.727
C3 carnitine (nM)						
Male	457.03±44.21	578.30±20.06	478.70±19.28	0.010$	0.627	<0.001$
Female	363.23±33.05	503.54±16.14	409.39±15.65	<0.001$	0.220	<0.001$
All	410.13±28.54	537.30±13.05	440.69±12.57	<0.001$	0.305	<0.001$
C4 carnitine (nM)						
Male	276.21±44.40	275.93±15.03	264.04±14.29	0.994	0.736	0.233
Female	238.48±30.33	235.99±11.96	225.03±11.11	0.933	0.633	0.194
All	257.35±26.63	254.03±9.57	242.65±9.01	0.889	0.519	0.076
C5 carnitine (nM)						
Male	143.07±12.26	173.70±5.49	140.34±5.19	0.018$	0.822	<0.001$
Female	106.98±8.16	138.08±4.62	116.65±4.14	0.005$	0.330	<0.001$
All	125.03±8.02	154.16±3.88	127.35±3.42	0.001$	0.776	<0.001$
C5-DC carnitine (nM)						
Male	55.41±2.73	58.47±1.79	56.12±1.76	0.429	0.852	0.142
Female	43.89±2.18	46.99±1.39	44.97±1.41	0.338	0.743	0.071
All	49.65±2.04	52.17±1.22	50.01±1.21	0.360	0.898	0.021$
C6 carnitine (nM)						
Male	55.33±6.18	68.82±2.89	62.74±3.14	0.043$	0.293	0.018$
Female	67.35±9.70	66.39±3.22	61.53±3.46	0.779	0.414	0.038$
All	62.03±5.79	67.49±2.19	62.08±2.36	0.310	0.994	0.002$
C8:0 carnitine (nM)						
Male	186.64±24.17	184.00±9.91	180.45±12.39	0.910	0.823	0.720
Female	269.34±53.29	188.07±14.09	195.58±18.12	0.041$	0.117	0.499
All	227.99±29.79	186.23±8.90	188.75±11.38	0.077	0.160	0.738
C10:0 carnitine (nM)						

Male	440.34±58.46	382.54±21.83	390.33±29.07	0.274	0.445	0.751
Female	620.96±115.87	398.45±30.75	425.17±39.43	0.011[$]	0.057	0.277
All	530.64±66.01	391.27±19.47	409.44±25.25	0.008[$]	0.051	0.295
C8:0 carnitine-to-C10:0 carnitine ratio ($x10^{-1}$)						
Male	4.29±0.08	4.87±0.06	4.72±0.05	<0.001[$]	<0.001[$]	0.008[$]
Female	4.37±0.11	4.79±0.05	4.66±0.05	0.001[$]	0.027[$]	0.012[$]
All	4.33±0.07	4.83±0.04	4.69±0.04	<0.001[$]	<0.001[$]	<0.001[$]
C8:1 carnitine (nM)						
Male	207.35±26.72	312.45±16.20	239.51±13.57	0.004[$]	0.291	<0.001[$]
Female	217.20±23.85	286.67±13.75	248.03±13.22	0.034[$]	0.325	0.007[$]
All	212.28±17.60	298.31±10.52	244.18±9.46	<0.001[$]	0.142	<0.001[$]
C10:1 carnitine (nM)						
Male	241.86±23.32	256.93±12.21	244.57±13.34	0.579	0.926	0.204
Female	286.74±42.83	234.38±12.66	231.55±13.16	0.126	0.116	0.749
All	264.30±24.31	244.56±8.89	237.43±9.38	0.370	0.242	0.273
C12:0 carnitine (nM)						
Male	81.54±10.65	62.63±3.38	57.58±3.55	0.031[$]	0.009[$]	0.138
Female	83.28±11.43	61.04±3.56	55.81±4.03	0.020[$]	0.009[$]	0.076
All	82.41±7.67	61.76±2.47	56.61±2.72	0.001[$]	<0.001[$]	0.020[$]
C12:1 carnitine (nM)						
Male	85.49±10.27	79.71±4.79	73.88±4.58	0.597	0.273	0.163
Female	89.82±10.95	78.47±4.34	71.19±4.04	0.295	0.071	0.025[$]
All	87.66±7.38	79.03±3.20	72.41±3.02	0.258	0.037[$]	0.010[$]
C12-OH carnitine (nM)						
Male	10.82±1.58	9.32±0.64	8.91±0.55	0.321	0.161	0.414
Female	9.00±1.28	8.20±0.42	7.60±0.37	0.461	0.164	0.063
All	9.91±1.01	8.70±0.37	8.19±0.33	0.190	0.041[$]	0.072
C14:0 carnitine (nM)						
Male	29.57±3.04	29.10±1.16	25.30±1.17	0.866	0.129	0.003[$]
Female	30.11±3.03	28.12±1.08	24.26±1.09	0.473	0.038[$]	<0.001[$]
All	29.83±2.11	28.56±0.79	24.73±0.80	0.513	0.010[$]	<0.001[$]
C14:1 carnitine (nM)						
Male	99.03±13.86	86.73 ±4.94	85.86±5.64	0.310	0.322	0.872
Female	105.20±13.55	85.66±5.31	80.81±5.34	0.143	0.069	0.268
All	102.11±9.53	86.14±3.65	83.09±3.87	0.075	0.043[$]	0.370
C14:2 carnitine (nM)						
Male	59.22±6.94	48.39±2.68	48.62±3.14	0.094	0.144	0.931
Female	56.84±7.11	45.24±2.79	43.17±2.90	0.098	0.059	0.320
All	58.03±4.88	46.66±1.94	45.63±2.13	0.017[$]	0.015[$]	0.532
C14-OH carnitine (nM)						
Male	6.04±0.77	6.39±0.35	6.08±0.30	0.660	0.953	0.334
Female	5.05±0.63	5.59±0.25	5.33±0.24	0.375	0.630	0.216
All	5.54±0.50	5.95±0.21	5.67±0.19	0.414	0.782	0.125

C16:0 carnitine (nM)						
Male	98.08±6.25	108.50±2.79	105.54±3.47	0.107	0.331	0.359
Female	93.57±5.28	99.59±2.19	97.19±2.33	0.268	0.525	0.280
All	95.83±4.04	103.61±1.78	100.96±2.05	0.066	0.278	0.160
C16:1 carnitine (nM)						
Male	35.61±4.22	38.84±1.74	37.36±1.84	0.428	0.680	0.446
Female	41.75±4.39	40.60±1.71	38.84±1.67	0.787	0.488	0.239
All	38.68±3.04	39.81±1.22	38.17±1.23	0.703	0.865	0.171
C16-OH carnitine (nM)						
Male	4.66±0.43	5.07±0.24	5.21±0.26	0.432	0.333	0.657
Female	4.52±0.47	4.78±0.22	4.54±0.20	0.625	0.970	0.183
All	4.59±0.32	4.91±0.16	4.84±0.16	0.385	0.504	0.693
C18:0 carnitine (nM)						
Male	45.12±3.93	37.57±1.19	39.95±1.56	0.017$	0.165	0.071
Female	37.18±2.16	33.83±1.05	34.05±1.02	0.187	0.205	0.785
All	41.15±2.33	35.52±0.80	36.71±0.93	0.006$	0.051	0.107
C18:1 carnitine (nM)						
Male	136.75±11.97	146.16±4.39	156.28±6.30	0.378	0.166	0.097
Female	137.10±10.65	133.75±4.01	143.55±4.50	0.740	0.559	0.010$
All	136.92±7.86	139.36±3.00	149.30±3.79	0.740	0.162	0.004$
C18:2 carnitine (nM)						
Male	52.56±4.26	51.49±1.69	54.83±2.11	0.790	0.633	0.078
Female	46.84±3.66	43.73±1.44	45.96±1.63	0.387	0.824	0.058
All	49.70±2.81	47.23±1.15	49.96±1.36	0.373	0.934	0.010$
C18:2-OH carnitine(nM)						
Male	4.11±0.56	4.44±0.22	4.47±0.28	0.522	0.569	0.919
Female	4.34±0.44	4.45±0.22	4.23±0.22	0.845	0.830	0.310
All	4.23±0.35	4.44±0.15	4.34±0.17	0.553	0.776	0.552
C20:0 carnitine (nM)						
Male	4.02±0.41	2.63±0.09	2.92±0.12	<0.001$	<0.001$	0.009$
Female	2.73±0.20	2.28±0.08	2.38±0.08	0.020$	0.087	0.127
All	3.38±0.26	2.43±0.06	2.62±0.08	<0.001$	<0.001$	0.003$
C20:1 carnitine (nM)						
Male	9.66±0.62	8.23±0.24	12.30±0.69	0.016$	0.067	<0.001$
Female	8.91±0.54	7.46±0.27	9.92±0.41	0.030$	0.284	<0.001$
All	9.28±0.41	7.81±0.19	11.00±0.40	0.001$	0.048$	<0.001$
C20:2 carnitine (nM)						
Male	3.85±0.24	3.70±0.13	5.34±0.28	0.608	0.011$	<0.001$
Female	3.48±0.25	3.22±0.11	4.25±0.15	0.341	0.036$	<0.001$
All	3.66±0.17	3.44±0.09	4.74±0.16	0.260	0.002$	<0.001$
C20:3 carnitine (nM)						
Male	2.15±0.20	2.37±0.09	2.66±0.12	0.282	0.049$	0.006$
Female	1.84±0.14	2.06±0.07	2.15±0.07	0.169	0.078	0.154

All	1.99±0.12	2.20±0.06	2.38±0.07	0.116	0.016$	0.002$
C20:4 carnitine (nM)						
Male	2.10±0.23	2.39±0.10	2.63±0.12	0.221	0.059	0.012$
Female	1.68±0.13	1.87±0.08	2.06±0.09	0.305	0.073	0.001$
All	1.89±0.14	2.10±0.07	2.32±0.08	0.169	0.162	0.002$
Long-chain acylcarnitine-to-FFA ratios	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
C16:0 carnitine-to-palmitic acid ratio (x10^{-4})						
Male	7.69±0.88	6.01±0.30	6.36±0.35	0.027$	0.114	0.399
Female	5.22±0.69	4.70±0.18	5.22±0.24	0.309	0.996	0.024$
All	6.45±0.60	5.29±0.18	5.74±0.21	0.014$	0.177	0.047$
C18:1 carnitine-to-oleic acid ratio (x10^{-4})						
Male	6.45±0.58	5.83±0.25	6.32±0.31	0.281	0.853	0.167
Female	4.97±0.52	4.58±0.14	5.18±0.22	0.331	0.702	0.005$
All	5.71±0.41	5.15±0.15	5.70±0.19	0.125	0.975	0.005$
C3 and C5 carnitine-to-substrate ratios	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
C3 carnitine-to-valine ratio (x10^{-3})						
Male	1.78±0.16	2.03±0.07	1.77±0.07	0.102	0.988	<0.001$
Female	1.71±0.14	2.03±0.06	1.75±0.06	0.035$	0.764	<0.001$
All	1.74±0.10	2.03±0.05	1.76±0.04	0.007$	0.854	<0.001$
C3 carnitine-to-ketovaline ratio (x10^{-2})						
Male	3.65±0.44	3.99±0.15	3.52±0.15	0.348	0.733	0.004$
Female	2.85±0.23	3.87±0.13	3.32±0.12	0.001$	0.095	<0.001$
All	3.25±0.26	3.93±0.10	3.41±0.09	0.005$	0.486	<0.001$
C5 carnitine-to-isoleucine+leucine ratio (x10^{-4})						
Male	6.99±0.53	7.69±0.30	6.77±0.24	0.286	0.691	0.001$
Female	6.65±0.44	7.63±0.24	6.85±0.22	0.087	0.697	0.006$
All	6.81±0.34	7.66±0.19	6.81±0.16	0.051	0.991	<0.001$
C5 carnitine-to-ketoisoleucine+ketoleucine ratio (x10^{-3})						

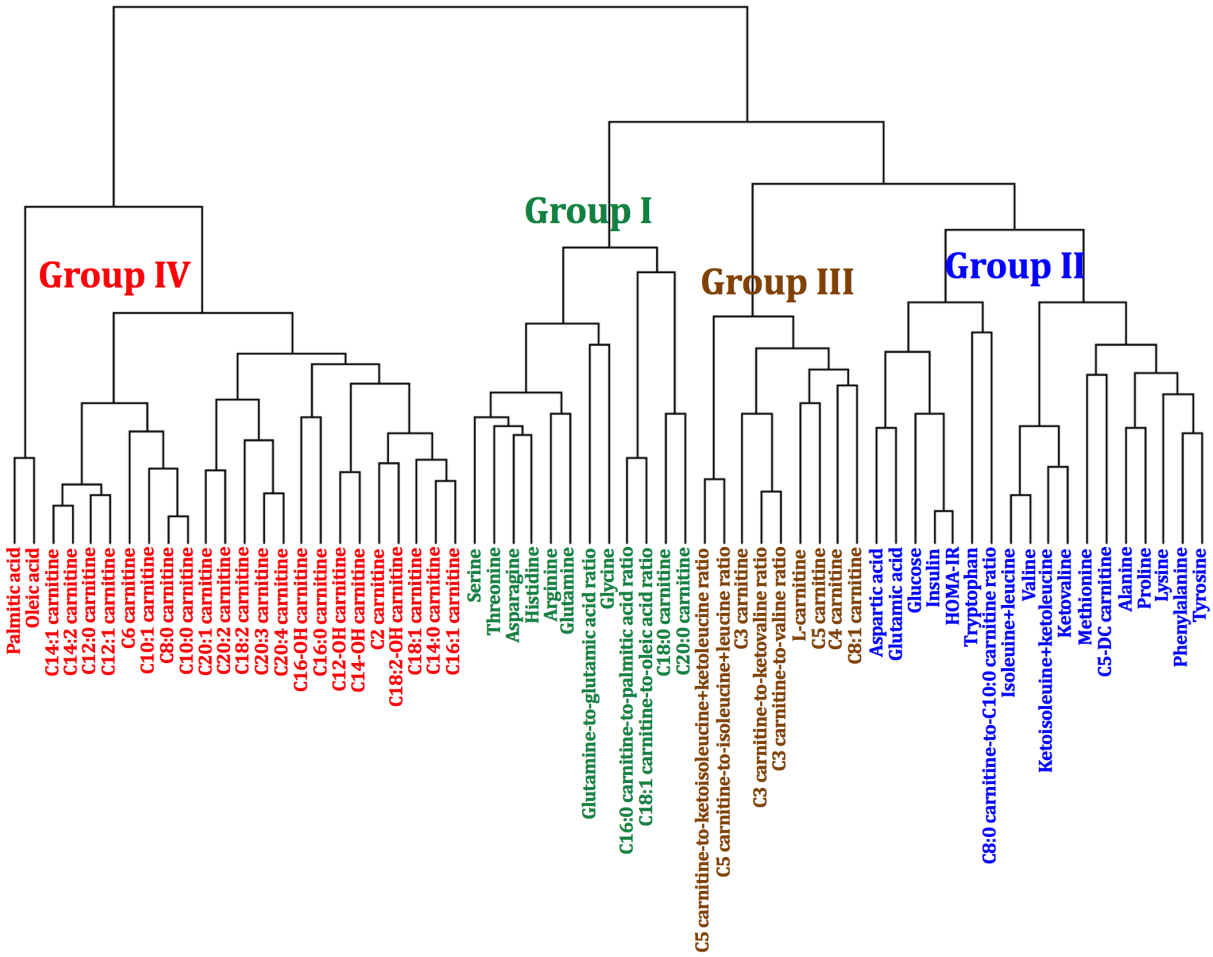
Male	2.58±0.26	2.98±0.13	2.65±0.11	0.167	0.787	0.009[$]
Female	2.23±0.16	3.09±0.11	2.74±0.09	<0.001[$]	0.022[$]	0.006[$]
All	2.40±0.15	3.04±0.08	2.70±0.07	<0.001[$]	0.079	<0.001[$]

Table 4.3 Groups of metabolites and insulin resistance profiles according to their correlations with each other

Groups	Lists of metabolites and insulin resistance profiles
I	• Arginine, asparagine, glutamine, glutamine-to-glutamic acid ratio, glycine, histidine, serine, threonine • C18:0 carnitine, C20:0 carnitine • C16:0 carnitine-to-palmitic acid ratio, C18:1 carnitine-to-oleic acid ratio
II	• Glucose, insulin, HOMA-IR • Alanine, aspartic acid, glutamic acid, isoleucine+leucine, lysine, methionine, proline, phenylalanine, tryptophan, tyrosine, valine • Ketoisoleucine+ketoleucine, ketovaline • C5-DC carnitine • C8:0-to-C10:0 carnitine ratio
III	• L-carnitine, C3 carnitine, C4 carnitine, C5 carnitine, C8:1 carnitine • C3 carnitine-to-valine ratio, C3 carnitine-to-ketovaline ratio, C5 carnitine-to-isoleucine+leucine ratio, C5 carnitine-to-ketoisoleucine+ketoleucine ratio
IV	• Palmitic acid, oleic acid • C2 carnitine, C6 carnitine, C8:0 carnitine, C10:0 carnitine, C10:1 carnitine, C12:0 carnitine, C12:1 carnitine, C12-OH carnitine, C14:0 carnitine, C14:1 carnitine, C14:2 carnitine, C14-OH carnitine, C16:0 carnitine, C16:1 carnitine, C16-OH carnitine, C18:1 carnitine, C18:2 carnitine, C18:2-OH carnitine, C20:1 carnitine, C20:2 carnitine, C20:3 carnitine, C20:4 carnitine

Figure 4.1 Cluster dendrogram (a) and heatmap (b) of group of metabolites and insulin resistance profiles

a



b

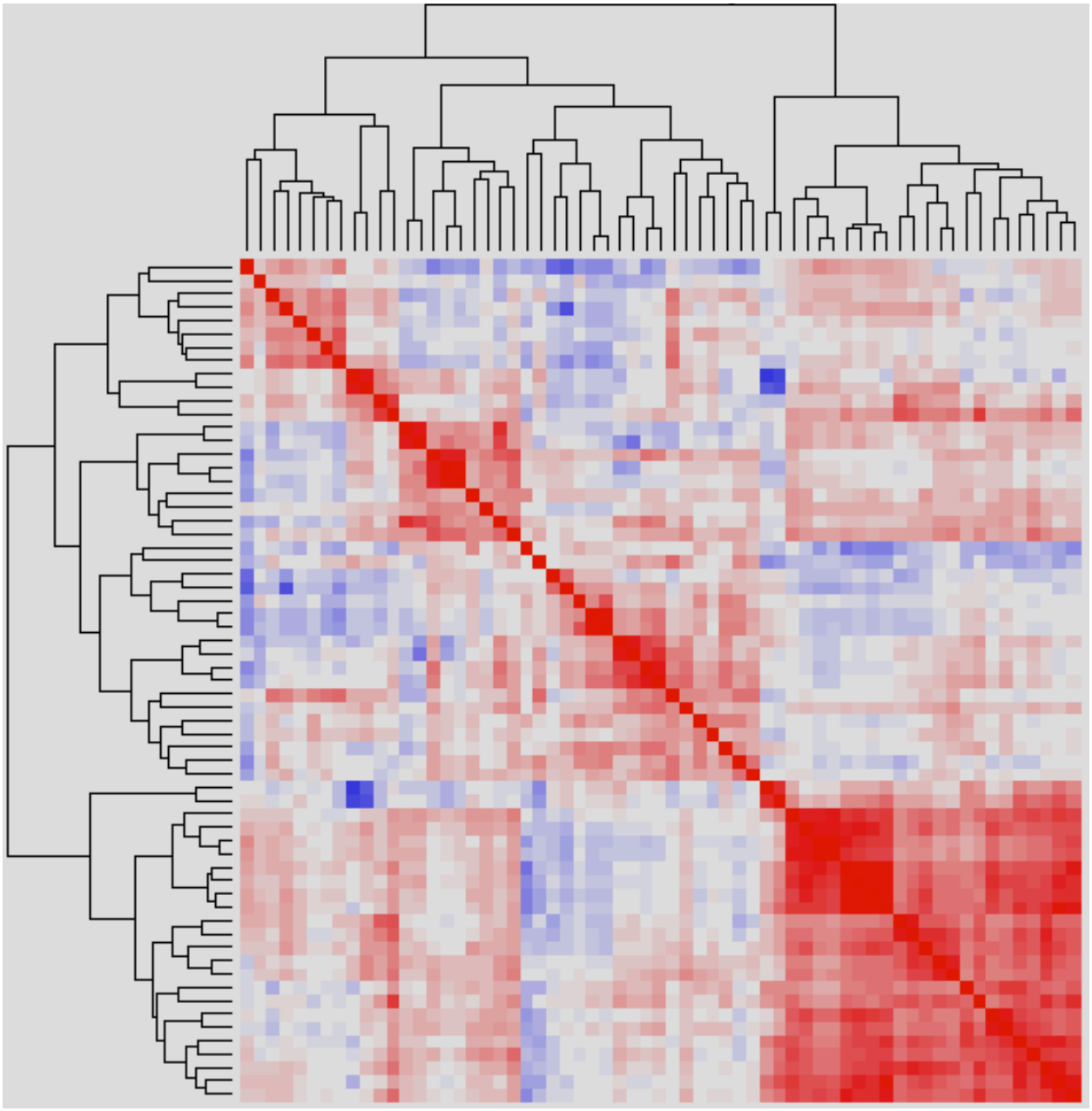
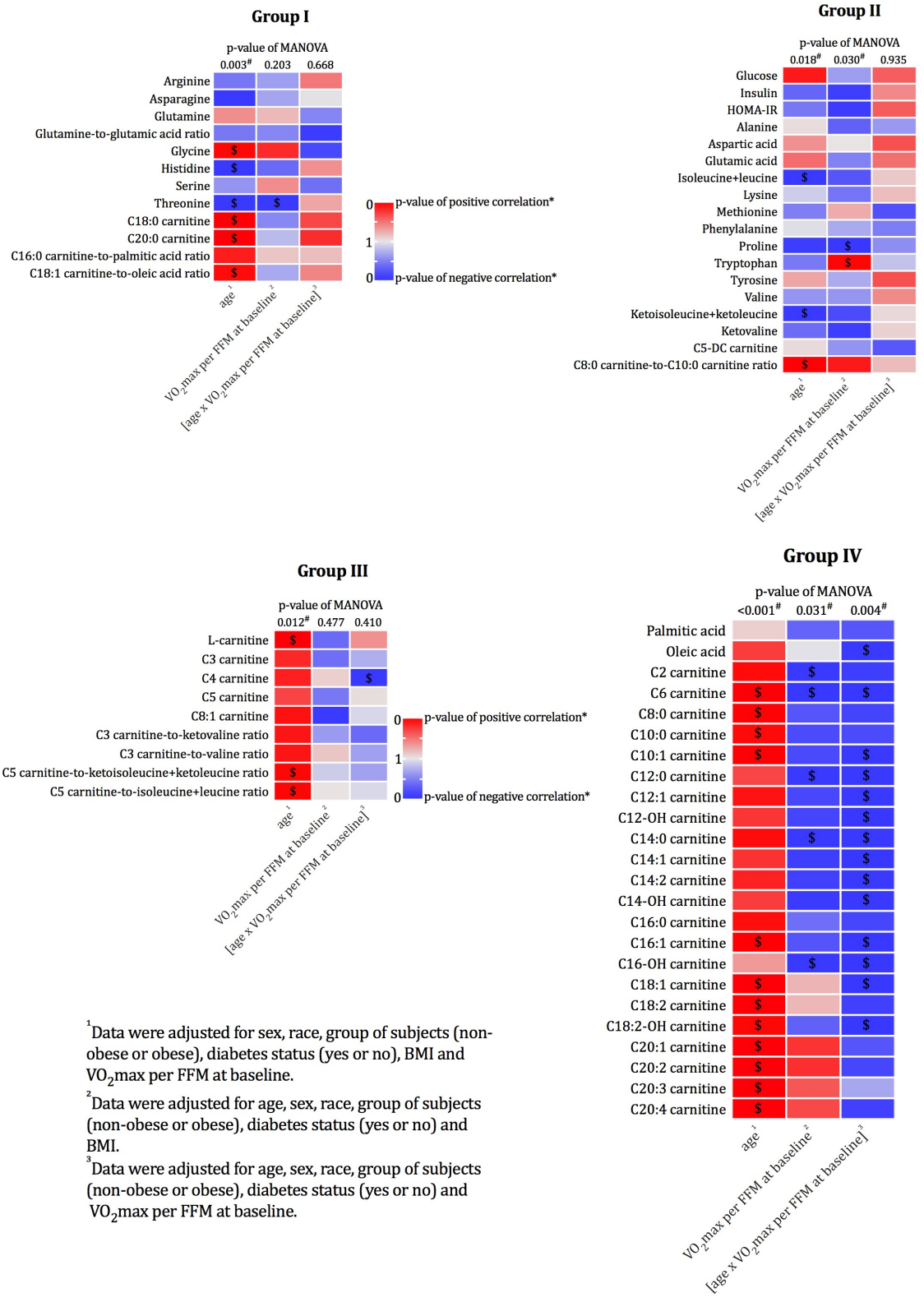


Figure 4.2 Correlations of metabolites and insulin resistance profiles at baseline (non-obese and obese subjects) vs. age, VO_2 max per FFM at baseline and [age x VO_2max per FFM at baseline]. * = p-value of ANOVA; $ = p-value of ANOVA <0.050; # = p-value of MANOVA <0.050.

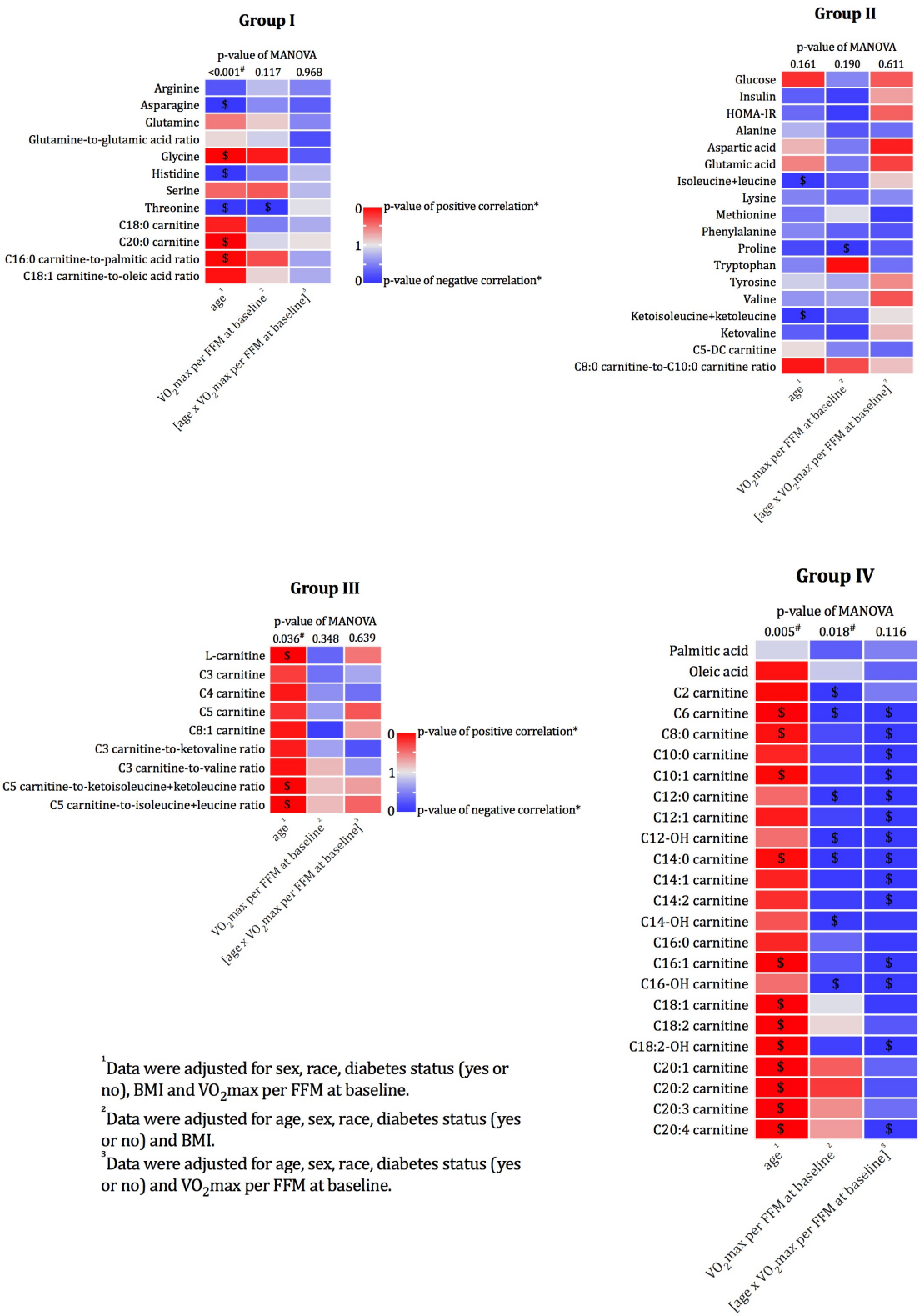


[1]Data were adjusted for sex, race, group of subjects (non-obese or obese), diabetes status (yes or no), BMI and VO_2max per FFM at baseline.

[2]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), diabetes status (yes or no) and BMI.

[3]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), diabetes status (yes or no) and VO_2max per FFM at baseline.

Figure 4.3 Correlations of metabolites and insulin resistance profiles in obese subjects at baseline vs. age, VO_2 max per FFM at baseline and [age x VO_2max per FFM at baseline]. * = p-value of ANOVA; $ = p-value of ANOVA <0.050; # = p-value of MANOVA <0.050.

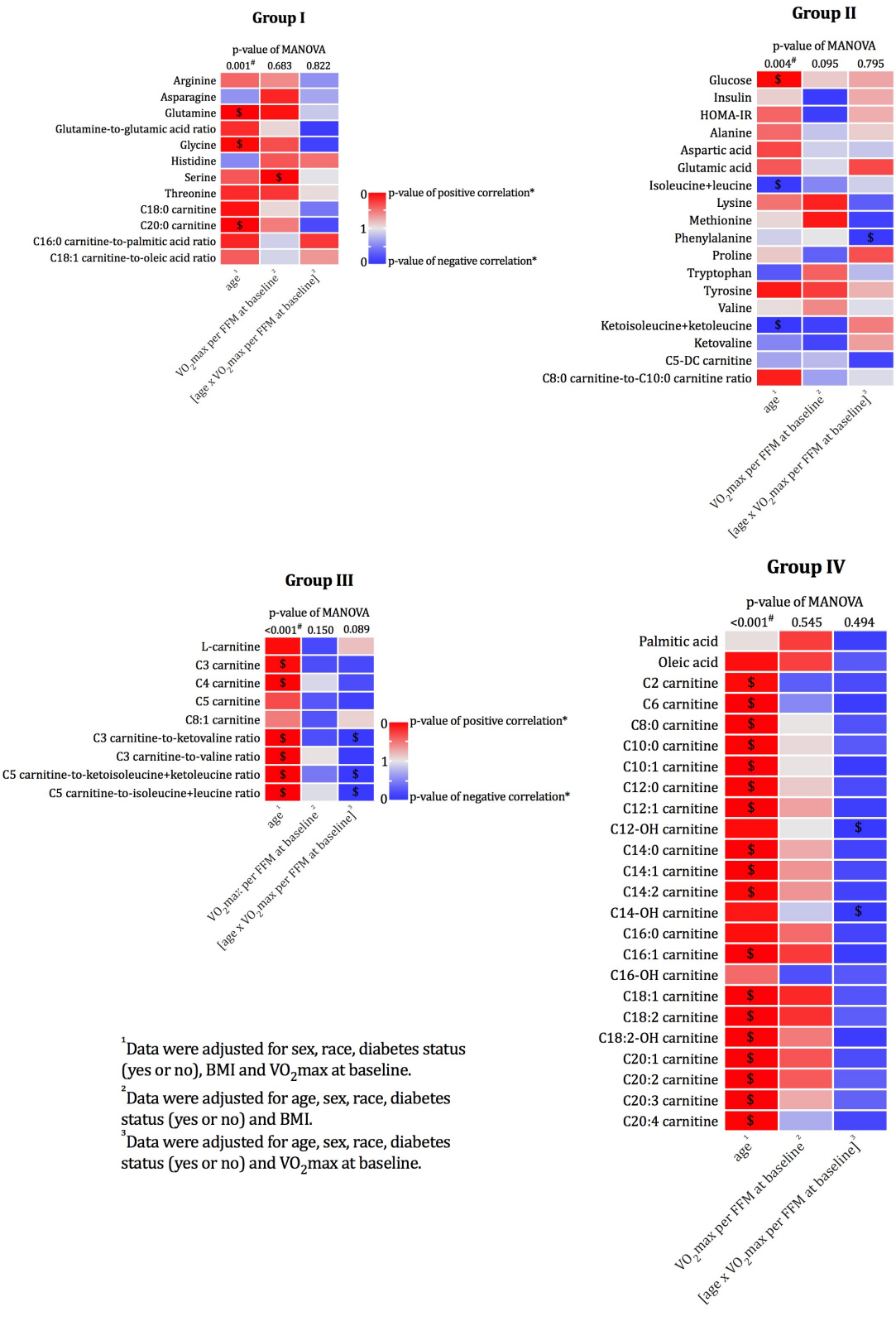


[1]Data were adjusted for sex, race, diabetes status (yes or no), BMI and VO_2max per FFM at baseline.

[2]Data were adjusted for age, sex, race, diabetes status (yes or no) and BMI.

[3]Data were adjusted for age, sex, race, diabetes status (yes or no) and VO_2max per FFM at baseline.

Figure 4.4 Correlations of metabolites and insulin resistance profiles in obese subjects after CR vs. age, VO_2 max per FFM at baseline and [age x VO_2max per FFM at baseline]. * = p-value of ANOVA; $ = p-value of ANOVA <0.050; # = p-value of MANOVA <0.050.

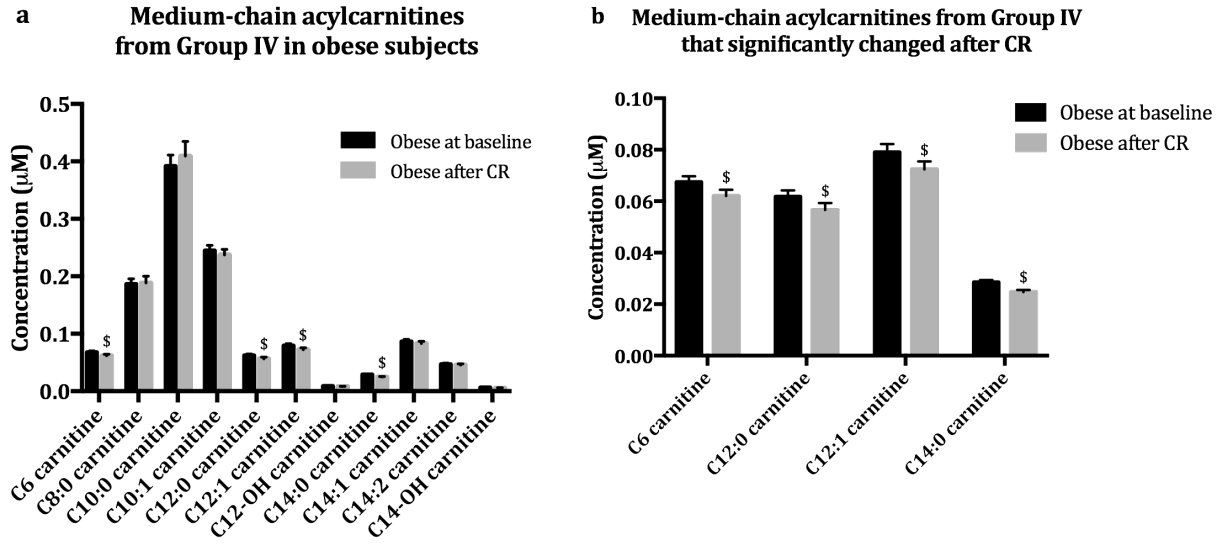


[1]Data were adjusted for sex, race, diabetes status (yes or no), BMI and VO_2max at baseline.

[2]Data were adjusted for age, sex, race, diabetes status (yes or no) and BMI.

[3]Data were adjusted for age, sex, race, diabetes status (yes or no) and VO_2max at baseline.

Figure 4.5 Medium-chain acylcarnitines from Group IV in obese subjects. Data were reported as average±SEM. $ = p-value <0.050



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Chapter 5
Effects of intrinsic cardiorespiratory fitness and caloric restriction on mitochondrial DNA and metabolites in human skeletal muscle

5.1 Introduction

The studies presented in chapter 4 demonstrate that 1) metabolism as reflected in the metabolome, changes with age, 2) high intrinsic cardiorespiratory fitness (VO_2max) was directly associated with more complete fatty acid oxidation (FAO) and delayed age-related incomplete FAO, and 3) caloric restriction (CR) was associated with more complete FAO, i.e. delayed metabolic aging. In brief, the effects of high VO_2max and CR are consistent with a younger metabolic state. To understand how metabolism decreases with age and how high VO_2max and CR delay metabolic aging, in-depth study of skeletal muscle, a major organ that influences VO_2max and is responsible for nutrient metabolism, is necessary. Here we studied mitochondrial DNA (mtDNA) deletion ratio because mtDNA can be damaged (deleted) by aging-induced oxidative stress (1-4). We also studied mtDNA count number, which is an indicator of mitochondrial biogenesis (5) and declines with age (6-9).

Previous work in humans has found that mtDNA deletion increases with age, especially at the major arc region, possibly due to aging-induced oxidative stress (10-17). A study in human plasma demonstrated that higher VO_2max was associated with lower markers of oxidative stress (MDA and 8-iso-PGF2α) as well as markers of oxidative DNA damage (8-OHdG) (18). In the rat genetic model of aerobic treadmill running capacity, rats selected for increased VO_2max (HCR rats) exhibited lower 4-HNE (19) and 8dOHG (20), markers of oxidative stress and oxidative DNA damage, respectively, in skeletal muscle. CR prevented aging-induced oxidative damage to mouse skeletal muscle mitochondria as well

(21). In addition, livers of CR rats displayed lower 8-oxo-dG, a marker of oxidative DNA damage, than those of the control rats (22).

Research in human skeletal muscle (23), rat skeletal muscle (24) and rat liver (24) have demonstrated that mtDNA count number, an indicator of mitochondrial biogenesis (5), decreases with age. A positive correlation between mtDNA count number and VO_2max was observed in human skeletal muscle (23). MtDNA count number was higher in skeletal muscle of HCR rats as well (25). Life-long CR reversed the aging-induced decrease in mtDNA count number in liver and skeletal muscle of rats and increased mtDNA count number in rat brain (24).

In this study, we tested the hypothesis that our observations in chapter 4 that high VO_2max and CR delay metabolic aging by slowing aging-induced mtDNA deletion and aging-induced decreases mitochondrial biogenesis, which potentially leads to good metabolic health and longevity. In addition to metabolomic analyses, we evaluated mtDNA deletion ratio and mtDNA count number in skeletal muscle of obese participants both before and after CR. Non-obese subjects served as controls.

5.2 Materials and methods

Enrollment criteria, CR protocol, body composition assessment, exercise testing, mixed meal tolerance test (MMTT) as well as plasma glucose and plasma insulin analyses techniques were described in chapter 4.

5.2.1 Skeletal muscle biopsy and metabolite extraction in skeletal muscle

Vastus lateralis muscle was obtained via suction-modified Bergström muscle biopsy technique at 120-min of MMTT, flash frozen in liquid nitrogen and stored at -80 °C. Nine to 14 mg of frozen skeletal muscle tissues were extracted by adding 270 to 420 µl (approximately 30 µl per 1 mg of skeletal muscle tissue) of extraction solvent (7:2:1 methanol:water:chloroform) containing an internal standard mixture (see Table A5.1 of appendices). The samples were sonicated for 30 seconds, allowed to rest on ice for 5 min, and then centrifuged at 4 °C for 10 min. The supernatant was transferred to an autosampler vial for analysis via mass spectrometry. The tissue pellet was dried at 45 °C for data normalization and mtDNA analysis.

5.2.2 Mass spectrometry (MS) protocols for metabolomics

A targeted metabolomic approach was used to study metabolites listed in Table A5.2 of appendices. All targeted metabolites were analyzed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 LC connected to an Agilent 6410 triple quadrupole MS. MS parameters were as follows: capillary voltage 4000 V, gas temperature 325 °C, gas flow 10 l/min, and nebulizer pressure 40 psi. For analysis of amino acids (AAs), oleic acid, glycolysis metabolites, TCA cycle metabolites and nucleotides, hydrophilic interaction chromatography was performed using a Phenomenex Luna NH2 column, 3 μm particle size, 150 mm x 1 mm inner diameter (i.d.) (Torrance, CA). The flow rate was 0.07 ml/min at 0 to 21 min and 0.09 ml/min at 21 to 27 min. The gradient consisted of a 10-min linear ramp from 80 to 0% B, 4 min at 0% B, and 13 min of re-equilibration at 80% B. Detection was performed using multiple reaction monitoring (MRM) in negative ion mode described in Table A5.3 of appendices. Mobile phase A was 5 mM ammonium acetate in water with pH 9.9 and mobile phase B was acetonitrile. For acylcarnitine analysis, reversed-phase liquid chromatography (RPLC) was performed using a Waters Acquity HSS T3 column, 1.8 μm particle size, 2.1 x 100 mm i.d. (Milford, MA), with a flow rate of 0.25 ml/min. The gradient consisted of a 7-min linear ramp from 0 to 80% B, 3 min at 100% B, and 5 min of re-equilibration at 0% B. MRM in positive ion mode was used with precursor/product ion transitions specified elsewhere (26). Mobile phase A was 0.1% of formic acid in water and mobile phase B was 0.1% of formic acid in acetonitrile.

All untargeted metabolites were analyzed by LC-MS using an Agilent 1260 infinity LC connected to an Agilent 6520 quadrupole time-of-flight MS. MS parameters were as follows: full-scan negative ion mode (m/z 50 to 1,200), acquisition rate 1 spectrum/sec, capillary voltage 3500 V, gas temperature 350 °C, drying gas 10 l/min, nebulizer pressure 20 psig, and reference mass correction enabled. RPLC was performed using a Waters Acquity HSS T3 column, 1.8 μm particle size, 2.1 x 100 mm i.d. (Milford, MA), with a flow rate of 0.25 ml/min. The gradient consisted of a 7-min linear ramp from 0 to 99% B, 3 min at 99% B, and 5 min of re-equilibration at 0% B. Mobile phase A was 0.1% of formic acid in water and mobile phase B was 0.1% of formic acid in 8:2 of isopropanol:acetonitrile.

5.2.3 Targeted metabolite quantification

Peak areas of targeted metabolites were obtained using Agilent Masshunter Quantitative Analysis software for triple quadrupole MS version B.07.00. Peaks were identified by comparison of accurate mass and retention time with those of authentic standards analyzed using the same method. Peaks were quantified by peak area using the 'Agile2' peak integrator. Concentrations of metabolites other than acylcarnitines with exact-matching internal standards were then quantified by calibration curves using peak area ratios to their internal standards. Concentrations of metabolites other than acylcarnitines without exact-matching internal standards were quantified by method of external calibration (see appendices) after instrument drift correction of their peak areas using the quadratic drift-correction (method described in chapter 6). Metabolite concentrations used for calibration curves are listed in Table A5.4 of appendices. Concentrations of acylcarnitine species presented in the internal standard mix (NSK-B) were quantified using peak area of unlabeled compound-to-peak area of internal standard multiplied by concentration of internal standard. Concentrations of other acylcarnitine species were quantified using peak area of biological compound-to-peak area of internal standard with the closest matching retention time multiplied by concentration of the internal standard. The concentrations of all targeted metabolites as μM (pmol/μl) were then multiplied by the volume (µl) of extraction solvent added to the sample and divided by the dried skeletal muscle tissue mass. The finalized data were reported as pmol/mg.

5.2.4 Untargeted metabolite selection, quantification and annotation

Untargeted features were initially identified and their peak areas were initially quantified using Profinder version B.08.00 (Agilent Technologies, Santa Clara, CA). To select candidate features for exact quantification, Pearson's correlation was then used to identify those features that correlated with age and metabolites, as well as between VO_2max per FFM. Features that were correlated with either age or VO_2max per FFM at baseline with p-value $\leq$0.10 were re-quantified using Agilent Masshunter Quantitative Analysis software for quadrupole time-of-flight MS version B.07.00. Peaks were re-quantified by peak area using the 'Agile2' or 'spectrum summation' peak integrator. Duplicated features and noise spectra were visually identified and discarded during re-

quantitation. Peak areas of finally selected untargeted metabolites were corrected for instrument drift using LOESS drift-correction method described elsewhere (27). Then, the corrected peak area was multiplied by the volume of extraction solvent added to the sample and divided by the dried skeletal muscle tissue mass, the result of which was termed the 'normalized peak area'. Metabolite annotation was completed using Human Metabolome Database (www.hmdb.ca) and LIPID MAPS Lipidomics Gateway (www.lipidmaps.org).

5.2.5 mtDNA deletion ratio and mtDNA count number analyses

MtDNA deletion ratio and mtDNA count number were assessed in the dried skeletal muscle tissue pellet. Total DNA was extracted using a DNeasy Blood and Tissue Kit purchased from Qiagen (Valencia, CA). Using real-time qPCR (Thermo Fisher Scientific Applied Biosystems® 7500), the cycling time of mtDNA at the major arc (mtDNA major arc; targeted DNA), mtDNA at the minor arc (mtDNA minor arc; controlled DNA) and nuclear DNA at the β2M gene (nDNA; controlled DNA) were measured in duplicate in each sample. The mixture for PCR analysis of each specific DNA consisted of 2 µl of extracted total DNA, 10 µl of Thermo Fisher Scientific SYBR Green Real-Time PCR Master Mixes, 6 µl of water, and 2 µl of 2 mM (1 mM of forward sequence and 1 mM of reverse sequence) of specific DNA primers (see Table A5.5 of appendices). The cycling time from each batch (42 samples) was then normalized by the median cycling time of a human pooled sample (generated by combining aliquots of total DNA extracted from human skeletal muscle samples), which was analyzed in duplicate in each batch. Finally, the amount of each specific DNA was calculated using the formula $1/(2^{\text{average cycling time}})$. MtDNA deletion ratio was calculated as 'the amount of mtDNA minor arc-the amount of mtDNA major arc/the amount of mtDNA minor arc' (5) and mtDNA count number was calculated as 'the amount of mtDNA major arc/the amount of nDNA' (5).

5.2.6 Statistical analyses

Comparison between the non-obese and the obese groups were completed using unpaired-two-tailed student's t tests. Paired-two-tailed student's t tests were utilized to compare baseline to post-CR within obese subjects. All metabolomics data are reported as

average ±standard error of mean (SEM). Correlations between independent variables (age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]) vs. dependent variables (metabolite levels and mtDNA profiles) were evaluated by ANOVA using RStudio version 1.0.136 (©2009-2016 RStudio, Inc.). All figures were created using Prism 7 Version 7.0a (©1994-2016 GraphPad Software, Inc.). P-value <0.050 was considered statistically significant.

5.3 Results

5.3.1 Participant characteristics

Participant characteristics are detailed in Tables 5.1 and 5.2. Ninety-nine obese (40 males and 59 females) and 15 non-obese (7 males and 8 females) individuals participated in this study. Most of them were Caucasian (92.9% of obese and 93.3% of non-obese subjects). Age was not significantly different between groups. Obese individuals were heavier (39.5 ± 0.5 vs. 23.1 ± 0.5 kg/m^2). Among the 99 obese subjects, 35 of them had type 2 diabetes and 28 were prescribed 1 or more anti-diabetic drugs prior to enrollment. Forty-one of 99 obese individuals completed muscle biopsies after CR. Among these 41 participants, body weight decreased by 17.2 ± 1.3% with CR (range 4.3-34.4%) and 1 person stopped anti-diabetic drugs.

5.3.2 Non-metabolite parameters

Non-metabolite parameters are listed in Table 5.1 (all participants at baseline and 41 obese participants after CR) and Table 5.2 (41 obese participants at baseline vs. 41 obese participants after CR). Lean mass, fat mass and total body adiposity (% fat) was higher in obese vs. non-obese. Resting energy expenditure (REE), fasting plasma glucose and insulin, as well as HOMA-IR were also higher in obese participants. All of these parameters changed significantly with CR and became closer in value to those of non-obese subjects. After correcting REE by lean+bone (fat-free) mass (FFM), REE did not differ between obese subjects at baseline and non-obese and remained decreased after CR. Absolute VO_2max (l/min) did not differ between groups. After correcting for FFM, however, VO_2max was lower in the obese group. When compared to baseline, VO_2max per

FFM was higher after CR. Baseline and post-CR VO_2max per FFM were significantly positively correlated (r = 0.727, p-value <0.001). Respiratory quotient at rest (RQR) and respiratory quotient at VO_2max (RQE) were not significantly different between groups.

5.3.3 Targeted metabolites and their differences between groups

Targeted metabolites in this study included AAs, branched-chain amino acid (BCAA) intermediates (C3 and C5 carnitines), oleic acid, fatty acid (FA) intermediates (acylcarnitines), glycolysis pathway metabolites, TCA cycle metabolites and nucleotides. Concentrations of metabolites are listed in Table 5.3 (all participants at baseline and 41 obese participants after CR) and Table 5.4 (41 obese participants at baseline vs. 41 obese participants after CR).

Lysine was higher in the obese group, and decreased after CR in females. Valine was higher in obese males and non-significantly trended (p-value = 0.060) higher in all obese vs. non-obese participants. C3 carnitine, an degradation product valine, was higher in obese males as well. Isoleucine+leucine was significantly decreased in obese female after CR. Asparagine, glutamine, glycine, histidine, and serine were significantly lower in the obese group, and CR increased their levels. Glutamine-to-glutamic acid ratio was lower in obese males and non-significantly trended (p-value = 0.070) lower in all obese vs. non-obese participants. Methionine was lower in obese vs. non-obese females.

To evaluate mitochondrial capacity of BCAA catabolism, we inferred a flux parameter by calculating the ratio of C3 and C5 carnitines to their substrates, which are valine and isoleucine+leucine, respectively. A higher ratio implies lower mitochondrial capacity for BCAA catabolism reflected in the greater accumulation of intermediate metabolites. The C3 carnitine-to-valine ratio was significantly higher in obese males compared to non-obese males; CR decreased C5 carnitine-to-isoleucine+leucine ratio in both sexes.

Oleic acid level did not differ between groups. C20:0 carnitine was significantly lower in obese subjects. CR significantly increased C16-OH carnitine level. C18:0 carnitine, C20:0 carnitine, C20:2 carnitine and C20:4 carnitines non-significantly trended (p-value = 0.068-0.082) higher after CR. To evaluate mitochondrial capacity of in-coming oleic acid, we inferred a flux by calculating the ratio of C18:1 carnitine, which is converted from oleic

acid during its carnitine shuttle, to the levels of oleic acid. A higher ratio represents represents an increased uptake of oleic acid into the mitochondria. We found the C18:1 carnitine-to-oleic acid ratio was significantly lower in obese subjects and was not changed by CR.

Several medium- (C6-C14) and short -chain (C2 and C4) acylcarnitines were lower in obese participants, including C2 carnitine, C4 carnitine, C6 carnitine, C8:0 carnitine, C10:0 carnitine and C10:1 carnitine. After CR, C12-OH carnitine and C14-OH carnitine were significantly higher whereas other medium and short even-chain acylcarnitines remained unchanged.

Glycolysis metabolites did not differ between groups. To evaluate glycolysis capacity, we inferred a flux by calculating the ratio of fructose 1,6-bisphosphate (FBP) to glucose-6-phosphate+fructose-6-phosphate (G6P+F6P), which is a rate-limiting step of glycolysis via phosphofructokinase (PFK) enzyme (28). This inferred flux did not differ between groups.

Among TCA cycle metabolites, citrate was lower in OB subjects. CR increased citrate level such that it no longer differed from NOB participants.

AMP, ADP and NAD^+ were significantly lower in the obese group, and CR increased AMP level. ATP non-significantly trended (p-value = 0.070) lower in obese subjects. NADH was significant lower in obese males. NAD^+-to-NADH ratio decreased significantly after CR. FAD was significantly lower in the obese group and was increased in obese males after CR.

5.3.4 Untargeted metabolites and their differences between groups

Out of 4,150 features identified in Profinder, 1,700 of them were present in more than 70% of all quality control (QC) samples and were selected for inclusion in Pearson's correlation. Of these 1,700 features, 410 (~24%) were correlated with either age or VO_2max per FFM at baseline ($p \leq 0.10$) and were then re-quantified using Agilent Masshunter Quantitative Analysis software. Duplicate features and noise spectra were discarded during re-quantification, and 212 remaining features were included in final analysis (Table A5.6 of appendices). To test reliability of re-quantification, we calculated Pearson's correlation of 6 metabolites that were detected in both targeted and untargeted

metabolomics. Significantly positive correlations were found in all 6 metabolites (r = 0.716-0.975, p-value <0.001), suggesting that re-quantification was reliable.

Of these 212 features, 60 differed between obese and non-obese participants (Table 5.5). Features that were significantly lower in obese subjects include glycyl-phenylalanine, N-(4-aminobutyryl)-l-histidine, 3-hydroxypentadecanoic acid, ketooleic acid, lysoPA(16:0), lysoPA(18:0), lysoPA(18:2), PA(36:3), PA(O-42:6), lysoPC(20:3), lysoPC(22:2), lysoPE(20:0), lysoPE(20:1), lysoPE(20:4), lysoPE(P-16:0), PE(36:3), PE(36:4), PE(38:4)OH, PE(P-36:4), PE(P-38:5), PE(O-44:6), PG(34:1), PG(P-36:5), PS(O-36:3), PI(38:4), PI-ceramide(d28:2), ceramide(d40:1), ceramide(t42:0), lactosyl sphingosine(d16:1), fumarate, nicotinate beta-d-ribonucleotide, 2-keto-6-acetamidocaproate, 3-hydroxydodecanedioic acid, 5,6-dihydroxyindole-2-carboxylic acid, calcitroic acid, neuromedin N, phosphocreatine, reduced glutathione, vanillin 4-sulfate and 17 unknown features. Features that were significantly higher in the obese group included carnosine, tryptophyl-valine and 2 unknown metabolites.

Of these 212 features, 34 were significantly changed after CR (Table 5.6). Features that were significantly lower after CR included tryptophyl-valine, arachidonic acid, PA(36:1)OH, PE(36:5), PE(P-34:2), PE(P-36:2), PE(P-36:3), 1,25-dihydroxyvitamin D_3 3-glycoside, glycocholic acid and 6 unknown metabolites. Features that were significantly higher after CR included alanyl-glutamine, ketopalmitic acid, PA(O-42:6), PC(28:0), PC(36:4), lysoPE(18:0), lysoPE(20:1), PE(P-38:5), PG(34:1), PG(P-36:5), lactosyl sphingosine(d16:2), neuromedin N, pantothenic acid and 6 unknown metabolites.

5.3.5 mtDNA profiles and their differences between groups

MtDNA deletion ratio and mtDNA count number did not differ between groups (Table 5.3) and did not change with CR (Table 5.4).

5.3.6 Skeletal muscle metabolite levels and mtDNA profiles change with age.

Among the NOB and OB groups, targeted metabolite levels were analyzed by ANOVA, adjusted for race, sex, diabetes status (yes or no), group (NOB or OB), HOMA-IR, BMI and baseline VO_2max adjusted for FFM. We found that age was negatively correlated with the concentration of several AAs (Figure 5.1a and Table A5.7 of appendices).

Correlations were similar when only the 41 obese subjects who received muscle biopsies both before and after CR were evaluated (Figure 5.1b,c and Table A5.8, A5.9 of appendices).

When the 212 untargeted metabolites at baseline were included, ANOVA adjusted for the factors above, we found that 1 unknown metabolite was positively correlated with age, and 11 metabolites were inversely correlated with age, including lysoPE(22:4), PE(38:2)OH, PE(P-36:2), PG(36:1), PG(P-36:5), lysoPS(18:1), lysoPS(20:4), PS(38:3), dehydroepisterone sulfate (DHEAS) and xanthine, as well as an unknown metabolite (Figure 5.2a and Table A5.10 of appendices). When only the 41 obese subjects who received muscle biopsies both before and after CR were evaluated, 3 metabolites at baseline were positively correlated with age, which were lysoPA(16:0), PE(38:4) and PE(O-44:6), and 6 metabolites at baseline were negatively correlated with age, including tryptophyl-valine, PA(36:3), PS(38:3) and UDP-n-acetylglucosamine, as well as 2 unknown metabolites (Figure 5.2b and Table A5.11 of appendices). After CR, 10 metabolites were significantly positively correlated with age, including eicosapentaenoic acid, PE(38:4), PE(P-38:5), PI(38:5), 11(R)-hydroxyeicosatetraenoic acid, allopregnanolone, calcitroic acid and urocanic acid, as well as 2 unknown metabolites, and 22 metabolites were significantly negatively correlated with age, including cardiolipin(72:8), lysoPE(20:0), lysoPE(20:2), lysoPE(P-16:0), PE(38:4)OH, PE(O-34:3), PI-ceramide(d28:2), lysoPS(20:4), PS(40:6), DHEAS, lactosyl sphingosine(d16:1), pregnenolone, and 10 unknown metabolites (Figure 5.2c and Table A5.12 of appendices).

ANOVA showed no significant correlations between age and mtDNA profiles (Figure 5.1a,b,c and Table A5.7, A5.8, A5.9 of appendices). However, when the 31 oldest subjects (55-67 years of age, average 58) were compared to the 31 youngest subjects (26-44 years of age, average 39) at baseline (p-value of age <0.001), the oldest group had significantly lower mtDNA count number (1077$\pm$76 vs. 1379 $\pm$104 counts, p-value = 0.023) and non-significantly trended towards a higher mtDNA deletion ratio (69.58$\pm$2.09 vs. 64.52$\pm$2.71%, p-value = 0.144).

5.3.7 VO_2max affects skeletal muscle metabolite levels and mtDNA count number independently of age.

Because VO_2max is highly genetically determined (as described in chapter 2), is a function of FFM (29, 30) and is not affected by fat mass (30), baseline VO_2max adjusted for FFM (VO_2max per FFM at baseline) was used for the analysis.

Among targeted metabolomics at baseline, ANOVA adjusted for age, race, sex, diabetes status (yes or no), group (non-obese or obese), HOMA-IR and BMI, revealed that VO_2max per FFM at baseline was negatively correlated with most AA and BCAA intermediate (C3 and C5 carnitines) levels as well as the ratios of C3 and C5 carnitines to their substrates (Figure 5.1a and Table A5.7 of appendices). Conversely, VO_2max per FFM at baseline was positively correlated with short even-, medium- and long-chain chain acylcarnitines as well as C18:1 carnitine-to-oleic acid ratio (Figure 5.1a and Table A5.7 of appendices). VO_2max per FFM at baseline was positively correlated with TCA cycle metabolites as well (Figure 5.1a and Table A5.7 of appendices). Correlations were similar when only the 41 obese subjects who completed muscle biopsies both before and after CR were evaluated (Figure 5.1b,c and Table A5.8, A5.9 of appendices). However, the correlations of VO_2max per FFM at baseline vs. medium- and long-chain acylcarnitines were more significant after CR (Figure 5.1b,c and Table A5.8, A5.9 of appendices).

Among the selected 212 untargeted metabolites, ANOVA adjusted for age, race, sex, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR and BMI revealed that 5 metabolites were significantly positively correlated with VO_2max per FFM at baseline, including PA(O-42:6), PG(34:1), PI(38:4), reduced glutathione and an unknown metabolite, and 11 metabolites were significantly negatively correlated with VO_2max per FFM at baseline, including 3-hydroxypentadecanoic acid, PC(28:0), PE(34:1), PE(36:1), PE(36:2), PE(P-36:2), PE(P-36:3), PE(P-40:6) and lysoPS(18:1), as well as 2 unknown metabolites (Figure 5.2a and Table A5.10 of appendices). When only the 41 obese subjects who completed muscle biopsies both before and after CR were evaluated, 3 metabolites at baseline were significantly positively correlated with VO_2max per FFM at baseline, which were UDP-n-acetylglucosamine, oxidized glutathione and reduced glutathione, and 1 baseline metabolite, beta-citryl-l-glutamic acid, was significantly negatively correlated with VO_2max per FFM (Figure 5.2b and Table A5.11 of appendices). After CR, 4 metabolites were

significantly positively correlated with VO_2max per FFM at baseline, including DHEAS, urocanic acid and 2 unknown metabolites, and 2 metabolites, beta-citryl-l-glutamic acid and PE(P-36:2), were significantly negatively correlated with VO_2max per FFM at baseline, (Figure 5.2c and Table A5.12 of appendices).

ANOVA also revealed a positive correlation between VO_2max per FFM at baseline and mtDNA count number (Figure 5.1a,b and Table A5.7, A5.8 of appendices), but the correlation disappeared after CR (Figure 5.1c and Table A5.9 of appendices). VO_2max per FFM at baseline and mtDNA deletion ratio were not correlated. When the 31 least fit people (VO_2max per FFM at baseline 22.62-36.64 ml/(kg x min), average 32.92 ml/(kg x min)) were compared to the 31 most fit people (VO_2max per FFM at baseline 44.78-77.22 ml/(kg x min), 50.71 ml/(kg x min)) (p-value of VO_2max per FFM at baseline <0.001), there was also no difference in mtDNA deletion ratio at baseline between groups (60.31$\pm$2.69% vs. 64.96$\pm$3.17%, p-value = 0.271).

5.3.8 VO_2max did not modulate age-related changes in skeletal muscle metabolites.

To identify whether VO_2max alters the effect of age on changes in skeletal muscle metabolite levels, we calculated the correlations between [age x VO_2max per FFM at baseline] and metabolite levels using ANOVA adjusted for age, race, sex, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR, BMI and VO_2max per FFM at baseline. If VO_2max alters the effect of age on changes in metabolite levels, metabolites that were significantly correlated with age must be also significantly correlated with [age x VO_2max per FFM at baseline]. However, the expected results were not found, suggesting that VO_2max does not modulate age-related changes in skeletal muscle metabolite levels.

5.3.9 The effect of VO_2max on skeletal muscle metabolite levels is partly mediated by mtDNA count number.

Because VO_2max was positively correlated with mtDNA count number as described in 5.3.7, it was necessary to determine whether the effect of VO_2max on metabolite levels is mediated by the difference in mtDNA count number. Here we used ANOVA adjusted for age, race, sex, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR and BMI to evaluate the relationship between mtDNA count number and metabolite levels. If

mtDNA count number mediates the effect of VO_2max on metabolite levels, the significant correlation between mtDNA and metabolite levels must be in the same direction as the significant correlation between VO_2max per FFM at baseline and metabolite levels as described in 5.3.8. The results suggest that the effect of VO_2max on 9 metabolites is mediated by the difference in mtDNA count number, including asparagine, beta-citryl-l-glutamic acid, 3-hydroxypentadecanoic acid, PC(28:0), PE(36:2), PE(P-40:6), oxidized glutathione and reduced glutathione, as well as an unknown metabolite (Figure 5.3a,b and 5.4a,b).

5.4 Discussion

Our study investigated the effects of obesity and CR on metabolite levels, mtDNA deletion ratio and mtDNA count number in postprandial (120 min) skeletal muscle of obese subjects at baseline and after CR as well as non-obese controls at baseline. In addition, we evaluated the effects of age and VO_2max per FFM at baseline on metabolites and mtDNA profiles and adjusted for multiple covariables via ANOVA. Analysis after CR enabled us to investigate the ability of CR to mitigate aging-induced changes, its relationship to VO_2max and its effect on metabolites and mtDNA profiles. Additionally, we sought to determine if the effect of VO_2max on metabolite levels is mediated by mtDNA. To our knowledge, this is the first study using ANOVA to document the effect of age and VO_2max on metabolite levels in human skeletal muscle.

VO_2max per FFM was lower in the obese group. This is consistent with several previous cross-sectional and longitudinal studies, all of which found that higher VO_2max is associated with lower BMI in both non-obese and obese humans (31-36). CR increased VO_2max per FFM, which was previously shown in humans (37, 38). The increase in VO_2max per FFM after CR could be explained by improvement of pulmonary (39-42) and cardiac (39, 43-45) function owing to weight loss. The high positive correlation between baseline and post-CR VO_2max per FFM VO_2max has a significant genetic determined (46-56).

BCAAs (isoleucine, leucine and valine) are insulin resistance-related AAs (57-64). Therefore, it is not surprising that obese subjects exhibited higher levels of valine, or that isoleucine+leucine decreased significantly after CR in females. Asparagine, glutamine, glycine and serine, markers of insulin sensitivity (57, 61, 65, 66), were lower in obese

subjects and increased with CR, consistent with lower insulin sensitivity in obese subjects and the improvement after CR. Glutamine-to-glutamic acid ratio, another marker of insulin sensitivity (61), was lower in obese participants, consistent with lower insulin sensitivity in this group. Histidine was lower in obese subjects and methionine was lower in obese females, which is in agreement with a previous report in human skeletal muscle (67). In that study, the authors suggested that obese subjects had lower histidine and methionine levels because those AAs are being used to replenish TCA cycle metabolites and maintain oxidative metabolism (67). The C3 carnitine-to-valine ratio was significantly higher in obese males, suggesting that obesity and insulin resistance are associated with decreased mitochondrial capacity for BCAA catabolism. The BCAA are thought to provide anaplerotic intermediates, which could lead to the use of alternative substrates. (68). CR decreased the C5 carnitine-to-isoleucine+leucine ratio, suggesting that CR improves mitochondrial capacity of BCAA catabolism. This effect of CR is probably not only due to improved insulin sensitivity, but also increased mitochondrial protein deacetylation since mitochondrial protein deacetylation was found to be associated with increased mitochondrial capacity of BCAA catabolism (69) and because CR can increase mitochondrial protein deacetylation via SIRT1 (70) and SIRT3 (71) activation.

Long-chain acylcarnitine reflects the influx of FAs into the mitochondria via the carnitine shuttle (72). In this study, C20:0 carnitine was significantly lower in obese subjects and CR increased levels of several long-chain acylcarnitines. Our results suggest that obesity is associated with decreased FA uptake into the mitochondria and that CR attenuates this pathology. This evidence of lower levels is consistent with our findings in plasma (chapter 4). The C18:1 carnitine-to-oleic acid ratio reflects influx of FAs into the mitochondria. The ratio was significantly lower in obese subjects, further supporting the obesity-associated decreased FA uptake into the mitochondria. The decrease in FA uptake into the mitochondria may be explained by the obesity-induced reduction in AMPK (73-75) and CPT-1 activity (76, 77).

Medium (C6-C14) and short even-chain (C2 and C4) acylcarnitine levels reflect FAO in the mitochondria (72). Several medium and short even-chain acylcarnitines were lower in obese participants and CR increased C12-OH carnitine and C14-OH carnitine levels, suggesting that obesity is associated with decreased FAO, which is consistent with previous

studies in human skeletal muscle (77-79). The reduction in FAO is probably a consequence of the obesity-induced decrease in mitochondrial FA uptake. Similar to our findings, increased FAO after CR has been previously observed in rats (80).

Citrate, a TCA cycle metabolite, was lower in obese subjects and increased after CR. This may be due to decreased mitochondrial capacity of BCAA catabolism and decreased FAO in obesity, resulting in diminished acetyl-CoA production and consequently lower production of TCA cycle metabolites, a condition which can be improved by CR. Lower levels of TCA cycle metabolites have been documented in skeletal muscle of high-fat diet (HFD)-induced obese rats (81) and in humans with insulin resistance (68).

The TCA cycle is a major source of ATP production. Therefore, the lower ATP level in obese subjects was consistent with lower citrate levels and could be explained by diminished TCA cycle metabolite production caused by an obesity-induced decrease in mitochondrial capacity for BCAA catabolism and decreased FAO. Oxidative phosphorylation (OXPHOS) also produces ATP. HFD-induced obesity has been associated with lower expression of genes involved in OXPHOS in human skeletal muscle (82), therefore decreased OXPHOS is another potential cause of the lower ATP level in obese participants (83). Lower AMP and ADP levels in obese subjects may be associated with less ATP production. NADH is a product of the TCA cycle, whereas NAD^+ is a product of OXPHOS. Lower NADH and NAD^+ levels in obese subjects may be due to lower production of TCA cycle metabolites and lower OXPHOS, respectively. Increased NAD^+-to-NADH ratio can activate SIRT1 thus increasing mitochondrial protein deacetylation, resulting in decreased susceptibility to metabolic syndrome (70). The NAD^+-to-NADH ratio increased in skeletal muscle of CR mice (84). Surprisingly, the NAD^+-to-NADH ratio decreased after CR in this study. Several enzymes involving FAO and AA catabolism are FAD-dependent, including acetyl-CoA dehydrogenases (FAO), short/branched-chain acyl-CoA dehydrogenase (isoleucine catabolism), isovaleryl-CoA dehydrogenase (leucine catabolism), isobutyryl-CoA dehydrogenase (valine catabolism) and glutaryl-CoA dehydrogenase (lysine catabolism) (85). In addition, FAD is required within the TCA cycle and for succinate dehydrogenase (complex II in the electron transport chain (ETC), which is the site of OXPHOS) to catalyze the oxidation of succinate to fumarate (86). Thus, lower FAD levels in obese subjects may be associated with decreased mitochondrial capacity of

BCAA catabolism, decreased FAO, decreased production of TCA cycle metabolites and lower OXPHOS.

Untargeted metabolomics revealed that glycyl-phenylalanine and N-(4-aminobutyryl)-l-histidine were lower in obese subjects, consistent with lower levels of glycine and histidine, respectively. Conversely, tryptophyl-valine was higher in the obese group, consistent with the higher valine level. Skeletal muscle carnosine is associated with cardiometabolic risk factors in humans, It is positively correlated with total body fat percentage but inversely correlated with insulin sensitivity and HDL cholesterol level (87). . CR decreased tryptophyl-valine, which was consistent with a reduction in tryptophan and valine after CR. The increase in alanyl-glutamine after CR was consistent with the increased glutamine level after CR as well. 3-hydroxypentadecanoic acid, a long-chain hydroxyl FA, and ketooleic acid were significantly lower in the obese group, and CR increased ketopalmitic acid level. These oxidized FAs can arise from microbial metabolism of FA and have biological activity, including inhibition of colinesterases (88). The higher level of FAs in non-obese subjects and in obese individuals after CR may be caused by insulin-induced FAT/CD36 translocation to the plasma membrane to facilitate FA uptake (89). However, arachidonic acid, a polyunsaturated fatty acid (PUFA), was lower after CR. This has also been observed in rat liver (90) and various mouse tissues including skeletal muscle (91), consistent with the CR-induced decrease in mitochondrial membrane lipid unsaturation (90-92), which is associated with decreased susceptibility to age-related lipid peroxidative damage to mitochondria (6, 93, 94), i.e. it delays aging.

Phospholipids, which are major components of cell and mitochondrial membranes (95, 96), were lower in the obese group. Skeletal muscle PC and PE were lower in obese subjects and were positively correlated with insulin sensitivity (97), and the authors believed that these findings were partly PGC-1α dependent (97). Phospholipids also play various important roles in cell and mitochondrial function, including mitochondrial fusion and fission (PA), lipid to protein interactions (PC), OXPHOS (PE), lipid signaling (PI), vesicle trafficking (PI), actin rearrangement (PI), calcium regulation (PI), O_2 transfer mechanism of complex IV of OXPHOS (PG) and cell apoptosis (PS) (95, 98-102). Therefore, lower phospholipid levels in obese subjects may be associated with the obesity-related decrease in cell and mitochondrial function. After CR, changes in the level and saturation

composition of phospholipids varied, consistent with previous research in mouse liver (103). Surprisingly, ceramides were lower in our obese participants, which was inconsistent with previous studies that found higher (104) or similar (105) ceramide level in skeletal muscle of obese vs. non-obese humans. Nicotinate beta-d-ribonucleotide is part of nicotinate and nicotinamide metabolism pathways (106), therefore the lower level found in obese participants was consistent with the lower NAD^+ level previously discussed as well. Calcitroic acid, a metabolite of 1,25-dihydroxyvitamin D_3 (107), was lower in the obese group. The agrees with previous studies that documented lower 1,25-dihydroxyvitamin D_3 level in serum of obese subjects (108, 109), which could be explained by the decrease in 25-hydroxyvitamin D (a precursor of 1,25-dihydroxyvitamin D_3) by multiple possible mechanisms, including lower dietary intake, reduced cutaneous synthesis, reduced intestinal absorption, and sequestration of 25-hydroxyvitamin D in adipose tissue (110). Reduced glutathione is the most abundant endogenous antioxidant that protects mitochondria against oxidative stress (111); its level decreases with aging in humans (112-114). In this study, reduced glutathione was lower in obese participants, consistent with previous analysis of human blood (115-117) and rat adipose tissue (118). However, we did not see a significant relationship with age in our study, after adjusting for multiple clinical factors. Because reduced glutathione deficiency is associated with decreased FAO and insulin resistance (111), our results suggest that reduced glutathione level may play a role in the reduction of FAO. Essentially, obesity mimics aging.

In a longitudinal study in humans (119), several bile acids in plasma were found to be associated with lower odds of longevity. Lower glycocholic acid, a bile acid, after CR in this study is consistent with decreased markers of short lifespan, again, providing some indirect evidence that CR delays metabolic aging. To our knowledge, the increase in pantothenic acid level after CR has not been previously documented. Obese mice receiving pantothenic acid derivatives exhibited lower blood glucose and serum insulin (120), suggesting that the higher level of pantothenic acid after CR observed in this study may be associated with improved insulin sensitivity.

MtDNA count number did not differ between groups, consistent with a previous study in human skeletal muscle (105). However, to identify whether CR can prevent the age-related decrease in mtDNA count number in human skeletal muscle, like previous

research in rat skeletal muscle (24), a study with long-term CR and long-term follow up is needed. This type of study may also demonstrate the attenuating effect of CR on age-related mtDNA deletion, as previous research in mouse skeletal muscle found that CR prevented aging-induced oxidative damage to mitochondria (21).

ANOVA from targeted metabolomics demonstrated that aging was associated with lower AA levels in skeletal muscle. This was not caused by sarcopenia of aging since metabolite levels were adjusted by skeletal muscle tissue mass. Rather, it may be explained by an aging-induced impairment of dietary AA delivery to skeletal muscle (121) caused by several mechanisms, including diminished response to protein ingestion (122-124), as well as impaired skeletal muscle protein anabolic response to insulin and muscle contraction (125-129). However, the impairment of dietary AA delivery to skeletal muscle likely plays a role in the sarcopenia of aging (130-132).

ANOVA of untargeted metabolomics demonstrated that aging was associated with lower tryptophyl-valine levels in skeletal muscle, which agrees with the previously discussed lower AA levels with age. 11(R)-hydroxyeicosatetraenoic acid is a pro-inflammatory substance (133). Similar to this study, a previous research in human plasma found that several pro-inflammatory hydroxyeicosatetraenoic acids increase with age (134), consistent with age-related inflammation. Eicosapentaenoic acid, an omega-3 FA, was higher with age, consistent previous studies of human plasma and erythrocytes (135-144). However, mechanisms responsible for the correlation are still unclear; some studies suggest that this is caused by higher intake of fish and omega-3 FA supplements in older people (143, 144). Our study did not include dietary records and therefore did not document habitual fish intake. Omega-3 FA supplement did not explain the correlation because the age range (40 - 62 years of age, average 49 years) of participants who reported fish oil supplement usage (n = 11) was narrow.

As previously discussed, PA, PC, PE, PG, PI and PS are phospholipids that are major components of cell and mitochondrial membranes; they play various important roles in cell and mitochondrial function. Cardiolipins are also phospholipids and are exclusively located in the inner mitochondrial membrane (145-147). They are involved in several mitochondrial bioenergetic processes and optimize the activity of proteins involved in OXPHOS (148-151). Thus, the age-associated decrease of most phospholipids observed in

our study may be associated with age-related decrease in cell and mitochondrial function. However, we found that several polyunsaturated phospholipids were positivity correlated with age, suggesting that aging is also associated with higher lipid unsaturation of the mitochondrial membrane, which is associated with increased susceptibility to lipid peroxidation, consistent with the age-related increase in lipid peroxidative damage to mitochondria (6, 93, 94). Because phospholipid levels were lower in obese subjects, the effect of obesity on phospholipids is similar to the effect of aging, i.e. obesity mimics aging. UDP-n-acetylglucosamine plays a crucial role in intracellular signaling (152). Thus, the reduction in UDP-n-acetylglucosamine with age documented in this study may link to age-related decreased in cellular function. However, further studies are needed to identify how UDP-n-acetylglucosamine decreases with age. It is widely accepted that the adrenal production of DHEAS and pregnenolone, which are a steroid hormone and steroid hormone precursor, respectively, declines with age (153-158). Therefore, it is not surprising that age and DHEAS as well as age and pregnenolone were negatively correlated in this study.

Xanthine is catalyzed by the enzyme xanthine oxidase (159). Previous research demonstrated that xanthine oxidase expression and activity increased with age in several mouse tissues (160), in skeletal muscle and plasma of rats (161), as well as in human plasma (161), resulting in age-related oxidative stress caused by production of hydrogen peroxide and superoxide anion from xanthine catabolism (162). Thus, the negative correlation between age and xanthine found in our study may be associated with the age-related increase in xanthine catabolism due to increased xanthine oxidase activity.

ANOVA showed no significant correlation between age and mtDNA profiles. However, there were baseline mtDNA deletion ratio and mtDNA count number differed between the 31 oldest and 31 youngest subjects. Previous research in human skeletal muscle (16) reported significantly higher mtDNA deletion in older individuals when compared to their younger counterparts, but the difference between age groups in our study did not reach statistical significance. This may be due to a larger age difference of between groups in the previous study (54-78 years of age, average 69 years vs. 19-40 years, average 29 years) compared with our study (55-67 years of age, average 58 years vs. 26-44 years of age, average 39 years), we just may not have the power to detect small differences across the groups. mtDNA deletion analysis suggests that the pattern of age-

related mtDNA deletion is not linear and may be mediated by other factors. Previous research in human skeletal muscle (23) demonstrated significant negative correlation between age and mtDNA count number, which was not documented in this study. However, that study did not adjust for any other covariables, suggesting that the effect of age on mtDNA count number may be mediated by other factors. Further research is necessary to identify mediators of the effects of age on mtDNA profiles.

ANOVA from targeted metabolomics revealed that high VO_2max was associated with lower AA and BCAA intermediate levels at 120 min after high-protein liquid ingestion (MMTT). Although several AAs are associated with insulin sensitivity status, the negative correlations between VO_2max and AAs are not due to insulin sensitivity status as ANOVAs were adjusted by HOMA-IR. Rather, the negative correlations may be explained by the higher mitochondrial capacity for AA catabolism in people with high VO_2max, which was confirmed by the negative correlations between VO_2max and BCAA intermediate-to-substrate ratios, consistent with previous work in rats (69). Because CR was also associated with increased mitochondrial capacity for BCAA catabolism, this effect of high VO_2max is comparable to the effect of CR. Unlike AAs, high VO_2max was associated with higher long-chain acylcarnitines and C18:1 carnitine-to-oleic acid ratio, suggesting that people with high VO_2 max have higher FA uptake into the mitochondria for FAO. Since CR also increased the levels of long-chain acylcarnitines, the effect of high VO_2max on increased FA uptake into the mitochondria for FAO is comparable to the effect of CR. Positive correlations between VO_2max and short-even chain acylcarnitines as well as medium-chain acylcarnitines suggest that high VO_2max is associated with higher FAO, which agrees with previous research in rats (25, 69, 163-166) and humans (167-169). Because CR also increased medium-chain acylcarnitine levels, the effect of high VO_2max on increased FAO is comparable to the effect of CR as well. Moreover, the positive correlations were more significant after CR, suggesting that obesity overwhelms the effect of VO_2max on FAO. Higher mitochondrial capacity for AA catabolism and FAO in people with high VO_2max resulted in increased TCA cycle production, consistent with positive correlations between VO_2max and TCA cycle metabolites found in this study as well as previous work in mouse skeletal muscle (170), which is again comparable with the effect of CR.

To our knowledge, human studies that identify mechanisms by which high VO_2max

is associated with higher mitochondrial capacity for AA catabolism and FA metabolism have not yet been published. However, studies in the rat genetic model of aerobic treadmill running capacity demonstrated that higher mitochondrial capacity for AA catabolism in high capacity running (HCR) rats was associated with higher mitochondrial protein deacetylation (69) and higher mitochondrial capacity for FA metabolism in HCR rats was associated with higher mitochondrial protein deacetylation (69) as well as higher expression of genes and proteins responsible for FA metabolism as described in chapter 2.

Unlike this skeletal muscle study, the study in fasting plasma described in chapter 4 demonstrated that VO_2max was negatively correlated with short even-, medium- and long-chain acylcarnitine levels. Therefore, we calculated correlations between fasting plasma acylcarnitines and acylcarnitines in skeletal muscle at 120 min of MMTT from 61 (14 non-obese and 47 obese) subjects who enrolled in both fasting plasma study (chapter 4) and this skeletal muscle study. There were not any highly positive correlations between acylcarnitines in fasting plasma and postprandial skeletal muscle ($r = -0.143$ to 0.320). We also calculated correlations between acylcarnitines in plasma and skeletal muscle collected at 120 min of MMTT from 36 (11 non-obese and 25 obese) subjects who participated in both study in plasma over MMTT (described in chapter 6) and this skeletal muscle study. Like fasting plasma, there were no highly positive correlations between any acylcarnitines in plasma and skeletal muscle at 120 min of MMTT ($r = -0.313$ to 0.382). The poor correlations between fasting acylcarnitines in plasma and skeletal muscle of humans has been previously documented (171) though a separate study in humans demonstrated that most acylcarnitines in fasting plasma originated from skeletal muscle (172). A possible explanation for the poor correlations is that acylcarnitines produced during FAO are only briefly present in skeletal muscle; incomplete FAO associated with low VO_2max leads to accumulation of acylcarnitines in plasma, and thus there were negative correlations between VO_2max and FA-derived plasma acylcarnitines.

ANOVA in untargeted metabolomics demonstrated that PE and VO_2max were negatively correlated. This is at odds with previous research in human skeletal muscle that observed higher PE in the group of people with high VO_2max who had higher insulin sensitivity and lower BMI (97). However, the results from that previous study were not adjusted by insulin sensitivity status or BMI, thus the higher level of PE may instead be

caused by higher insulin sensitivity and lower BMI. Further in-depth research is necessary to identify the interaction among skeletal muscle PE, obesity, insulin resistance, and VO_2max. We found that levels of PA, PG and PI were positively correlated with high VO_2max; the results suggest that high VO_2max is associated with better cell and mitochondrial function since these metabolites play various important roles in cell and mitochondrial function.

As previously discussed, UDP-n-acetylglucosamine has a crucial role in intracellular signaling. Therefore, the positive correlation between VO_2max and UDP-n-acetylglucosamine observed in this study may be related to high VO_2max-associated improved cellular function. Because aging was associated with lower UDP-n-acetylglucosamine level, the age independent positive correlation between VO_2max and UDP-n-acetylglucosamine suggests that VO_2max is associated with delay aging. We also found that DHEAS was positively correlated with VO_2max, which agrees with previous findings in human serum (158, 173). However, the mechanisms responsible for this positive correlation remain unclear (173). Interestingly, several observational studies in humans have demonstrated that low serum DHEAS is associated with cardiovascular diseases (CVD) (174-182). Thus, the protective effect of high VO_2max against CVD may in part be mediated by DHEAS. Since aging was associated with lower DHEAS, the positive correlation between VO_2max and DHEAS independent of age further suggests that VO_2max is associated with delayed aging. It has been previously documented antioxidant enzyme glutathione peroxidase activity was higher in plasma (18) of people with higher VO_2max. In addition, the increased antioxidant enzyme glutathione reductase activity in human plasma was associated with increased VO_2max after training (183, 184). Therefore, the positive correlations between VO_2max and oxidized glutathione as well as VO_2max and reduced glutathione found in our study may be associated with activity of glutathione peroxidase and glutathione reductase, respectively, suggesting that high VO_2max is associated with lower oxidative stress, consistent with previous studies in the rat genetic model of aerobic treadmill running capacity and humans as described in chapter 2.

ANOVA also demonstrated a positive correlation between VO_2max and mtDNA count number, consistent with previous skeletal muscle research in humans (23) and rats (25). This positive correlation disappeared after CR, suggesting that CR diminishes the

effect of VO_2max on mtDNA count number.

In contrast to fasting plasma (described in chapter 4), VO_2max does not modulate age-related changes in skeletal muscle metabolite levels. Rather, it directly affects metabolite levels in skeletal muscle. The agrees with the fact that skeletal muscle is a major determinant of VO_2max (185). Moreover, the modulating effect of VO_2max on age-related changes in plasma metabolite levels does not reflect current skeletal muscle metabolite levels.

Our study revealed that the effect of VO_2max on a few metabolites in skeletal muscle is mediated by mtDNA count number, suggesting that the effect of VO_2max on skeletal muscle metabolite levels is in part mediated by mtDNA. Other possible mediators include mitophagy, mitochondrial proteostasis, mitochondrial membrane lipid composition and ETC efficiency (6). Further research is necessary to identify related mediators.

Our study has some limitations, including the small number of non-obese subjects and limited amount of skeletal muscle tissue available for mitochondrial isolation. Further research on isolated skeletal muscle from non-obese subjects would determine the effects of age and VO_2max on mitochondria-specific metabolites in skeletal muscle under physiological condition. Additionally, the duration of CR in this study was not long enough to identify the changes in metabolite levels and mtDNA profiles in older individuals. Future research that includes a longer duration of CR may reveal the dynamic effect of CR on age-related changes in metabolite levels and mtDNA profiles.

5.5 Summary

Analysis of human skeletal muscle via targeted and untargeted metabolomics approaches revealed that metabolism changes with age. Obesity was associated with lower levels of reduced glutathione and phospholipids, conditions that also decreased with age, suggesting that obesity mimics aging. CR decreased mitochondrial membrane lipid unsaturation by reducing the levels of PUFAs, which is associated with decreased susceptibility to age-related lipid peroxidative damage to mitochondria, consistent with delayed metabolic aging. CR also delays aging by decreasing the level of glycocholic acid, a marker of short lifespan. Unlike plasma metabolites, VO_2max did not modulate the effect of age-related changes in skeletal muscle metabolite levels. Rather, it was directly associated

with changes in metabolite levels in skeletal muscle, and this effect of VO_2max at baseline was partly mediated by mtDNA count number. However, CR diminished the effect of VO_2max on mtDNA count number. High VO_2max was associated with higher levels of UDP-n-acetyl glucosamine, glutathione, and DHEAS, all of which decrease with age, suggesting that high VO_2max delays metabolic aging as well. Moreover, CR and high VO_2max were associated with improved mitochondrial capacity for AA and FA utilization, resulting in increased TCA cycle metabolite production. Because both CR and high VO_2max can delay metabolic aging and improve mitochondrial substrate utilization, the effects of CR and high VO_2max are comparable with each other. These benefits of CR and high VO_2max are consistent with a 'younger' metabolic state, and may enhance metabolic health and longevity.

Table 5.1 Characteristics and non-metabolite parameters of all participants at baseline and 41 obese participants after CR. Data reported as mean (minimum-maximum) unless otherwise noted. $ = p-value <0.050; NA = not available

Characteristics	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
Number of subjects	15	99	41	NA	NA
Male/female	7/8	40/59	21/20	NA	NA
Race (n)					
Caucasian	14	92	39	NA	NA
African American	1	6	2	NA	NA
Asian	0	1	0	NA	NA
Age (years)					
Male	47 (27-60)	52 (37-67)	51 (37-67)	0.185	0.342
Female	45 (34-59)	48 (26-62)	47 (30-62)	0.318	0.463
All	46 (27-60)	49 (26-67)	49 (30-67)	0.134	0.210
BMI					
Male	23.8 (19.2-26.0)	38.8 (30.0-32.0)	31.9 (26.1-44.4)	<0.001$	<0.001$
Female	22.5 (20.9-24.4)	40.0 (30.9-48.5)	32.8 (26.0-39.3)	<0.001$	<0.001$
All	23.1 (19.2-26)	39.5 (30.0-48.5)	32.4 (26.0-44.4)	<0.001$	<0.001$
Type 2 DM (n)					
Male	0	18		7	NA
Female	0	17		6	NA
All	0	35		13	NA
Prescribed anti-diabetic drugs (n)					
Male	0	15	5	NA	NA
Female	0	13	5	NA	NA
All	0	28	10	NA	NA
Lean mass (kg)					
Male	53.0 (39.4-62.1)	70.4 (51.7-95.1)	67.3 (49.9-78.0)	<0.001$	NA
Female	40.6 (31.6-47.0)	52.4 (41.5-62.3)	50.3 (43.1-60.1)	<0.001$	NA
All	46.4 (31.6-62.1)	59.7 (41.5-95.1)	59.0 (43.1-78.0)	<0.001$	NA
Lean+bone (fat-free) mass (kg)					
Male	57.0 (48.2-65.8)	73.9 (54.6-99.2)	70.8 (52.9-82.0)	<0.001$	<0.001$
Female	41.9 (33.6-47.3)	54.9 (43.6-65.6)	53.0 (45.7-63.3)	<0.001$	<0.001$
All	49.0 (33.6-65.8)	62.6 (43.6-99.2)	62.1 (45.7-82.0)	<0.001$	<0.001$
Fat mass (kg)					
Male	16.1 (5.1-23.3)	48.3 (33.3-70.9)	33.3 (18.7-61.5)	<0.001$	<0.001$
Female	18.7 (10.2-24.5)	53.6 (33.0-77.3)	37.9 (19.8-58.4)	<0.001$	<0.001$
All	17.5 (5.1-24.5)	51.5 (33.0-77.3)	35.5 (18.7-61.5)	<0.001$	<0.001$
Total body fat (%)					
Male	22.2 (10.0-29.4)	40.5 (31.4-50.5)	32.5 (22.3-44.7)	<0.001$	<0.001$
Female	31.9 (19.1-39.0)	50.2 (37.5-59.3)	42.4 (30.1-50.6)	<0.001$	<0.001$
All	27.3 (10.0-39.0)	46.3 (31.4-59.3)	37.3 (22.3-50.6)	<0.001$	<0.001$
Fasting plasma glucose (mg/dl)					

Male	89 (75-106)	116 (73-245)	95 (69-131)	0.063	0.269
Female	87 (78-99)	103 (80-146)	97 (83-148)	0.002[$]	0.069
All	88 (75-106)	108 (73-245)	96 (69-148)	0.004[$]	0.034[$]
Fasting plasma insulin (mIU/l)					
Male	8.4 (4.9-11.6)	23.8 (10.1-48.6)	13.6 (5.7-24.8)	<0.001[$]	0.007[$]
Female	8.7 (5.1-14.5)	21.5 (5.8-53.0)	14.1 (7.7-22.5)	<0.001[$]	0.003
All	8.6 (4.9-14.5)	22.4 (5.8-53.0)	13.8 (5.7-24.8)	<0.001[$]	<0.001[$]
HOMA-IR ((fasting plasma glucose x fasting plasma insulin) /405)					
Male	1.9 (1.1-2.8)	7.0 (2.1-17.4)	3.3 (1.4-7.1)	<0.001[$]	0.025[$]
Female	1.9 (1.0-3.4)	5.7 (1.2-13.7)	3.4 (1.6-5.2)	<0.001[$]	0.002[$]
All	1.9 (1.0-3.4)	6.2 (1.2-17.4)	3.3 (1.4-7.1)	<0.001[$]	<0.001[$]
Resting energy expenditure (REE) (kCal)					
Male	1748 (1212-2101)	2274 (1573-2909)	2004 (1246-2578)	<0.001[$]	0.069
Female	1378 (967-1866)	1800 (1304-2502)	1605 (1144-2031)	<0.001[$]	0.069
All	1551 (967-2101)	1989 (1304-2909)	1804 (1144-2578)	<0.001[$]	0.021[$]
REE per fat-free mass (kCal/kg)					
Male	30.6 (25.2-34.6)	30.8 (25.9-37.0)	28.2 (23.2-35.6)	0.875	0.089
Female	32.8 (24.2-40.8)	32.8 (25.5-44.2)	30.1 (23.2-36.7)	0.959	0.138
All	31.7 (24.2-40.8)	32.0 (25.5-44.2)	29.1 (23.2-36.7)	0.787	0.026[$]
Respiratory quotient at rest (RQR)					
Male	0.80 (0.72-0.85)	0.81 (0.74-0.93)	0.79 (0.68-1.06)	0.540	0.935
Female	0.80 (0.67-0.89)	0.81 (0.71-0.94)	0.80 (0.70-0.91)	0.428	0.934
All	0.81 (0.67-0.91)	0.79 (0.66-0.94)	0.80 (0.67-1.06)	0.308	0.987
Respiratory quotient at VO_2max (RQE)					
Male	1.21 (1.04-1.36)	1.17 (0.99-1.37)	1.15 (0.85-1.37)	0.343	0.317
Female	1.16 (1.03-1.34)	1.12 (0.84-1.30)	1.14 (0.96-1.35)	0.257	0.666
All	1.18 (1.03-1.36)	1.14 (0.84-1.37)	1.15 (0.85-1.37)	0.120	0.304
VO_2max (l/min)					
Male	3.09 (2.13-4.26)	3.00 (1.69-4.30)	3.23 (1.77-4.69)	0.740	0.657
Female	2.13 (1.66-2.49)	2.14 (1.10-2.94)	2.25 (1.57-3.64)	0.946	0.529
All	2.58 (1.66-4.26)	2.49 (1.10-4.30)	2.75 (1.57-4.69)	0.624	0.452
VO_2max per fat-free mass (ml/(kg x min))					
Male	53.99 (38.49-70.22)	40.61 (28.29-53.98)	45.32 (30.52-58.83)	<0.001[$]	0.032[$]
Female	51.06 (42.23-59.70)	39.08 (22.62-55.57)	42.79 (27.08-70.71)	<0.001[$]	0.044[$]
All	52.43 (38.49-70.22)	39.70 (22.62-55.57)	44.09 (27.08-70.71)	<0.001[$]	0.003[$]

Table 5.2 Characteristics and non-metabolite parameters of 41 obese participants who completed muscle biopsies both before and after CR. Data were reported as "average (minimum-maximum)". $ = p-value <0.050; NA = not available

Characteristics	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Number of subjects	41		NA
Male/female	21/20		NA
Race			
Caucasian	39		NA
African American	2		NA
Asian	0		NA
Age (years)			
Male	51 (37-67)		NA
Female	47 (30-62)		NA
All	49 (30-67)		NA
BMI			
Male	38.8 (30.0-32.0)	31.9 (26.1-44.4)	<0.001$
Female	40.0 (30.9-48.5)	32.8 (26.0-39.3)	<0.001$
All	39.5 (30.0-48.5)	32.4 (26.0-44.4)	<0.001$
% of weight loss after CR			
Male	18.1 (4.3-34.4)		NA
Female	16.3 (5.5-27.5)		NA
All	17.2 (4.3-34.4)		NA
Number of type 2 DM			
Male	7	7	NA
Female	6	6	NA
All	13	13	NA
Number of subjects who received anti-diabetic drugs			
Male	6	5	NA
Female	5	5	NA
All	11	10	NA
Lean mass (kg)			
Male	70.0 (51.7-82.6)	67.3 (49.9-78.0)	0.002$
Female	52.9 (46.9-60.1)	50.3 (43.1-60.1)	<0.001$
All	61.2 (46.9-82.6)	59.0 (43.1-78.0)	<0.001$
Lean+bone (fat-free) mass (kg)			
Male	73.5 (54.6-86.5)	70.8 (52.9-82.0)	0.001$
Female	55.6 (49.4-63.7)	53.0 (45.7-63.3)	<0.001$
All	64.8 (49.4-86.5)	62.1 (45.7-82.0)	<0.001$
Fat mass (kg)			
Male	49.9 (33.4-70.8)	33.3 (18.7-61.5)	<0.001$
Female	52.0 (33.0-73.8)	37.9 (19.8-58.4)	<0.001$
All	50.9 (33.0-73.8)	35.5 (18.7-61.5)	<0.001$
Total body fat percentage			
Male	41.4 (31.3-50.5)	32.5 (22.3-44.7)	<0.001$
Female	49.0 (37.5-58.3)	42.4 (30.1-50.6)	<0.001$
All	45.1 (31.3-58.3)	37.3 (22.3-50.6)	<0.001$
Fasting plasma glucose (mg/dl)			
Male	100 (73-137)	95 (69-131)	0.419
Female	105 (80-146)	97 (83-148)	0.021$
All	102 (73-146)	96 (69-148)	0.059

Fasting plasma insulin (mIU/l)			
Male	22.5 (11.0-33.2)	13.6 (5.7-24.8)	<0.001[$]
Female	21.0 (9.9-38.1)	14.1 (7.7-22.5)	<0.001[$]
All	21.7 (9.9-38.1)	13.8 (5.7-24.8)	<0.001[$]
HOMA-IR ((Fasting plasma glucose x Fasting plasma insulin)/405)			
Male	5.7 (2.1-10.7)	3.3 (1.4-7.1)	0.001[$]
Female	5.6 (2.2-11.0)	3.4 (1.6-5.2)	<0.001[$]
All	5.6 (2.1-11.0)	3.3 (1.4-7.1)	<0.001[$]
Resting energy expenditure (REE) (kCal)			
Male	2237 (1573-2886)	2004 (1246-2578)	<0.001[$]
Female	1773 (1304-2293)	1605 (1144-2031)	0.005[$]
All	2005 (1304-2886)	1804 (1144-2578)	<0.001[$]
REE per fat-free mass (kCal/kg)			
Male	30.3 (25.9-35.1)	28.2 (23.2-35.6)	0.002[$]
Female	31.9 (25.5-37.1)	30.1 (23.2-36.7)	0.054
All	31.1 (25.5-37.1)	29.1 (23.2-36.7)	<0.001[$]
Respiratory quotient at rest (RQR)			
Male	0.80 (0.74-0.93)	0.79 (0.68-1.06)	0.605
Female	0.82 (0.77-0.93)	0.80 (0.70-0.91)	0.091
All	0.81 (0.74-0.93)	0.77 (0.67-1.06)	0.198
Respiratory quotient at VO_2max (RQE)			
Male	1.17 (1.02-1.37)	1.15 (0.85-1.37)	0.385
Female	1.11 (0.97-1.25)	1.14 (0.96-1.35)	0.304
All	1.14 (0.97-1.37)	1.15 (0.85-1.37)	0.934
VO_2max (l/min)			
Male	3.19 (1.69-4.30)	3.23 (1.77-4.69)	0.963
Female	2.18 (1.69-2.91)	2.25 (1.57-3.64)	0.365
All	2.70 (1.69-4.30)	2.75 (1.57-4.69)	0.541
VO_2max per fat-free mass (ml/(kg x min))			
Male	43.20 (30.94-53.98)	45.32 (30.52-58.83)	0.211
Female	39.40 (27.96-43.84)	42.79 (27.08-70.71)	0.037[$]
All	41.35 (27.96-53.98)	44.09 (27.08-70.71)	0.014[$]

Table 5.3 Concentrations and ratios of targeted metabolites and mtDNA profiles of all participants at baseline and 41 obese participants after CR. Data were reported as average±SEM. $ = p-value <0.050

AAs	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
Alanine (nmol/mg)					
Male	10.07±1.02	12.03±0.65	14.33±1.76	0.236	0.186
Female	13.85±1.25	12.02±0.58	11.11±0.39	0.268	0.011$
All	12.09±0.94	12.03±0.43	12.76±0.94	0.958	0.688
Arginine (nmol/mg)					
Male	1.45±0.09	1.59±0.09	1.58±0.14	0.540	0.618
Female	1.69±0.17	1.91±0.09	1.87±0.09	0.418	0.347
All	1.58±0.10	1.78±0.07	1.72±0.09	0.275	0.376
Asparagine (nmol/mg)					
Male	8.32±0.75	7.23±0.28	8.08±0.48	0.148	0.801
Female	9.70±0.86	6.67±0.21	6.68±0.26	<0.001$	<0.001$
All	9.05±0.59	6.89±0.17	7.40±0.29	<0.001$	0.008$
Aspartic acid (pmol/mg)					
Male	892.04±124.15	1195.72±132.04	1051.36±148.84	0.352	0.560
Female	1027.11±223.24	1203.57±90.15	1230.68±124.74	0.497	0.408
All	964.07±129.41	1200.40±73.30	1138.83±97.36	0.241	0.334
Glutamic acid (nmol/mg)					
Male	8.85±1.53	9.34±0.42	10.71±1.01	0.684	0.353
Female	10.874±1.16	9.27±0.28	10.29±0.57	0.070	0.619
All	9.93±0.95	9.30±0.24	10.50±0.58	0.374	0.611
Glutamine (nmol/mg)					
Male	52.61±1.71	45.52±1.67	53.81±2.19	0.090	0.765
Female	55.32±2.02	47.02±1.14	51.71±2.15	0.012$	0.333
All	54.06±1.34	46.41±0.96	52.78±1.53	0.003$	0.634
Glutamine-to-glutamic acid ratio					
Male	6.94±1.02	5.20±0.28	6.23±0.85	0.036$	0.663
Female	5.45±0.51	5.32±0.20	5.17±0.21	0.821	0.546
All	6.15±0.56	5.27±0.17	5.72±0.45	0.070	0.602
Glycine (nmol/mg)					
Male	4.15±0.27	3.61±0.15	4.73±0.30	0.153	0.303
Female	4.39±0.43	3.66±0.17	4.16±0.25	0.144	0.634
All	4.28±0.25	3.64±0.12	4.45±0.20	0.048$	0.639
Histidine (nmol/mg)					
Male	12.70±0.68	10.74±0.41	14.18±0.93	0.063	0.384
Female	11.02±0.93	9.23±0.24	9.94±0.42	0.017$	0.229
All	11.80±0.61	9.84±0.23	12.11±0.61	0.003$	0.777
Isoleucine+leucine (pmol/mg)					
Male	49.77±1.83	58.48±2.02	59.08±2.92	0.084	0.086

Female	49.97±1.77	50.78±1.06	49.03±1.68	0.787	0.748
All	49.88±1.23	53.89±1.09	54.18±1.86	0.164	0.182
Lysine (nmol/mg)					
Male	27.94±4.22	38.11±2.21	42.16±3.52	0.076	0.041[$]
Female	31.67±4.26	48.42±2.42	45.61±2.46	0.016[$]	0.007[$]
All	29.93±2.94	44.25±1.77	43.84±2.16	0.003[$]	<0.001[$]
Methionine (pmol/mg)					
Male	125.42±10.76	139.73±6.28	151.87±15.06	0.368	0.338
Female	152.73±11.26	122.40±3.51	113.91±3.85	0.005[$]	<0.001[$]
All	139.99±8.38	129.40±3.38	133.35±8.40	0.256	0.656
Phenylalanine (pmol/mg)					
Male	196.69±12.49	234.27±9.19	257.03±22.61	0.105	0.146
Female	221.20±11.19	209.46±5.01	192.45±6.04	0.413	0.023[$]
All	209.76±8.68	219.49±4.89	225.52±12.86	0.457	0.477
Proline (nmol/mg)					
Male	2.09±0.41	2.20±0.15	2.46±0.23	0.794	0.433
Female	1.98±0.27	2.06±0.09	2.04±0.11	0.768	0.816
All	2.04±0.23	2.12±0.08	2.26±0.13	0.719	0.396
Serine (pmol/mg)					
Male	1047.55±131.94	959.05±35.63	1246.13±95.22	0.384	0.286
Female	1429.25±136.84	961.87±39.91	1117.67±64.31	<0.001[$]	0.027[$]
All	1251.12±105.19	960.73±27.67	1183.47±58.17	<0.001[$]	0.560
Threonine (nmol/mg)					
Male	1.39±0.11	1.42±0.05	1.63±0.11	0.847	0.223
Female	1.72±0.16	1.37±0.04	1.46±0.06	0.009[$]	0.059
All	1.56±0.10	1.39±0.03	1.55±0.06	0.058	0.882
Tryptophan (pmol/mg)					
Male	672.23±91.45	737.77±24.23	690.01±49.30	0.346	0.861
Female	752.46±78.69	708.66±24.93	727.29±30.00	0.554	0.715
All	715.02±58.63	720.42±17.78	708.19±28.98	0.916	0.909
Tyrosine (pmol/mg)					
Male	259.65±21.59	302.87±9.41	279.59±17.52	0.081	0.551
Female	322.63±17.61	306.15±9.38	276.96±13.45	0.534	0.067
All	293.24±15.70	304.83±6.73	278.18±10.98	0.529	0.466
Valine (pmol/mg)					
Male	788.75±39.18	940.61±29.29	941.00±44.45	0.041[$]	0.071
Female	771.18±32.88	819.48±20.15	795.39±37.57	0.394	0.706
All	779.38±24.51	868.43±17.81	869.97±31.07	0.060	0.097
Glycolysis metabolites	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
Glucose (nmol/mg)					
Male	4.31±0.24	5.20±0.61	7.96±1.42	0.548	0.157
Female	4.25±0.42	5.24±0.31	4.32±0.34	0.326	0.920
All	4.28±0.23	5.23±0.31	5.99±0.76	0.274	0.213
Glucose-6-					

phosphate+fructose -6-phosphate (nmol/mg)					
Male	2.44±0.82	5.57±2.46	8.56±3.07	0.601	0.268
Female	3.60±1.24	4.24±1.63	2.87±1.19	0.886	0.727
All	3.06±0.75	4.78±1.38	5.79±1.72	0.632	0.349
Fructose 1,6-bisphosphate (nmol/mg)					
Male	3.28±0.84	5.94±1.69	7.96±2.78	0.518	0.349
Female	5.65±1.71	6.33±1.26	2.79±1.07	0.848	0.166
All	4.54±1.01	6.17±1.01	5.44±1.55	0.538	0.738
Glyceraldehyde-3-phosphate (nmol/mg)					
Male	28.84±7.71	22.37±3.08	19.15±5.60	0.424	0.374
Female	36.06±12.49	21.59±2.61	18.39±2.84	0.089	0.058
All	32.87±7.38	21.90±1.98	18.78±3.15	0.067	0.047$
Dihydroxyacetone phosphate (nmol/mg)					
Male	3.94±0.79	3.87±0.47	6.04±1.34	0.952	0.388
Female	6.22±1.54	4.23±0.47	4.34±0.62	0.159	0.496
All	5.16±0.92	4.09±0.34	5.21±1.03	0.256	0.976
2-phosphoglyceric acid+ 3-phosphoglyceric acid (pmol/mg)					
Male	775.01±326.88	1192.99±178.10	1340.91±390.45	0.358	0.432
Female	1518.10±456.81	1080.59±148.96	865.08±253.38	0.321	0.197
All	1171.32±294.67	1124.62±113.84	1108.80±235.23	0.882	0.885
Lactate (nmol/mg)					
Male	34.23±11.43	47.52±10.60	117.06±35.89	0.611	0.202
Female	56.12±21.34	61.79±12.20	27.11±9.45	0.869	0.159
All	45.90±12.49	56.03±8.43	73.19±20.03	0.651	0.428
Fructose 1,6-bisphosphate-to-glucose-6 phosphate+fructose -6-phosphate ratio					
Male	2.20±0.49	3.66±0.56	2.23±0.37	0.289	0.960
Female	1.89±0.36	4.30±0.74	1.84±0.34	0.242	0.925
All	2.03±0.29	4.04±0.50	2.04±0.25	0.121	0.991
FA	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
Oleic acid (pmol/mg)					
Male	65.55±35.31	247.72±101.77	706.07±292.87	0.463	0.224
Female	38.34±7.02	199.73±44.46	39.08±4.99	0.189	0.935
All	51.04±16.61	219.12±48.67	380.71±157.32	0.184	0.213

Acylcarnitines	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
L-carnitine (nmol/mg)					
Male	17.55±1.59	18.75±0.73	22.48±1.45	0.526	0.079
Female	21.85±1.83	17.59±0.52	19.35±0.75	0.008$	0.140
All	19.84±1.31	18.06±0.43	20.95±0.86	0.143	0.497
C2 carnitine (nmol/mg)					
Male	4.49±1.66	1.58±0.12	2.87±0.46	<0.001$	0.195
Female	3.55±0.82	1.60±0.12	2.25±0.29	<0.001$	0.069
All	3.99±0.86	1.59±0.09	2.57±0.27	<0.001$	0.044$
C3 carnitine (pmol/mg)					
Male	20.48±1.75	40.65±2.99	38.35±3.92	0.008$	0.016$
Female	38.15±9.87	32.53±1.68	32.20±2.77	0.331	0.435
All	29.90±5.67	35.81±1.61	35.35±2.44	0.209	0.307
C4 carnitine (pmol/mg)					
Male	42.01±21.04	9.47±0.96	10.15±1.55	<0.001$	0.013$
Female	27.16±8.70	17.88±4.86	11.15±2.15	0.499	0.018$
All	34.09±10.61	14.49±2.94	10.64±1.30	0.024$	<0.001$
C5 carnitine (pmol/mg)					
Male	4.26±0.37	7.72±0.94	3.72±0.43	0.136	0.486
Female	10.44±5.66	7.21±1.67	4.77±0.58	0.518	0.127
All	7.56±3.04	7.41±1.06	4.23±0.36	0.962	0.087
C5-DC carnitine (pmol/mg)					
Male	4.22±0.80	2.23±0.19	3.40±0.39	<0.001$	0.328
Female	4.51±0.94	2.15±0.12	3.04±0.37	<0.001$	0.087
All	4.37±0.60	2.18±0.11	3.23±0.28	<0.001$	0.051
C6 carnitine (pmol/mg)					
Male	13.33±3.46	4.24±0.73	6.81±1.62	<0.001$	0.068
Female	10.50±2.96	6.64±1.30	9.87±3.80	0.300	0.922
All	11.82±2.21	5.67±0.83	8.31±2.02	0.009$	0.333
C8:0 carnitine (pmol/mg)					
Male	4.00±0.91	1.35±0.25	2.14±0.63	<0.001$	0.139
Female	3.08±0.90	2.36±0.44	3.21±1.23	0.563	0.949
All	3.51±0.63	1.95±0.28	2.66±0.68	0.045$	0.478
C8:1 carnitine (pmol/mg)					
Male	1.91±0.24	2.09±0.18	2.07±0.26	0.690	0.747
Female	2.82±0.48	2.28±0.15	2.57±0.30	0.224	0.654
All	2.40±0.29	2.20±0.11	2.31±0.20	0.540	0.820
C10:0 carnitine (pmol/mg)					
Male	4.16±0.98	1.52±0.28	2.44±0.81	0.001$	0.270

Female	4.13±1.46	2.38±0.38	3.15±0.95	0.134	0.583
All	4.15±0.87	2.03±0.26	2.55±0.62	0.005[$]	0.243
C10:1 carnitine (fmol/mg)					
Male	1728.14±374.07	805.86±108.75	1095.66±303.08	0.004[$]	0.278
Female	1489.39±134.09	909.08±129.03	1097.02±250.85	0.134	0.419
All	1600.81±277.05	867.38±88.32	1096.32±195.21	0.004[$]	0.171
C12:0 carnitine (pmol/mg)					
Male	4.94±1.14	2.34±0.42	5.38±1.65	0.025[$]	0.883
Female	7.89±3.25	4.68±1.34	4.60±1.75	0.405	0.347
All	6.51±1.80	3.74±0.82	5.00±1.19	0.214	0.502
C12:1 carnitine (fmol/mg)					
Male	1156.70±347.68	615.92±143.96	937.82±280.41	0.155	0.682
Female	974.95±338.16	659.67±109.85	665.15±173.34	0.332	0.379
All	1059.76±235.07	641.99±87.14	804.81±166.02	0.087	0.414
C12-OH carnitine (fmol/mg)					
Male	668.19±251.18	400.19±128.18	2322.18±985.72	0.414	0.349
Female	353.11±173.39	745.70±460.17	265.46±60.85	0.868	0.073
All	597.22±144.74	606.10±278.63	1318.90±525.47	0.990	0.415
C14:0 carnitine (pmol/mg)					
Male	7.40±1.88	6.35±1.71	13.73±4.21	0.804	0.404
Female	8.55±2.77	6.94±1.53	4.91±1.51	0.709	0.228
All	8.01±1.66	6.70±1.14	9.43±2.36	0.664	0.728
C14:1 carnitine (pmol/mg)					
Male	6.04±1.83	4.29±1.62	7.65±2.53	0.662	0.726
Female	5.03±1.98	3.32±0.69	2.97±1.08	0.395	0.338
All	5.50±1.32	3.71±0.77	5.37±1.43	0.383	0.959
C14:2 carnitine (fmol/mg)					
Male	1982.21±468.78	1375.40±512.00	2390.08±803.43	0.629	0.778
Female	1953.16±775.63	956.88±191.60	1003.87±391.89	0.096	0.239
All	1966.71±472.10	1125.98±235.65	1713.88±461.41	0.186	0.757
C14-OH carnitine (fmol/mg)					
Male	447.63±155.91	243.52±60.74	1042.96±374.23	0.205	0.377
Female	405.39±75.26	221.30±55.48	199.64±32.87	0.236	0.007[$]
All	425.11±80.02	230.28±40.99	631.58±201.39	0.079	0.544
C16:0 carnitine (pmol/mg)					
Male	19.11±4.74	26.46±6.56	61.74±20.26	0.647	0.242
Female	27.74±10.31	29.89±6.12	16.46±4.24	0.900	0.235
All	23.71±5.84	28.50±4.49	39.65±11.05	0.685	0.398
C16:1 carnitine (pmol/mg)					
Male	10.03±2.90	15.17±5.27	34.17±12.80	0.689	0.293
Female	14.59±6.07	18.56±4.62	8.05±2.42	0.758	0.235
All	12.46±3.45	17.19±3.47	21.43±6.90	0.602	0.445
C16-OH carnitine (fmol/mg)					

Male	474.77±201.97	761.21±320.18	4320.21±1900.87	0.714	0.260
Female	515.43±158.03	580.97±213.40	217.99±47.23	0.911	0.023$
All	496.46±121.85	653.80±180.63	2319.13±1015.38	0.737	0.286
C18:0 carnitine (pmol/mg)					
Male	12.97±2.90	20.53±4.64	53.17±17.27	0.505	0.197
Female	28.12±13.13	22.52±4.90	10.02±2.13	0.693	0.049$
All	21.05±7.19	21.71±3.46	32.12±9.44	0.943	0.499
C18:1 carnitine (pmol/mg)					
Male	27.58±6.63	68.06±20.48	158.00±57.10	0.418	0.205
Female	67.48±31.25	85.93±19.34	33.37±8.30	0.734	0.155
All	48.86±17.25	78.71±14.15	97.21±30.79	0.422	0.359
C18:2 carnitine (pmol/mg)					
Male	6.83±1.39	18.47±5.56	48.73±19.48	0.390	0.231
Female	21.06±9.96	25.93±6.22	8.74±2.23	0.780	0.094
All	14.42±5.52	22.92±4.33	29.22±10.41	0.456	0.405
C20:0 carnitine (fmol/mg)					
Male	319.91±79.22	236.97±36.20	661.88±239.09	0.376	0.425
Female	404.74±174.43	198.48 ± 33.57	128.89±23.69	0.066	0.022$
All	365.15±97.45	214.04±24.73	401.88±128.62	0.043$	0.869
C20:1 carnitine (fmol/mg)					
Male	637.29±231.93	1353.97±517.28	4086.64±1578.56	0.570	0.227
Female	1369.86±651.80	1338.63±325.74	541.60±127.85	0.973	0.077
All	1027.99±365.50	1344.83±283.64	2348.13±848.32	0.671	0.359
C20:2 carnitine (fmol/mg)					
Male	235.40±76.78	432.43±131.66	1609.57±653.94	0.540	0.242
Female	559.14±304.52	578.05±137.86	241.36±59.36	0.578	0.142
All	408.06±166.65	519.21±97.69	942.15±349.32	0.670	0.369
C20:3 carnitine (fmol/mg)					
Male	117.28±29.09	414.77±135.34	1340.06±619.76	0.367	0.271
Female	455.56±252.06	631.72±163.16	171.26±63.12	0.700	0.136
All	297.70±138.37	544.06±111.58	769.99±328.37	0.401	0.396
C20:4 carnitine (fmol/mg)					
Male	249.03±101.45	932.53±287.58	3734.87±1623.85	0.331	0.232
Female	927.02±355.22	1346.46±371.69	306.19±110.22	0.684	0.052
All	610.62±232.31	1179.22±249.89	2062.35±866.92	0.384	0.321
TCA cycle metabolites	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
Citrate (pmol/mg)					
Male	534.42±101.47	361.90±21.25	591.40±81.79	0.011$	0.715
Female	445.13±40.78	333.79±18.05	337.39±27.94	0.034$	0.045$
All	486.80±51.42	345.15±13.76	467.49±47.93	<0.001$	0.152
Succinate					

(pmol/mg)					
Male	168.34±62.37	186.78±30.08	191.09±44.78	0.811	0.793
Female	163.71±32.82	227.25±33.82	128.69±24.31	0.498	0.431
All	165.87±32.64	210.90±23.52	160.65±25.98	0.468	0.913
Malate (pmol/mg)					
Male	864.85±213.55	1065.08±184.93	1782.77±435.58	0.661	0.246
Female	1748.22±507.89	1020.71±121.34	765.12±91.56	0.058	0.008[$]
All	1335.98±302.95	1038.63±103.43	1286.36±238.75	0.308	0.910
Nucleotides	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
AMP (pmol/mg)					
Male	76.81±7.49	68.33±3.53	81.95±5.86	0.352	0.647
Female	80.27±7.38	63.54±2.15	86.58±7.06	0.011[$]	0.608
All	78.65±5.09	65.47±1.92	84.21±4.53	0.015[$]	0.496
ADP (nmol/mg)					
Male	14.62±1.04	13.21±0.50	13.14±0.88	0.276	0.378
Female	16.61±0.71	13.17±0.36	14.95±0.48	0.001[$]	0.074
All	15.68±0.65	13.19±0.29	14.02±0.52	0.002[$]	0.088
ATP (nmol/mg)					
Male	14.88±0.43	12.70±0.68	11.02±1.32	0.193	0.109
Female	14.05±1.27	12.24±0.52	12.76±0.79	0.231	0.391
All	14.44±0.69	12.42±0.41	11.87±0.78	0.070	0.065
NAD^+ (nmol/mg)					
Male	17.46±1.26	13.18±0.78	11.46±1.60	0.032[$]	0.048[$]
Female	16.30±1.73	12.21±0.73	15.02±0.95	0.055	0.496
All	16.84±1.07	12.60±0.54	13.20±0.97	0.004[$]	0.041[$]
NADH (pmol/mg)					
Male	1580.92±297.09	918.56±114.63	862.98±183.30	0.046[$]	0.079
Female	1052.75±331.37	947.07±107.20	1048.93±169.52	0.740	0.991
All	1279.11±228.65	935.42±78.47	960.85±123.78	0.134	0.219
NAD^+-to-NADH ratio					
Male	16.06±4.55	145.17±80.94	52.24±17.37	0.544	0.286
Female	42.61±20.31	106.74±31.01	22.58±3.35	0.454	0.152
All	31.23±11.60	122.44±37.48	36.63±8.91	0.365	0.751
NADP (pmol/mg)					
Male	397.41±93.32	373.62±26.38	312.62±32.46	0.748	0.287
Female	328.83±58.79	354.79±23.52	301.23±30.64	0.702	0.654
All	360.83±52.41	362.40±17.54	306.92±22.07	0.975	0.271
FAD (pmol/mg)					
Male	202.91±22.82	165.18±7.01	210.52±11.75	0.055	0.756
Female	232.09±16.34	157.27±6.81	180.08±11.49	<0.001[$]	0.020[$]
All	218.47±13.80	160.47±4.94	195.67±8.47	<0.001[$]	0.168
Long-chain acylcarnitine-to-FA ratio	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
C18:1 carnitine-to-					

oleic acid ratio ($x10^{-1}$)					
Male	8.79±3.28	5.37±0.89	5.68±1.49	0.179	0.339
Female	15.64±5.00	4.43±0.77	8.71±2.04	<0.001$	0.134
All	12.44±3.10	4.81±0.58	7.16±1.26	<0.001$	0.064
C3 and C5 carnitine-to-substrate ratios	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
C3 carnitine-to-valine ratio ($x10^{-2}$)					
Male	2.62±0.25	4.40±0.32	4.10±0.39	0.026$	0.043$
Female	4.98±1.31	3.99±0.20	4.05±0.27	0.164	0.319
All	3.88±0.75	4.15±0.17	4.08±0.24	0.608	0.745
C5 carnitine-to-isoleucine+leucine ratio ($x10^{-2}$)					
Male	8.59±0.72	13.61±1.66	6.25±0.65	0.216	0.063
Female	21.30±11.84	14.39±3.33	9.68±1.08	0.492	0.132
All	15.37±6.35	14.07±2.09	7.92±0.67	0.827	0.064
mtDNA profiles	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR
mtDNA deletion ratio (%)					
Male	64.14±5.68	66.29±2.41	71.26±2.40	0.731	0.188
Female	64.93±7.75	61.15±1.98	68.58±1.78	0.534	0.517
All	64.56±4.74	63.23±1.54	69.95±1.50	0.760	0.158
mtDNA count number					
Male	1436±276	1178±83	1109±111	0.266	0.197
Female	1426±291	1442±88	1121±106	0.947	0.229
All	1431±194	1336±63	1115±76	0.599	0.071

Table 5.4 Concentrations and ratios of targeted metabolites and mtDNA profiles of 41 obese participants who completed muscle biopsies both before and after CR. Data were reported as average±SEM. $ = p-value <0.050

AAs	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Alanine (nmol/mg)			
Male	11.96±0.98	14.33±1.76	0.126
Female	11.43±0.55	11.11±0.39	0.695
All	11.70±0.56	12.76±0.94	0.230
Arginine (nmol/mg)			
Male	1.51±0.10	1.58±0.14	0.699
Female	2.00±0.14	1.87±0.09	0.325
All	1.75±0.09	1.72±0.09	0.770
Asparagine (nmol/mg)			
Male	7.17±0.40	8.08±0.48	0.119
Female	6.39±0.24	6.68±0.26	0.431
All	6.79±0.24	7.40±0.29	0.078
Aspartic acid (pmol/mg)			
Male	1215.73±221.25	1051.36±148.84	0.484
Female	1357.65±150.08	1230.68±124.74	0.453
All	1284.96±133.72	1138.83±97.36	0.306
Glutamic acid (nmol/mg)			
Male	9.90±0.58	10.71±1.01	0.487
Female	9.48±0.41	10.29±0.57	0.226
All	9.70±0.36	10.50±0.58	0.224
Glutamine (nmol/mg)			
Male	47.92±2.19	53.81±2.19	0.089
Female	47.84±1.63	51.71±2.15	0.242
All	47.88±1.36	52.78±1.53	0.037$
Glutamine-to-glutamic acid ratio			
Male	5.19±0.44	6.23±0.85	0.148
Female	5.14±0.18	5.17±0.21	0.891
All	5.17±0.24	5.72±0.45	0.152
Glycine (nmol/mg)			
Male	3.62±0.21	4.73±0.30	0.002$
Female	3.48±0.26	4.16±0.25	0.006$
All	3.55±0.17	4.45±0.20	<0.001$
Histidine (nmol/mg)			
Male	11.18±0.61	14.18±0.93	0.013$
Female	9.77±0.46	9.94±0.42	0.794
All	10.49±0.39	12.11±0.61	0.021$
Isoleucine+leucine (pmol/mg)			
Male	55.57±2.21	59.08±2.92	0.118
Female	53.41±2.09	49.03±1.68	0.043$
All	54.52±1.52	54.18±1.86	0.833
Lysine (nmol/mg)			
Male	38.97±3.49	42.16±3.52	0.497
Female	54.04±4.50	45.61±2.46	0.049$
All	46.32±3.04	43.84±2.16	0.439
Methionine (pmol/mg)			
Male	128.51±6.75	151.87±15.06	0.099

Female	120.08±4.73	113.91±3.85	0.312
All	124.40±4.16	133.35±8.40	0.256
Phenylalanine (pmol/mg)			
Male	233.86±11.94	257.03±22.61	0.200
Female	206.25±7.35	192.45±6.04	0.124
All	220.39±7.34	225.52±12.86	0.617
Proline (nmol/mg)			
Male	2.23±0.23	2.46±0.23	0.379
Female	2.29±0.14	2.04±0.11	0.093
All	2.26±0.14	2.26±0.13	0.991
Serine (pmol/mg)			
Male	918.92±44.38	1246.13±95.22	0.002[$]
Female	937.71±70.13	1117.67±64.31	0.015[$]
All	928.09±40.58	1183.47±58.17	<0.001[$]
Threonine (nmol/mg)			
Male	1.39±0.06	1.63±0.11	0.062
Female	1.45±0.07	1.46±0.06	0.946
All	1.42±0.05	1.55±0.06	0.108
Tryptophan (pmol/mg)			
Male	743.43±37.93	690.01±49.30	0.207
Female	767.46±38.26	727.29±30.00	0.334
All	755.15±26.67	708.19±28.98	0.107
Tyrosine (pmol/mg)			
Male	288.49±9.71	279.59±17.52	0.594
Female	311.63±14.89	276.96±13.45	0.058
All	299.78±8.88	278.18±10.98	0.078
Valine (pmol/mg)			
Male	904.69±33.40	941.00±44.45	0.286
Female	877.47±35.83	795.39±37.57	0.115
All	891.41±31.07	869.97±31.07	0.489
Glycolysis metabolites	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Glucose (nmol/mg)			
Male	4.56±0.66	7.96±1.42	0.547
Female	4.80±0.36	4.32±0.34	0.252
All	4.67±0.38	5.99±0.76	0.063
Glucose-6-phosphate+fructose-6-phosphate (nmol/mg)			
Male	6.93±4.12	8.56±3.07	0.601
Female	2.83±1.37	2.87±1.19	0.981
All	4.93±2.21	5.79±1.72	0.604
Fructose 1,6-bisphosphate (nmol/mg)			
Male	3.94±0.87	7.96±2.78	0.518
Female	2.91±0.63	2.79±1.07	0.922
All	3.44±0.54	5.44±1.55	0.209
Glyceraldehyde-3-phosphate (nmol/mg)			
Male	25.30±5.19	19.15±5.60	0.387
Female	20.07±3.55	18.39±2.84	0.692
All	22.75±3.16	18.78±3.15	0.335
Dihydroxyacetone phosphate (nmol/mg)			
Male	3.36±0.41	6.04±1.34	0.062

Female	3.63±0.62	4.34±0.62	0.687
All	3.49±0.36	5.21±1.03	0.123
2-phosphoglyceric acid+ 3-phosphoglyceric acid (pmol/mg)			
Male	1272.54±244.16	1340.91±390.45	0.898
Female	872.94±211.46	865.08±253.38	0.982
All	1067.62±162.39	1108.80±235.23	0.906
Lactate (nmol/mg)			
Male	47.31±15.38	117.06±35.89	0.036[$]
Female	34.47±11.27	27.11±9.45	0.644
All	41.05±9.54	73.19±20.03	0.090
Fructose 1,6-bisphosphate-to-glucose-6-phosphate+fructose-6-phosphate ratio			
Male	2.91±0.86	2.23±0.37	0.344
Female	2.01±0.44	1.84±0.34	0.616
All	2.47±0.49	2.04±0.25	0.277
FA	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Oleic acid (pmol/mg)			
Male	100.20±42.96	706.07±292.87	0.099
Female	46.35±6.17	39.08±4.99	0.244
All	73.93±22.35	380.71±157.32	0.053
Acylcarnitines	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
L-carnitine (nmol/mg)			
Male	20.37±1.07	22.48±1.45	0.099
Female	18.18±0.96	19.35±0.75	0.302
All	19.30±0.74	20.95±0.86	0.051
C2 carnitine (nmol/mg)			
Male	1.61±0.16	2.87±0.46	0.007[$]
Female	1.79±0.25	2.25±0.29	0.130
All	1.70±0.15	2.57±0.27	0.002[$]
C3 carnitine (pmol/mg)			
Male	37.73±3.44	38.35±3.92	0.786
Female	32.52±2.51	32.20±2.77	0.929
All	34.97±2.15	35.35±2.44	0.884
C4 carnitine (pmol/mg)			
Male	9.64±1.38	10.15±1.55	0.790
Female	21.78±10.36	11.15±2.15	0.255
All	15.56±5.13	10.64±1.30	0.286
C5 carnitine (pmol/mg)			
Male	7.88±1.40	3.72±0.43	0.003[$]
Female	6.52±1.42	4.77±0.58	0.228
All	7.21±0.99	4.23±0.36	0.003[$]
C5-DC carnitine (pmol/mg)			
Male	2.55±0.30	3.40±0.39	0.043[$]
Female	2.46±0.24	3.04±0.37	0.056
All	2.51±0.19	3.23±0.28	0.005[$]

C6 carnitine (pmol/mg)			
Male	4.63±1.07	6.81±1.62	0.343
Female	8.31±3.14	9.87±3.80	0.762
All	6.43±1.63	8.31±2.02	0.490
C8:0 carnitine (pmol/mg)			
Male	1.51±0.35	2.14±0.63	0.456
Female	2.71±0.98	3.21±1.23	0.767
All	2.10±0.51	2.66±0.68	0.534
C8:1 carnitine (pmol/mg)			
Male	2.16±0.25	2.07±0.26	0.747
Female	2.43±0.26	2.57±0.30	0.601
All	2.29±0.18	2.31±0.20	0.931
C10:0 carnitine (pmol/mg)			
Male	1.63±0.33	2.44±0.81	0.411
Female	2.25±0.64	3.15±0.95	0.477
All	1.93±0.36	2.55±0.62	0.275
C10:1 carnitine (fmol/mg)			
Male	960.63±147.13	1095.66±303.08	0.694
Female	1003.66±250.28	1097.02±250.85	0.796
All	981.63±141.67	1096.32±195.21	0.639
C12:0 carnitine (pmol/mg)			
Male	2.29±0.48	5.38±1.65	0.107
Female	2.64±0.90	4.60±1.75	0.901
All	2.46±0.50	5.00±1.19	0.073
C12:1 carnitine (fmol/mg)			
Male	664.41±127.68	937.82±280.41	0.362
Female	623.27±185.80	665.15±173.34	0.874
All	644.34±110.38	804.81±166.02	0.415
C12-OH carnitine (fmol/mg)			
Male	422.79±198.31	2322.18±985.72	0.049[$]
Female	231.01±54.10	265.46±60.85	0.687
All	329.24±104.78	1318.90±525.47	0.047[$]
C14:0 carnitine (pmol/mg)			
Male	6.84±2.56	13.73±4.21	0.086
Female	3.91±1.27	4.91±1.51	0.642
All	5.41±1.45	9.43±2.36	0.080
C14:1 carnitine (pmol/mg)			
Male	5.19±2.54	7.65±2.53	0.359
Female	2.58±0.86	2.97±1.08	0.787
All	3.92±1.37	5.37±1.43	0.340
C14:2 carnitine (fmol/mg)			
Male	1793.87±851.05	2390.08±803.43	0.480
Female	788.57±251.84	1003.87±391.89	0.662
All	1303.48±454.42	1713.88±461.41	0.399
C14-OH carnitine (fmol/mg)			
Male	285.80±104.11	1042.96±374.23	0.023[$]
Female	166.09±30.14	199.64±32.87	0.417
All	227.41±55.46	631.58±201.39	0.020[$]
C16:0 carnitine (pmol/mg)			
Male	26.03±8.68	61.74±20.26	0.070
Female	15.98±5.60	16.46±4.24	0.949
All	21.13±5.21	39.65±11.05	0.085
C16:1 carnitine (pmol/mg)			
Male	16.96±8.78	34.17±12.80	0.153

Female	7.86±2.85	8.05±2.42	0.962
All	12.53±4.70	21.43±6.90	0.166
C16-OH carnitine (fmol/mg)			
Male	794.71±493.86	4320.21±1900.87	0.036[$]
Female	230.70±69.22	217.99±47.23	0.878
All	519.58±256.04	2319.13±1015.38	0.038[$]
C18:0 carnitine (pmol/mg)			
Male	22.58±7.22	53.17±17.27	0.047[$]
Female	12.28±4.63	10.02±2.13	0.674
All	17.55±4.36	32.12±9.44	0.082
C18:1 carnitine (pmol/mg)			
Male	63.85±27.58	158.00±57.10	0.092
Female	37.97±15.63	33.37±8.30	0.804
All	51.23±15.99	97.21±30.79	0.126
C18:2 carnitine (pmol/mg)			
Male	18.89±8.61	48.73±19.48	0.116
Female	9.25±3.72	8.74±2.23	0.912
All	14.19±4.77	29.22±10.41	0.131
C20:0 carnitine (fmol/mg)			
Male	244.60±54.92	661.88±239.09	0.052
Female	144.72±36.50	128.89±23.69	0.725
All	195.88±33.81	401.88±128.62	0.069
C20:1 carnitine (fmol/mg)			
Male	1479.87±870.66	4086.64±1578.56	0.084
Female	659.33±284.70	541.60±127.85	0.714
All	1079.61±465.96	2348.13±848.32	0.106
C20:2 carnitine (fmol/mg)			
Male	429.41±186.86	1609.57±653.94	0.060
Female	254.63±96.81	241.36±59.36	0.911
All	344.15±106.32	942.15±349.32	0.068
C20:3 carnitine (fmol/mg)			
Male	388.06±181.60	1340.06±619.76	0.113
Female	211.27±93.94	171.26±63.12	0.737
All	301.83±103.37	769.99±328.37	0.134
C20:4 carnitine (fmol/mg)			
Male	913.86±425.39	3734.87±1623.85	0.074
Female	356.63±174.09	306.19±110.22	0.814
All	642.04±235.17	2062.35±866.92	0.082
TCA cycle metabolites	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Citrate (pmol/mg)			
Male	326.81±19.40	591.40±81.79	0.005[$]
Female	312.08±20.63	337.39±27.94	0.403
All	319.63±14.01	467.49±47.93	0.004[$]
Succinate (pmol/mg)			
Male	203.20±51.53	191.09±44.78	0.853
Female	104.04±14.66	128.69±24.31	0.407
All	154.83±28.13	160.65±25.98	0.871
Malate (pmol/mg)			
Male	1218.69±311.50	1782.77±435.58	0.151
Female	782.53±113.19	765.12±91.56	0.913

All	1005.93±170.31	1286.36±238.75	0.191
Nucleotides	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
AMP (pmol/mg)			
Male	69.40±6.11	81.95±5.86	0.046[$]
Female	61.82±2.29	86.58±7.06	0.003[$]
All	65.70±3.34	84.21±4.53	0.015[$]
ADP (nmol/mg)			
Male	13.85±0.60	13.14±0.88	0.475
Female	14.56±0.57	14.95±0.48	0.585
All	14.19±0.41	14.02±0.52	0.776
ATP (nmol/mg)			
Male	13.09±0.78	11.02±1.32	0.167
Female	13.65±0.70	12.76±0.79	0.356
All	13.36±0.52	11.87±0.78	0.092
NAD^+ (nmol/mg)			
Male	15.06±0.84	11.46±1.60	0.057
Female	15.33±0.86	15.02±0.95	0.771
All	15.19±0.60	13.20±0.97	0.070
NADH (pmol/mg)			
Male	828.09±152.06	862.98±183.30	0.986
Female	926.06±216.23	1048.93±169.52	0.695
All	875.88±129.67	960.85±123.78	0.737
NAD^+-to-NADH ratio			
Male	118.56±84.83	52.24±17.37	0.390
Female	175.62±74.96	22.58±3.35	0.053
All	146.40±56.26	36.63±8.91	0.048[$]
NADP (pmol/mg)			
Male	367.63±29.54	312.62±32.46	0.191
Female	311.93±36.08	301.23±30.64	0.802
All	340.46±22.33	306.92±22.07	0.303
FAD (pmol/mg)			
Male	181.99±8.79	210.52±11.75	0.015[$]
Female	177.92±15.47	180.08±11.49	0.905
All	180.00±8.68	195.67±8.47	0.139
Long-chain acylcarnitine-to-FA ratio	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
C18:1 carnitine-to-oleic acid ratio ($x10^{-1}$)			
Male	7.23±1.23	5.68±1.49	0.271
Female	5.77±1.82	8.71±2.04	0.310
All	6.52±1.08	7.16±1.26	0.684
C3 and C5 carnitine-to-substrate ratios	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
C3 carnitine-to-valine ratio ($x10^{-2}$)			

Male	4.14±0.36	4.10±0.39	0.922
Female	3.73±0.28	4.05±0.27	0.204
All	3.94±0.23	4.08±0.24	0.581
C5 carnitine-to-isoleucine+leucine ratio ($x10^{-2}$)			
Male	14.20±2.47	6.25±0.65	0.001[$]
Female	12.74±2.88	9.68±1.08	0.279
All	13.49±1.87	7.92±0.67	0.003[$]
mtDNA profiles	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
mtDNA deletion ratio (%)			
Male	69.79±2.78	71.26±2.40	0.401
Female	65.67±2.42	68.58±1.78	0.209
All	67.78±1.86	69.95±1.50	0.125
mtDNA count number			
Male	1093±97	1109±111	0.881
Female	1304±103	1121±106	0.075
All	1196±72	1115±76	0.266

Table 5.5 Normalized peak areas of untargeted metabolites that were different between non-obese and all obese participants after CR. Data were reported as average±SEM. Neutral mass and retention time were reported as Da and min, respectively. $ = p-value <0.050

Dipeptides (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
Carnosine (226.1024_1.06) (x10^8)			
Male	1.74±0.19	2.55±0.12	0.011$
Female	1.66±0.19	2.13±0.08	0.046$
All	1.70±0.13	2.30±0.07	0.002$
Glycyl-phenylalanine (222.0883_6.79) (x10^5)			
Male	2.33±0.21	2.20±0.13	0.696
Female	3.46±0.55	2.39±0.12	0.006$
All	2.93±0.34	2.31±0.09	0.020$
N-(4-aminobutyryl)-l-histidine (254.1507_6.85) (x10^6)			
Male	5.56±0.20	5.08±0.14	0.175
Female	5.90±0.14	5.19±0.10	0.013$
All	5.74±0.12	5.15±0.08	0.007$
Tryptophyl-valine (303.1412_4.17) (x10^6)			
Male	1.16±0.24	1.90±0.16	0.063
Female	1.90±0.25	2.34±0.15	0.300
All	1.55±0.19	2.16±0.11	0.042$
FAs (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
3-hydroxypentadecanoic acid (258.2186_8.37) (x10^7)			
Male	1.77±0.07	1.76±0.05	0.938
Female	2.01±0.07	1.71±0.03	0.002$
All	1.89±0.05	1.73±0.03	0.021$
Ketooleic acid (308.2030_8.61) (x10^6)			
Male	3.24±0.60	3.32±0.20	0.882
Female	4.48±0.69	2.81±0.18	0.003$
All	3.90±0.47	3.02±0.13	0.026$
Lipids (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
LysoPA(16:0) (410.2419_8.73) (x10^6)			
Male	3.89±0.82	2.16±0.14	<0.001$
Female	3.97±1.48	2.36±0.14	0.016$
All	3.93±0.85	2.28±0.10	<0.001$
LysoPA(18:0) (438.2734_9.11) (x10^6)			
Male	5.43±0.99	4.06±0.26	0.072
Female	5.83±1.14	3.86±0.17	0.002$

All	5.64±0.74	3.94±0.15	<0.001$
LysoPA(18:2) (434.2439_8.54) (x10^5)			
Male	15.43±3.99	8.30±0.48	<0.001$
Female	16.07±5.05	9.18±0.42	0.002$
All	15.77±3.16	8.82±0.32	<0.001$
PA(36:3) (698.4830_9.30) (x10^6)			
Male	1.85±0.35	1.06±0.07	0.001$
Female	2.31±0.33	1.30±0.06	<0.001$
All	2.10±0.24	1.20±0.05	<0.001$
PA(O-42:6) (762.5422_10.77) (x10^5)			
Male	12.50±1.15	9.78±0.32	0.004$
Female	14.90±1.38	9.22±0.28	<0.001$
All	13.78±0.93	9.45±0.21	<0.001$
LysoPC(20:3) (605.3634_8.88) (x10^6)			
Male	2.67±1.06	1.16±0.13	0.006$
Female	1.21±0.37	1.12±0.13	0.821
All	1.89±0.55	1.14±0.09	0.020$
LysoPC(22:2) (635.4111_8.97) (x10^5)			
Male	9.54±2.49	3.92±0.39	<0.001$
Female	4.49±1.13	4.66±0.44	0.894
All	6.85±1.43	4.36±0.31	0.011$
LysoPE(20:0) (561.3344_8.80) (x10^6)			
Male	2.36±0.46	1.66±0.12	0.051
Female	1.67±0.32	1.40±0.09	0.307
All	1.99±0.28	1.51±0.07	0.026$
LysoPE(20:1) (567.3513_8.44) (x10^7)			
Male	3.28±0.34	2.68±0.11	0.049$
Female	3.08±0.31	2.76±0.10	0.267
All	3.18±0.22	2.73±0.07	0.032$
LysoPE(20:4) (501.2856_8.25) (x10^7)			
Male	6.14±0.93	3.93±0.21	<0.001$
Female	5.37±1.26	4.05±0.22	0.082
All	5.73±0.78	4.00±0.16	<0.001$
LysoPE(P-16:0) (721.4498_8.68) (x10^6)			
Male	2.69±0.49	1.78±0.11	0.007$
Female	2.04±0.33	1.84±0.12	0.568
All	2.34±0.29	1.82±0.08	0.029$
PE(36:3) (741.5288_9.82) (x10^7)			
Male	2.78±0.24	1.92±0.07	<0.001$
Female	2.19±0.19	2.07±0.07	0.562
All	2.47±0.16	2.01±0.05	0.003$
PE(36:4) (739.5151_9.70) (x10^7)			
Male	4.63±0.15	3.83±0.12	0.007$

Female	4.33±0.37	3.65±0.10	0.023[$]
All	4.47±0.21	3.72±0.07	<0.001[$]
PE(38:4)OH (783.5330_9.50) (x10^6)			
Male	4.47±0.87	2.99±0.24	0.033[$]
Female	3.28±0.58	2.60±0.17	0.192
All	3.83±0.52	2.76±0.14	0.011[$]
PE(P-36:4) (723.5166_9.88) (x10^7)			
Male	4.20±0.64	2.92±0.11	<0.001[$]
Female	3.65±0.21	3.18±0.12	0.184
All	3.91±0.32	3.07±0.09	0.001[$]
PE(P-38:5) (749.5365_9.91) (x10^7)			
Male	4.06±0.37	3.82±0.14	0.502
Female	4.54±0.26	3.47±0.09	<0.001[$]
All	4.32±0.22	3.61±0.08	0.002[$]
PE(O-44:6) (833.6155_10.46) (x10^7)			
Male	6.30±0.37	5.19±0.15	0.006[$]
Female	6.51±0.27	5.03±0.10	<0.001[$]
All	6.41±0.22	5.09±0.08	<0.001[$]
PG(34:1) (748.5262_10.54) (x10^7)			
Male	4.30±0.34	3.63±0.14	0.064
Female	4.93±0.49	3.50±0.09	<0.001[$]
All	4.64±0.31	3.55±0.08	<0.001[$]
PG(P-36:5) (848.4519_10.55) (x10^5)			
Male	7.81±0.63	6.66±0.27	0.105
Female	8.87±0.81	6.84±0.20	0.002[$]
All	8.37±0.52	6.77±0.16	<0.001[$]
PS(O-36:3) (771.5340_9.57) (x10^6)			
Male	10.84±1.36	8.54±0.37	0.032[$]
Female	10.37±0.87	8.08±0.43	0.061
All	10.59±0.76	8.27±0.29	0.005[$]
PI(38:4) (886.5584_10.43) (x10^8)			
Male	4.92±0.26	4.78±0.17	0.735
Female	6.22±0.61	4.23±0.11	<0.001[$]
All	5.62±0.38	4.45±0.10	<0.001[$]
PI-ceramide(d28:2) (693.4124_8.74) (x10^6)			
Male	3.28±0.81	2.05±0.14	0.012[$]
Female	2.42±0.52	2.04±0.14	0.377
All	2.82±0.46	2.04±0.10	0.015[$]
Ceramide(d40:1) (681.6250_10.71) (x10^6)			
Male	2.01±0.30	1.92±0.13	0.788
Female	3.09±0.20	2.00±0.11	<0.001[$]
All	2.59±0.22	1.97±0.08	0.008[$]
Ceramide(t42:0) (667.6105_10.59) (x10^6)			

Male	5.15±0.62	4.45±0.22	0.230
Female	5.34±0.24	4.00±0.19	0.011[$]
All	5.26±0.30	4.18±0.14	0.006[$]
Lactosyl sphingosine(d16:1) (595.3681_8.76) (x10^6)			
Male	2.87±0.87	1.56±0.17	0.016[$]
Female	1.52±0.40	1.41±0.11	0.735
All	2.15±0.48	1.47±0.09	0.025[$]
TCA cycle metabolites (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
Fumarate (115.9962_0.91) (x10^7)			
Male	4.09±0.25	3.76±0.14	0.336
Female	5.11±0.41	3.67±0.10	<0.001[$]
All	4.63±0.23	3.71±0.08	<0.001[$]
Nucleotides (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
Nicotinate beta-d-ribonucleotide (336.0495_1.18) (x10^6)			
Male	15.12±2.63	9.44±0.76	0.010[$]
Female	15.84±4.26	9.29±0.75	0.012[$]
All	15.50±2.49	9.35±0.53	<0.001[$]
Other metabolites (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
2-keto-6-acetamidocaproate (187.0619_4.06) (x10^5)			
Male	13.08±3.49	7.10±0.16	0.146
Female	13.08±3.49	6.52±0.86	0.016[$]
All	13.08±2.38	6.76±0.81	0.007[$]
3-hydroxydodecanedioic acid (246.1450_5.69) (x10^7)			
Male	4.08±0.15	3.94±0.11	0.588
Female	4.56±0.18	3.87±0.08	0.002[$]
All	4.34±0.13	3.89±0.06	0.010[$]
5,6-dihydroxyindole-2-carboxylic acid (193.0250_1.17) (x10^7)			
Male	2.33±0.42	1.53±0.09	0.006[$]
Female	2.01±0.36	1.58±0.10	0.177
All	2.16±0.27	1.56±0.07	0.006[$]
Calcitroic acid (374.2597_9.28) (x10^6)			
Male	1.51±0.09	1.41±0.03	0.256
Female	1.56±0.07	1.37±0.04	0.086
All	1.53±0.05	1.38±0.03	0.038[$]
Neuromedin N (617.3690_8.48) (x10^6)			
Male	3.01±0.43	2.38±0.15	0.126
Female	2.74±0.52	2.13±0.10	0.065

All	2.86±0.33	2.23±0.08	0.014$
Phosphocreatine (477.0836_1.20) (x10^6)			
Male	11.51±1.61	8.01±0.52	0.016$
Female	10.24±2.21	8.26±0.55	0.245
All	10.83±1.36	8.16±0.39	0.019$
Reduced glutathione (432.0969_1.69) (x10^6)			
Male	2.16±0.32	1.93±0.15	0.531
Female	3.18±0.77	2.04±0.13	0.014$
All	2.71±0.44	1.99±0.10	0.021$
Vanillin 4-sulfate (232.0022_4.71) (x10^6)			
Male	5.03±1.29	3.09±0.39	0.079
Female	4.00±0.65	2.78±0.19	0.037$
All	4.48±0.68	2.90±0.20	0.006$
Unknown metabolites (neutral mass_retention time)	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
Unknown (287.9841_1.07) (x10^6)			
Male	3.45±0.67	2.16±0.20	0.024$
Female	4.99±1.41	2.51±0.20	0.001$
All	4.27±0.81	2.37±0.15	<0.001$
Unknown (379.9179_0.98) (x10^6)			
Male	4.13±0.24	3.72±0.18	0.368
Female	5.09±0.37	3.99±0.14	0.009$
All	4.65±0.25	3.88±0.11	0.014$
Unknown (474.1936_0.95) (x10^6)			
Male	2.73±0.43	4.44±0.26	0.012$
Female	2.69±0.47	3.60±0.21	0.138
All	2.71±0.31	3.94±0.17	0.008$
Unknown (547.0832_1.19) (x10^6)			
Male	10.04±1.74	5.30±0.51	0.001$
Female	9.53±2.96	5.66±0.53	0.034$
All	9.77±1.72	5.52±0.38	<0.001$
Unknown (591.3519_8.33) (x10^6)			
Male	10.19±0.93	8.23±0.45	0.094
Female	9.80±2.10	7.39±0.28	0.026$
All	9.98±1.17	7.73±0.25	0.004$
Unknown (609.3357_8.43) (x10^7)			
Male	1.89±0.12	1.56±0.06	0.044$
Female	1.66±0.15	1.47±0.05	0.178
All	1.77±0.10	1.51±0.04	0.015$
Unknown (619.3638_8.70) (x10^6)			
Male	2.43±0.80	1.38±0.13	0.021$
Female	1.43±0.37	1.21±0.10	0.489

All	1.89±0.43	1.28±0.08	0.020[$]
Unknown (633.0985_1.21) (x10^6)			
Male	17.93±4.83	9.10±1.11	0.010[$]
Female	14.01±5.72	11.01±1.32	0.471
All	15.84±3.69	10.24±0.91	0.042[$]
Unknown (635.3788_8.70) (x10^6)			
Male	3.88±1.27	1.57±0.18	0.001[$]
Female	2.03±0.80	1.58±0.18	0.426
All	2.89±0.74	1.58±0.13	0.003[$]
Unknown (672.0854_1.20) (x10^6)			
Male	2.58±0.54	1.59±0.12	0.010[$]
Female	2.95±0.68	1.72±0.11	0.003[$]
All	2.78±0.43	1.67±0.08	<0.001[$]
Unknown (693.0396_1.22) (x10^6)			
Male	2.62±0.20	2.19±0.10	0.085
Female	2.78±0.24	2.32±0.08	0.060
All	2.71±0.15	2.27±0.06	0.012[$]
Unknown (743.0656_1.51) (x10^6)			
Male	4.67±0.37	3.65±0.17	0.020[$]
Female	4.62±0.43	3.62±0.14	0.018[$]
All	4.64±0.28	3.63±0.11	<0.001[$]
Unknown (763.5656_10.32) (x10^6)			
Male	3.94±0.65	5.87±0.30	0.014[$]
Female	4.69±0.40	5.32±0.20	0.274
All	4.34±0.37	5.54±0.17	0.010[$]
Unknown (769.0324_1.22) (x10^6)			
Male	2.29±0.30	1.80±0.10	0.070
Female	2.50±0.34	1.78±0.09	0.009[$]
All	2.40±0.22	1.79±0.07	0.001[$]
Unknown (823.5687_8.79) (x10^6)			
Male	5.32±0.72	4.08±0.17	0.020[$]
Female	4.63±0.63	3.68±0.16	0.051
All	4.95±0.47	3.84±0.12	0.002[$]
Unknown (827.5675_9.76) (x10^7)			
Male	4.78±0.53	3.97±0.14	0.054
Female	4.25±0.38	3.60±0.09	0.023[$]
All	4.50±0.32	3.75±0.08	0.002[$]
Unknown (829.5778_9.82) (x10^7)			
Male	4.35±0.33	3.61±0.15	0.054
Female	4.21±0.22	3.76±0.11	0.150
All	4.28±0.19	3.70±0.09	0.016[$]
Unknown (831.5981_10.18) (x10^7)			
Male	11.76±0.69	9.81±0.30	0.014[$]

Female	12.34±0.59	9.66±0.22	<0.001[$]
All	12.07±0.44	9.72±0.18	<0.001[$]
Unknown (866.1155_1.21) (x10^6)			
Male	2.96±0.60	1.73±0.14	0.004[$]
Female	2.44±0.58	1.86±0.14	0.180
All	2.68±0.41	1.80±0.10	0.004[$]

Table 5.6 Normalized peak areas of untargeted metabolites that were significantly changed after CR in 41 obese participants. Data were reported as average±SEM. Neutral mass and retention time were reported as Da and min, respectively. $ = p-value <0.050

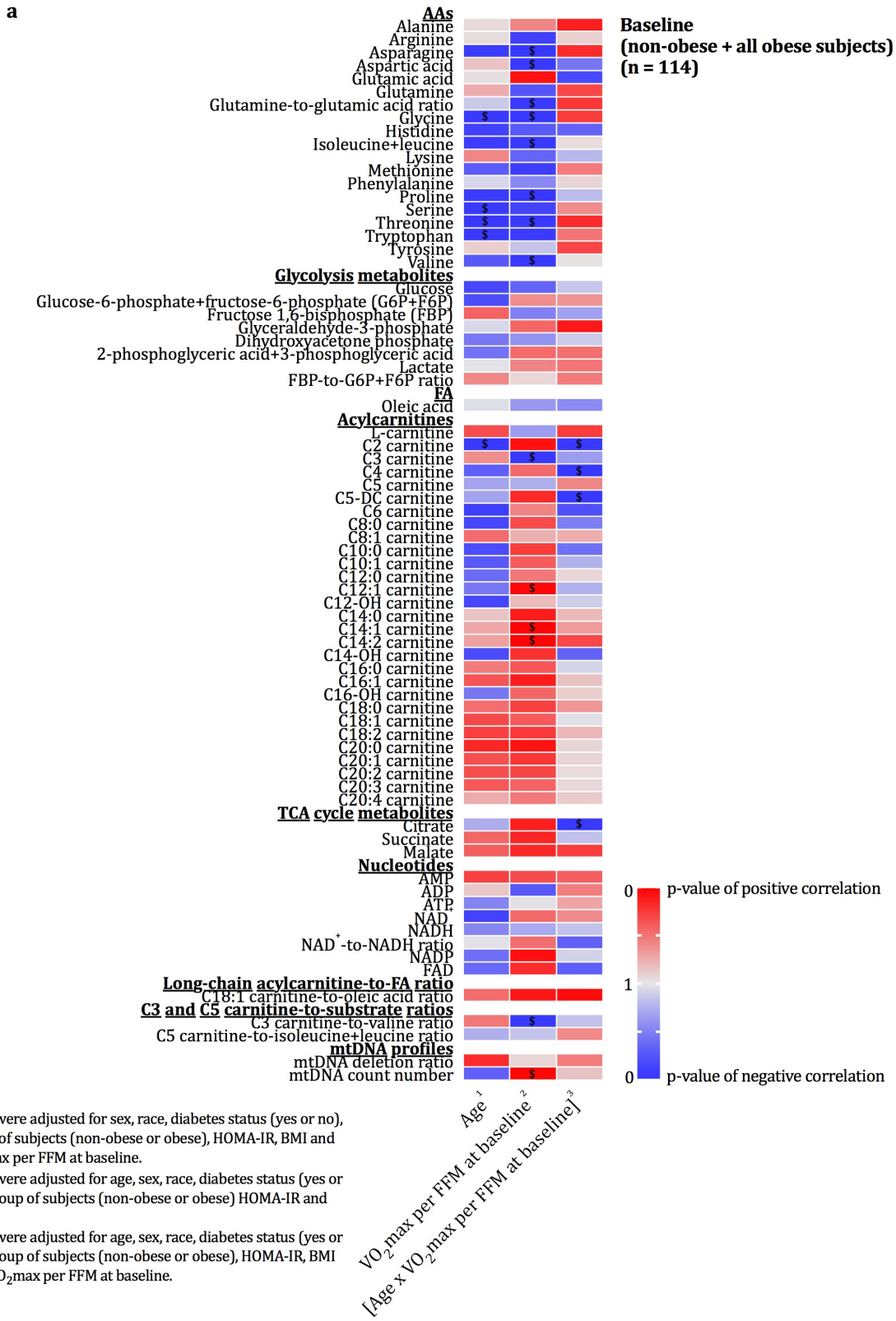
Dipeptides (neutral mass_retention time)	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Alanyl-glutamine (217.0934_2.83) (x10^5)			
Male	4.57±0.49	8.52±1.09	0.001$
Female	7.10±0.92	9.13±1.10	0.010$
All	5.81±0.55	8.81±0.77	<0.001$
Tryptophyl-valine (303.1412_4.17) (x10^6)			
Male	2.05±0.21	1.65±0.21	0.029$
Female	2.19±0.30	1.91±0.19	0.192
All	2.12±0.18	1.78±0.14	0.013$
FAs (neutral mass_retention time)	**Obese at baseline**	**Obese after CR**	**p-value of obese at baseline vs. obese after CR**
Arachidonic acid (446.3331_9.05) (x10^5)			
Male	4.25±0.90	2.83±0.38	0.141
Female	7.66±1.34	5.66±1.27	0.109
All	5.91±0.85	4.21±0.68	0.027$
Ketopalmitic acid (270.2158_8.58) (x10^6)			
Male	2.92±0.15	3.26±0.21	0.107
Female	2.69±0.11	2.92±0.15	0.232
All	2.81±0.09	3.10±0.13	0.042$
Lipids (neutral mass_retention time)	**Obese at baseline**	**Obese after CR**	**p-value of obese at baseline vs. obese after CR**
PA(O-42:6) (762.5422_10.77) (x10^5)			
Male	9.96±0.42	13.31±1.02	0.005$
Female	9.93±0.49	10.49±0.51	0.264
All	9.95±0.32	11.93±0.61	0.003$
PA(36:1)OH (718.5238_9.40) (x10^6)			
Male	9.38±0.35	8.85±0.61	0.385
Female	15.42±0.91	13.44±0.80	0.009$
All	12.33±0.67	11.09±0.61	0.011$
PC(28:0) (737.5373_9.98) (x10^6)			
Male	3.76±0.17	3.84±0.16	0.624
Female	4.11±0.27	4.60±0.35	0.026$
All	3.93±0.16	4.21±0.20	0.042$
PC(36:4) (841.5952_10.24) (x10^6)			
Male	6.81±0.36	7.33±0.32	0.127
Female	6.37±0.29	7.29±0.36	0.015$
All	6.60±0.23	7.31±0.24	0.004$
LysoPE(18:0) (481.3144_8.43) (x10^7)			

Male	5.65±0.28	20.89±7.97	0.040[$]
Female	5.92±0.31	6.65±0.35	0.085
All	5.79±0.21	15.09±4.24	0.034[$]
LysoPE(20:1) (567.3513_8.44) (x10^7)			
Male	2.52±0.14	3.12±0.24	0.022[$]
Female	2.82±0.16	3.19±0.32	0.314
All	2.66±0.11	3.15±0.20	0.027[$]
PE(36:5) (737.4261_8.77) (x10^6)			
Male	7.31±1.11	5.48±0.62	0.007[$]
Female	10.22±0.70	8.67±0.69	0.032[$]
All	8.73±0.69	7.03±0.52	<0.001[$]
PE(P-34:2) (699.5203_9.93) (x10^5)			
Male	9.61±0.57	8.40±0.54	0.004[$]
Female	20.26±2.46	16.26±2.00	<0.001[$]
All	14.81±1.48	12.23±1.18	<0.001[$]
PE(P-36:2) (727.5424_10.10) (x10^7)			
Male	3.28±0.19	2.63±0.19	<0.001[$]
Female	4.46±0.24	3.48±0.20	<0.001[$]
All	3.86±0.18	3.04±0.15	<0.001[$]
PE(P-36:3) (725.5296_9.95) (x10^7)			
Male	1.27±0.10	1.19±0.10	0.148
Female	2.68±0.27	2.34±0.28	0.004[$]
All	1.96±0.18	1.75±0.17	0.001[$]
PE(P-38:5) (749.5365_9.91) (x10^7)			
Male	3.81±0.14	4.13±0.17	0.157
Female	3.68±0.17	4.40±0.24	<0.001[$]
All	3.75±0.11	4.26±0.15	<0.001[$]
PG(34:1) (748.5262_10.54) (x10^7)			
Male	3.77±0.19	4.62±0.29	<0.001[$]
Female	3.84±0.15	3.94±0.20	0.534
All	3.81±0.12	4.29±0.18	0.002[$]
PG(P-36:5) (848.4519_10.55) (x10^5)			
Male	7.13±0.42	8.14±0.43	0.005[$]
Female	7.50±0.33	7.67±0.44	0.677
All	7.31±0.27	7.91±0.31	0.026[$]
Lactosyl sphingosine(d16:2) (593.3368_8.32) (x10^6)			
Male	2.52±0.13	3.56±0.43	0.021[$]
Female	2.07±0.07	2.73±0.16	<0.001[$]
All	2.30±0.08	3.16±0.24	<0.001[$]
Other metabolites (neutral mass_retention time)	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
1,25-dihydroxyvitamin D_3 3-glycoside (578.3776_9.31) (x10^5)			
Male	7.80±0.63	7.27±0.73	0.427

Female	7.12±0.53	5.54±0.57	<0.001$
All	7.46±0.41	6.43±0.48	0.009$
Glycocholic acid (468.3664_9.02) (x10^6)			
Male	1.80±0.23	1.42±0.18	0.142
Female	2.22±0.31	1.82±0.35	0.159
All	2.01±0.19	1.62±0.19	0.038$
Neuromedin N (617.3690_8.48) (x10^6)			
Male	2.39±0.23	2.99±0.30	0.134
Female	2.42±0.16	3.63±0.38	0.003$
All	2.40±0.14	3.30±0.25	0.002$
Pantothenic acid (219.1122_3.89) (x10^7)			
Male	3.92±0.57	7.18±0.71	<0.001$
Female	3.49±0.45	4.20±0.42	0.206
All	3.71±0.36	5.73±0.48	<0.001$
Unknown metabolites (neutral mass_retention time)	Obese at baseline	Obese after CR	p-value of obese at baseline vs. obese after CR
Unknown (287.9841_1.07) (x10^6)			
Male	2.34±0.27	2.97±0.29	0.057
Female	2.16±0.29	2.84±0.25	0.061
All	2.25±0.20	2.91±0.19	0.007$
Unknown (361.1471_4.02) (x10^6)			
Male	3.32±0.22	2.86±0.22	0.056
Female	3.79±0.33	3.59±0.29	0.354
All	3.55±0.20	3.22±0.19	0.036$
Unknown (434.3319_9.24) (x10^6)			
Male	1.63±0.08	1.41±0.09	0.025$
Female	1.69±0.14	1.53±0.13	0.354
All	1.66±0.08	1.47±0.08	0.044$
Unknown (591.3519_8.33) (x10^6)			
Male	8.40±0.64	9.02±0.46	0.356
Female	8.21±0.55	10.63±0.82	0.002$
All	8.31±0.42	9.80±0.48	0.003$
Unknown (598.3691_8.93) (x10^6)			
Male	3.54±0.75	1.73±0.35	0.015$
Female	3.29±0.69	2.41±0.43	0.224
All	3.42±0.51	2.06±0.28	0.008$
Unknown (606.4157_9.47) (x10^6)			
Male	2.23±0.54	2.97±0.44	0.058$
Female	1.67±0.25	2.52±0.37	0.002$
All	1.96±0.30	2.75±0.29	<0.001$
Unknown (619.3638_8.70) (x10^6)			
Male	1.24±0.11	1.69±0.34	0.226
Female	1.45±0.18	2.15±0.43	0.119

All	1.34±0.11	1.92±0.27	0.045[$]
Unknown (664.0899_0.98) (x10^6)			
Male	2.19±0.13	1.62±0.15	0.214
Female	1.76±0.16	1.69±0.15	0.725
All	1.98±0.12	1.65±0.11	0.022[$]
Unknown (672.0854_1.2) (x10^6)			
Male	1.51±0.17	2.12±0.18	0.013[$]
Female	1.68±0.10	2.15±0.31	0.151
All	1.59±0.10	2.14±0.18	0.006[$]
Unknown (693.0396_1.22) (x10^6)			
Male	2.18±0.14	2.59±0.13	0.033[$]
Female	2.17±0.09	2.29±0.09	0.337
All	2.18±0.08	2.44±0.08	0.019[$]
Unknown (743.0656_1.51) (x10^6)			
Male	3.91±0.18	3.08±0.31	0.014[$]
Female	4.05±0.15	3.88±0.24	0.391
All	3.98±0.12	3.47±0.20	0.010[$]
Unknown (801.5529_9.73) (x10^7)			
Male	5.42±0.13	4.77±0.17	0.002[$]
Female	6.59±0.24	5.85±0.24	0.004[$]
All	5.99±0.16	5.30±0.17	<0.001[$]

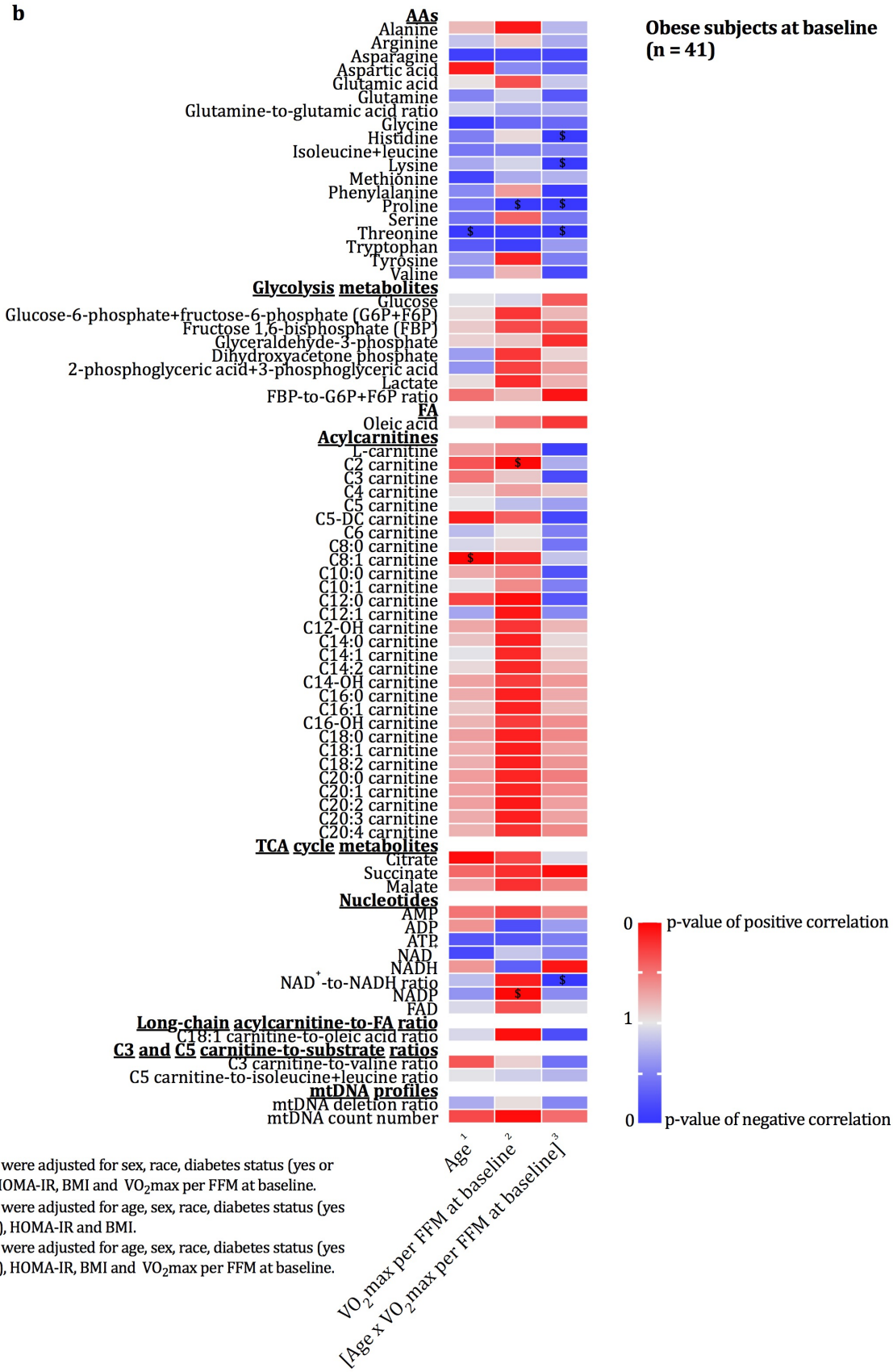
Figure 5.1 Correlations of targeted metabolites and mtDNA profiles vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]. $ = p-value <0.050.



[1]Data were adjusted for sex, race, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR, BMI and VO_2max per FFM at baseline.

[2]Data were adjusted for age, sex, race, diabetes status (yes or no), group of subjects (non-obese or obese) HOMA-IR and BMI.

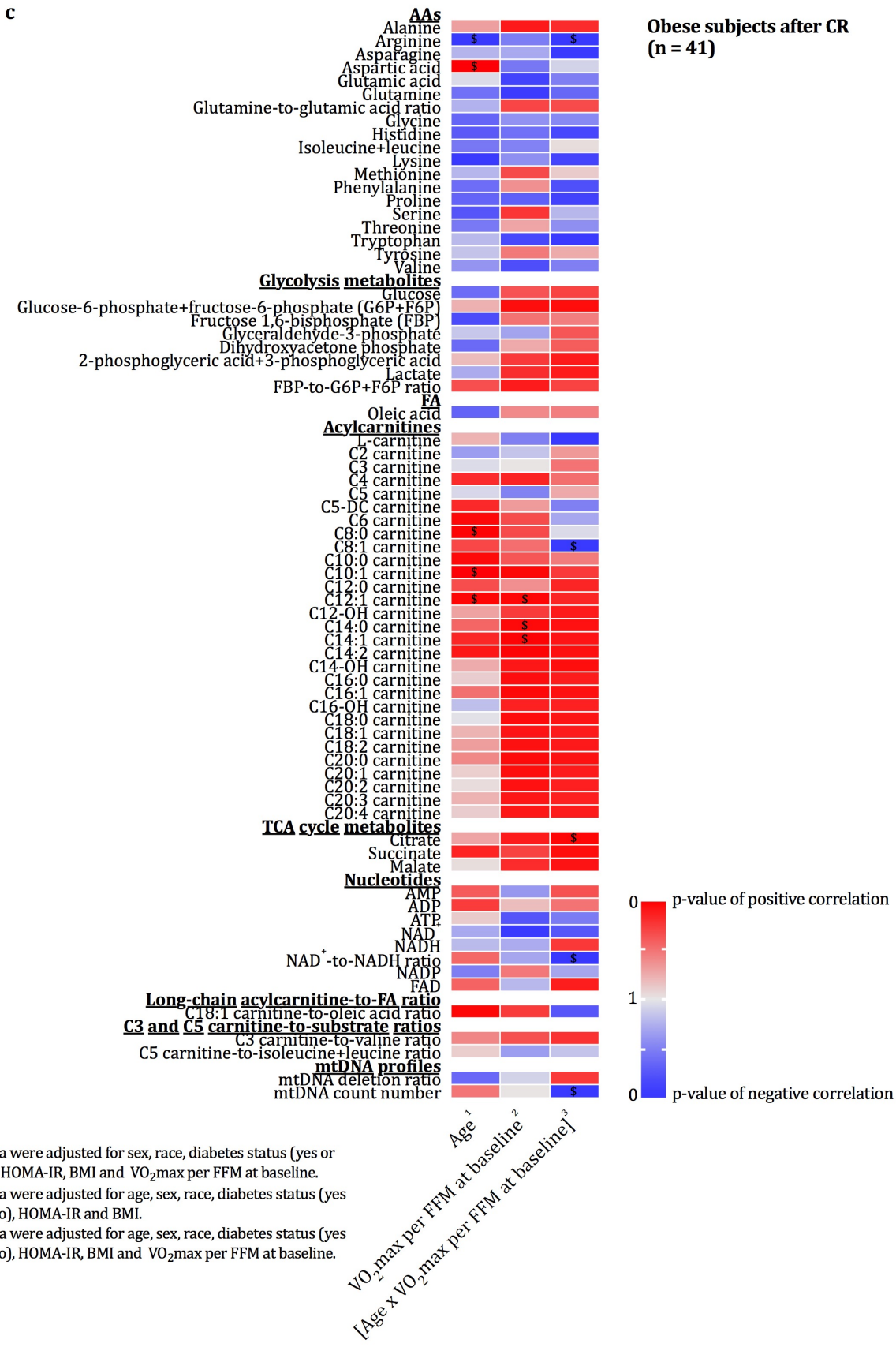
[3]Data were adjusted for age, sex, race, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR, BMI and VO_2max per FFM at baseline.



[1]Data were adjusted for sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.

[2]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.

[3]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.

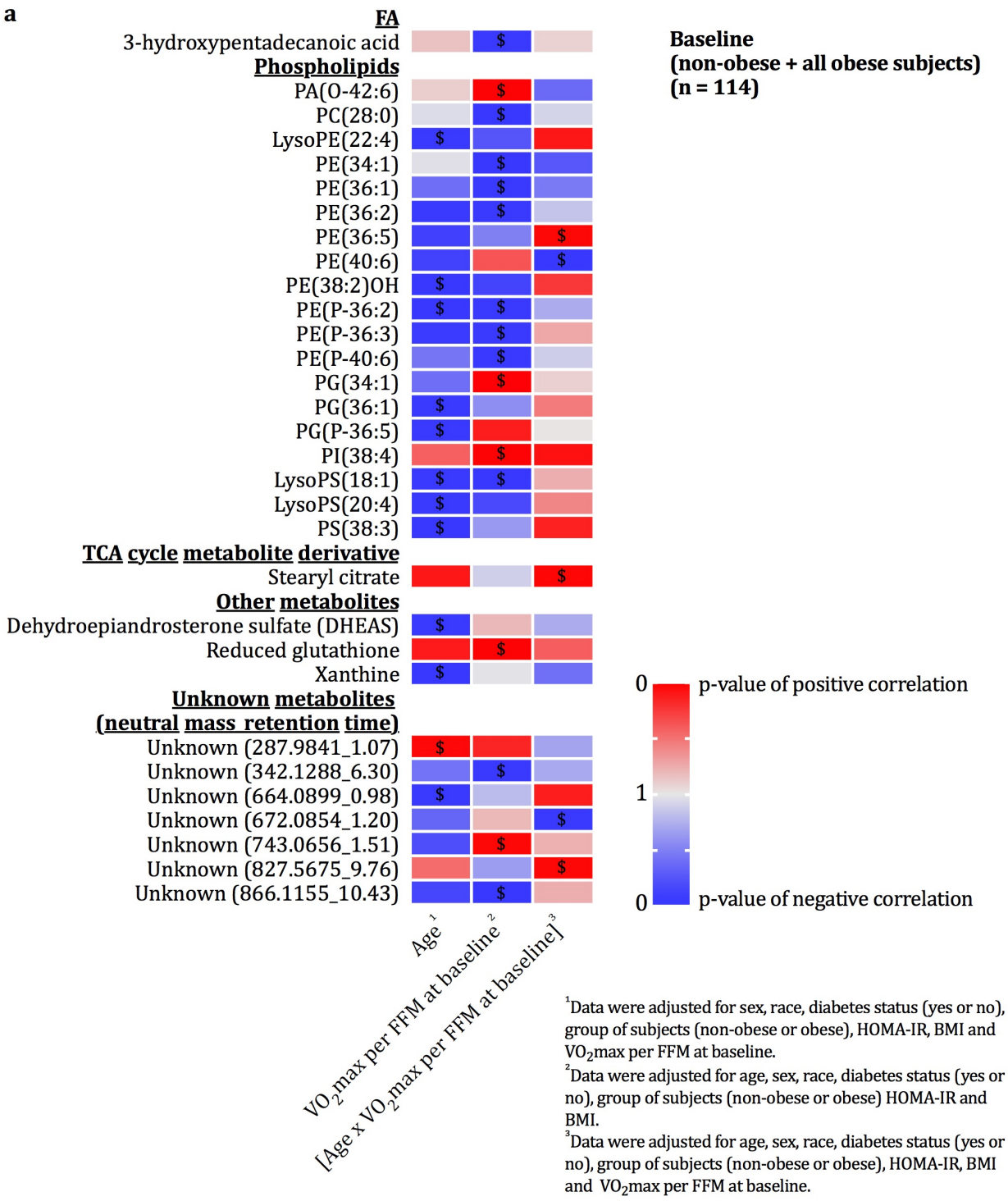


[1]Data were adjusted for sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.

[2]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.

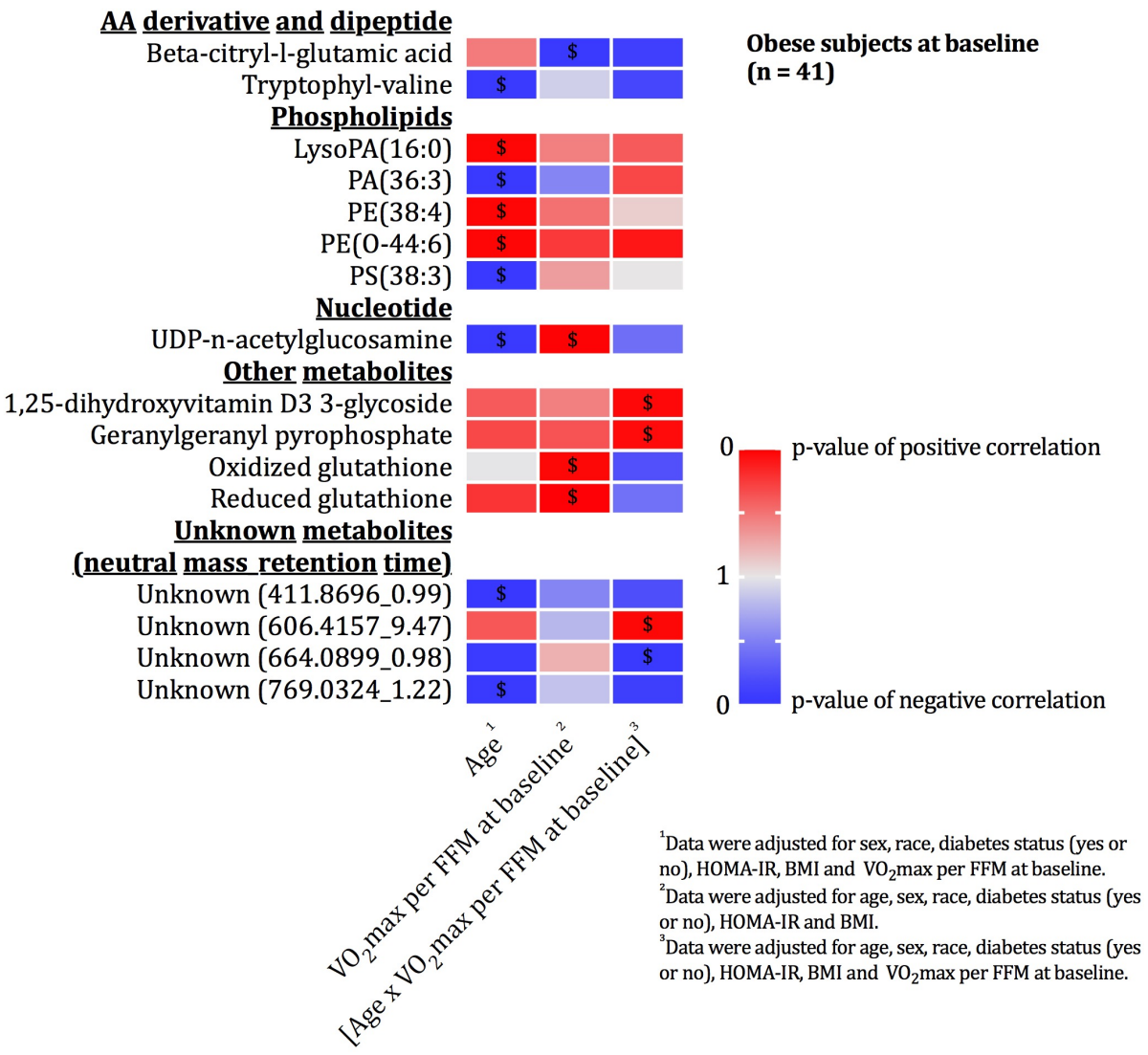
[3]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.

Figure 5.2 Correlations of untargeted metabolites and mtDNA profiles vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]. $ = p-value <0.050.



[1]Data were adjusted for sex, race, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR, BMI and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, diabetes status (yes or no), group of subjects (non-obese or obese) HOMA-IR and BMI.
[3]Data were adjusted for age, sex, race, diabetes status (yes or no), group of subjects (non-obese or obese), HOMA-IR, BMI and VO_2max per FFM at baseline.

b



[1]Data were adjusted for sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.
[3]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO_2max per FFM at baseline.

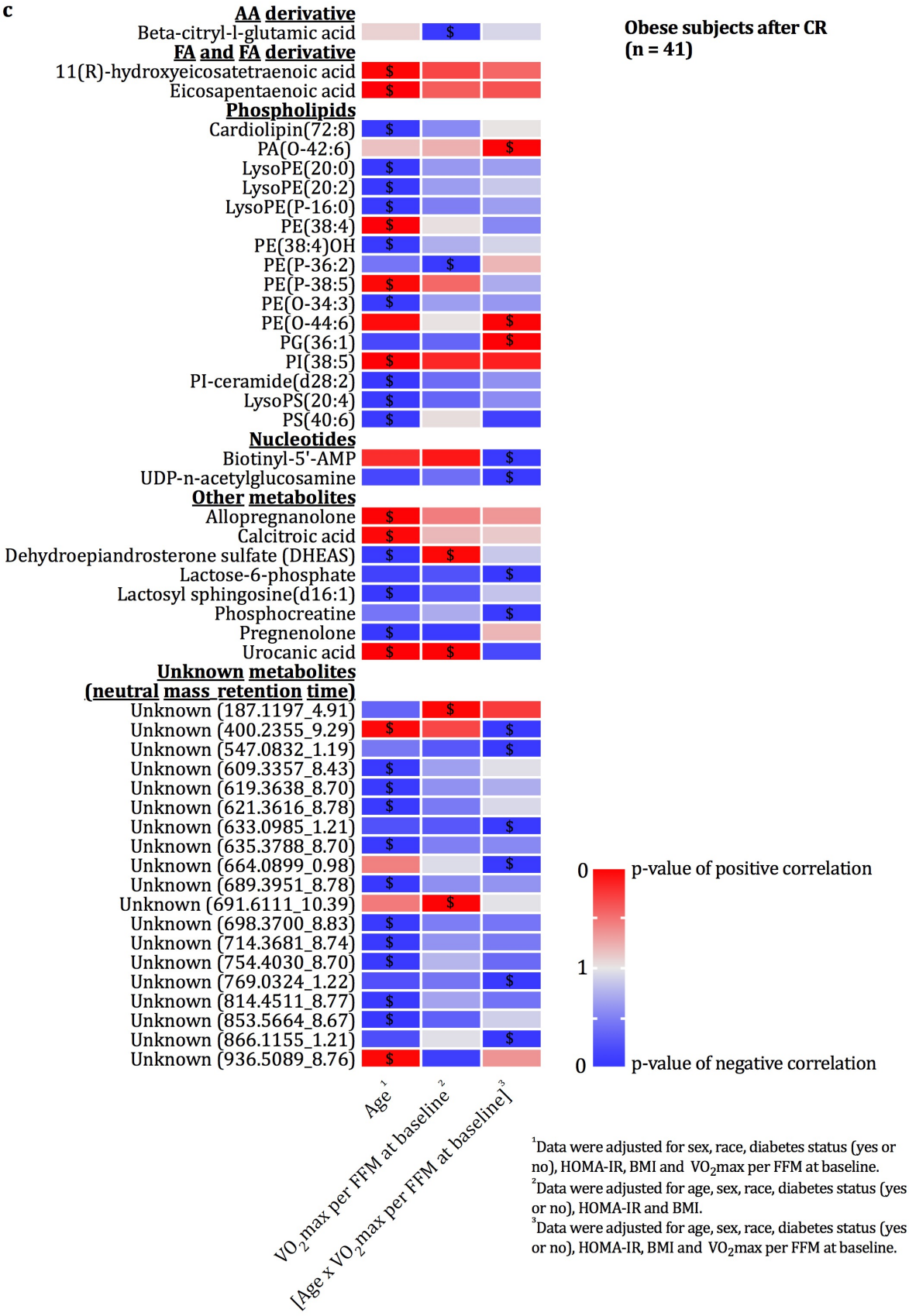
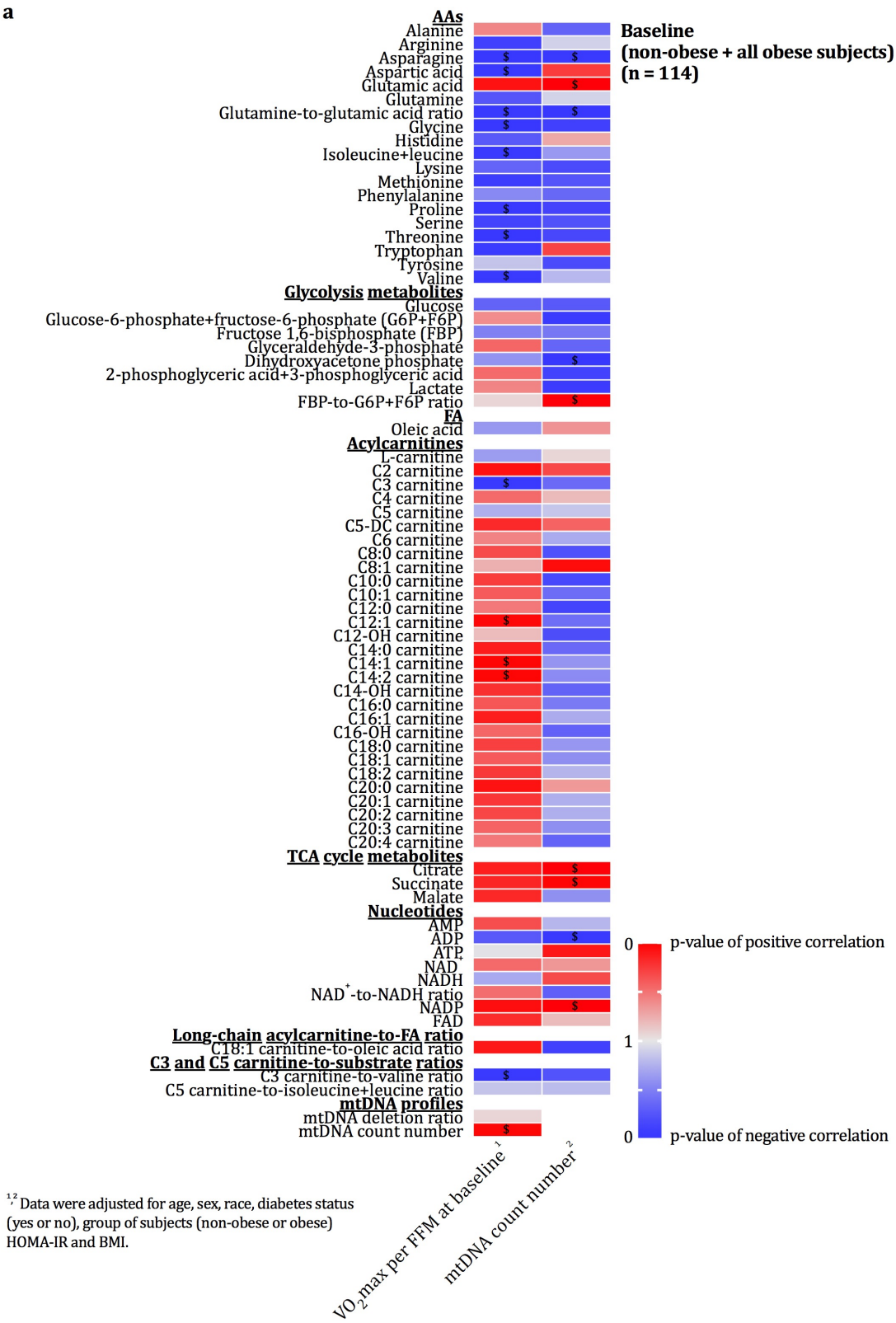
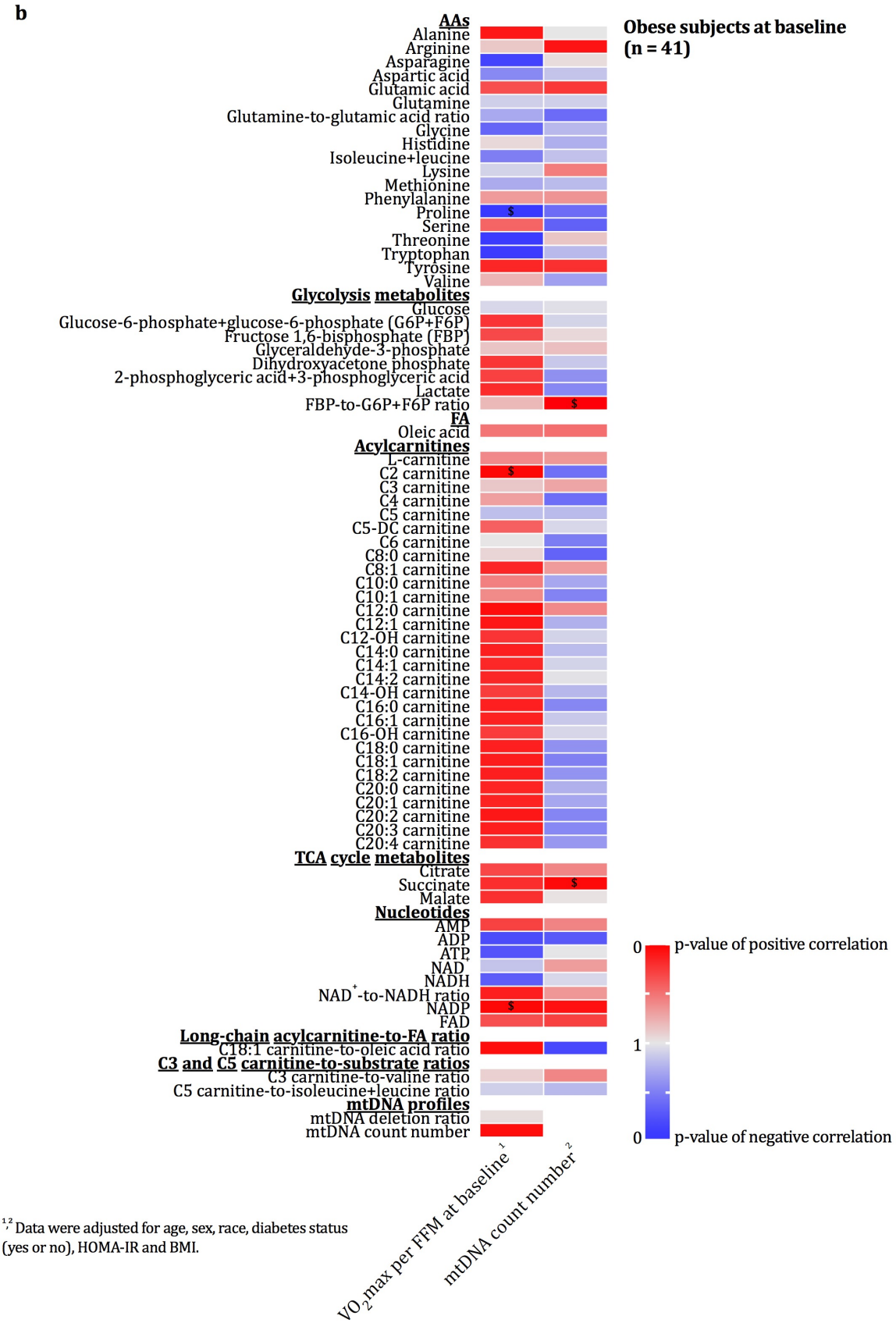
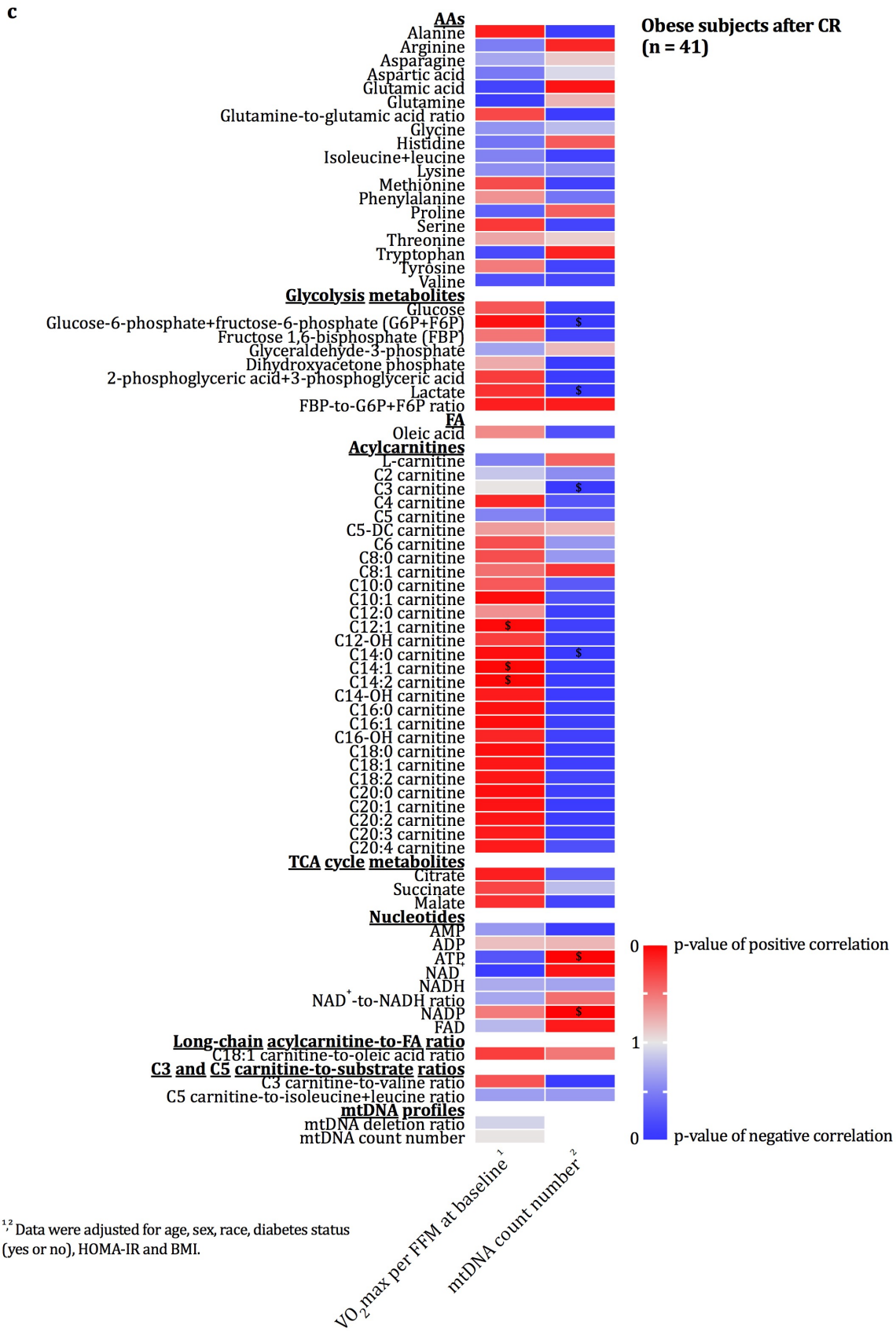

c
AA derivative
Beta-citryl-l-glutamic acid
FA and FA derivative
11(R)-hydroxyeicosatetraenoic acid
Eicosapentaenoic acid
Phospholipids
Cardiolipin(72:8)
PA(O-42:6)
LysoPE(20:0)
LysoPE(20:2)
LysoPE(P-16:0)
PE(38:4)
PE(38:4)OH
PE(P-36:2)
PE(P-38:5)
PE(O-34:3)
PE(O-44:6)
PG(36:1)
PI(38:5)
PI-ceramide(d28:2)
LysoPS(20:4)
PS(40:6)
Nucleotides
Biotinyl-5'-AMP
UDP-n-acetylglucosamine
Other metabolites
Allopregnanolone
Calcitroic acid
Dehydroepiandrosterone sulfate (DHEAS)
Lactose-6-phosphate
Lactosyl sphingosine(d16:1)
Phosphocreatine
Pregnenolone
Urocanic acid
Unknown metabolites (neutral mass_retention time)
Unknown (187.1197_4.91)
Unknown (400.2355_9.29)
Unknown (547.0832_1.19)
Unknown (609.3357_8.43)
Unknown (619.3638_8.70)
Unknown (621.3616_8.78)
Unknown (633.0985_1.21)
Unknown (635.3788_8.70)
Unknown (664.0899_0.98)
Unknown (689.3951_8.78)
Unknown (691.6111_10.39)
Unknown (698.3700_8.83)
Unknown (714.3681_8.74)
Unknown (754.4030_8.70)
Unknown (769.0324_1.22)
Unknown (814.4511_8.77)
Unknown (853.5664_8.67)
Unknown (866.1155_1.21)
Unknown (936.5089_8.76)
Age [1]
VO2max per FFM at baseline [2]
[Age x VO2max per FFM at baseline] [3]
Obese subjects after CR (n = 41)
0
p-value of positive correlation
1
0
p-value of negative correlation
[1]Data were adjusted for sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.
[3]Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR, BMI and VO2max per FFM at baseline.

Figure 5.3 Correlations of targeted metabolites and mtDNA profiles vs. VO_2max per FFM at baseline, as well as targeted metabolites vs. mtDNA count number. $ = p-value <0.050.



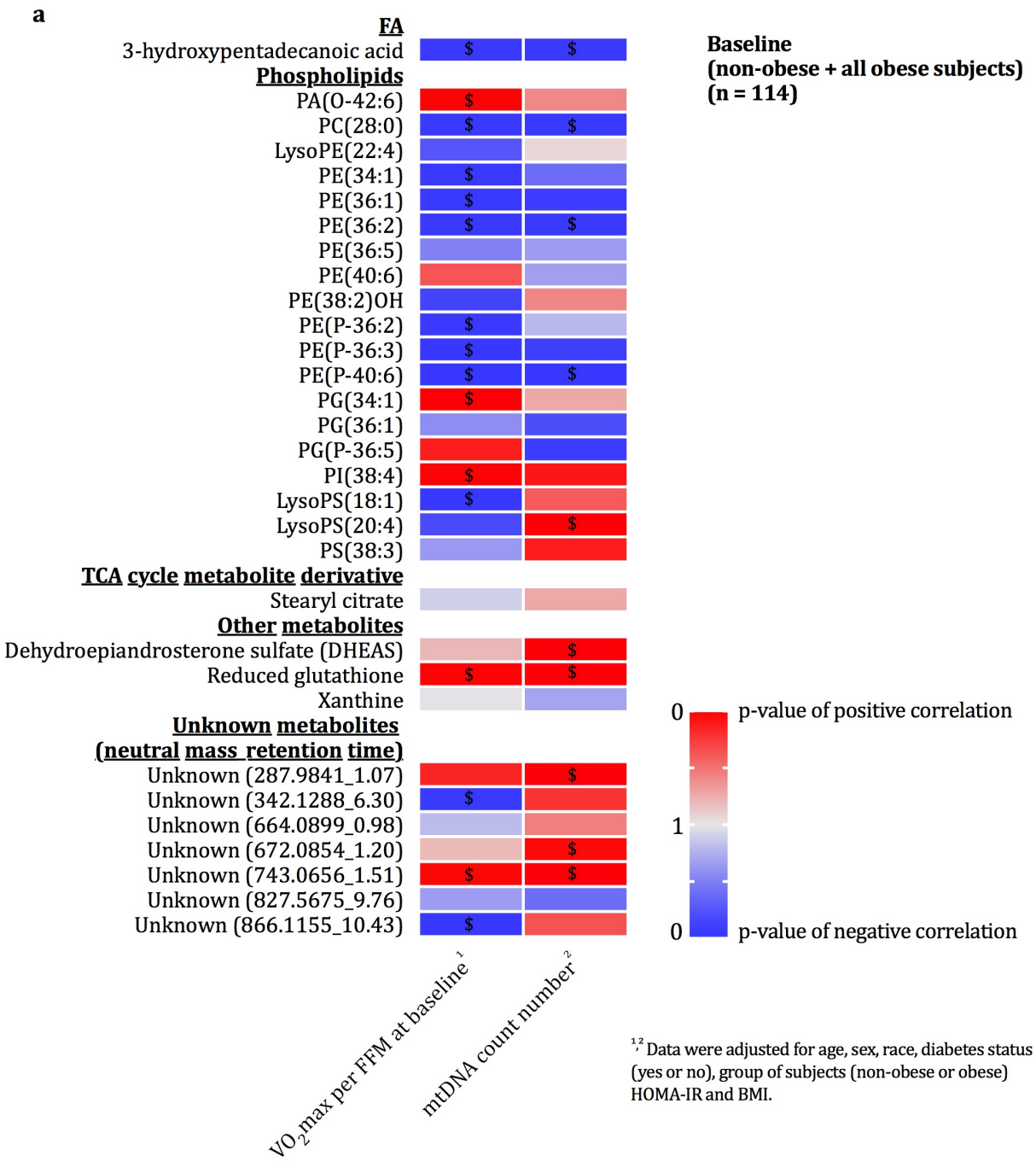


[1,2] Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.

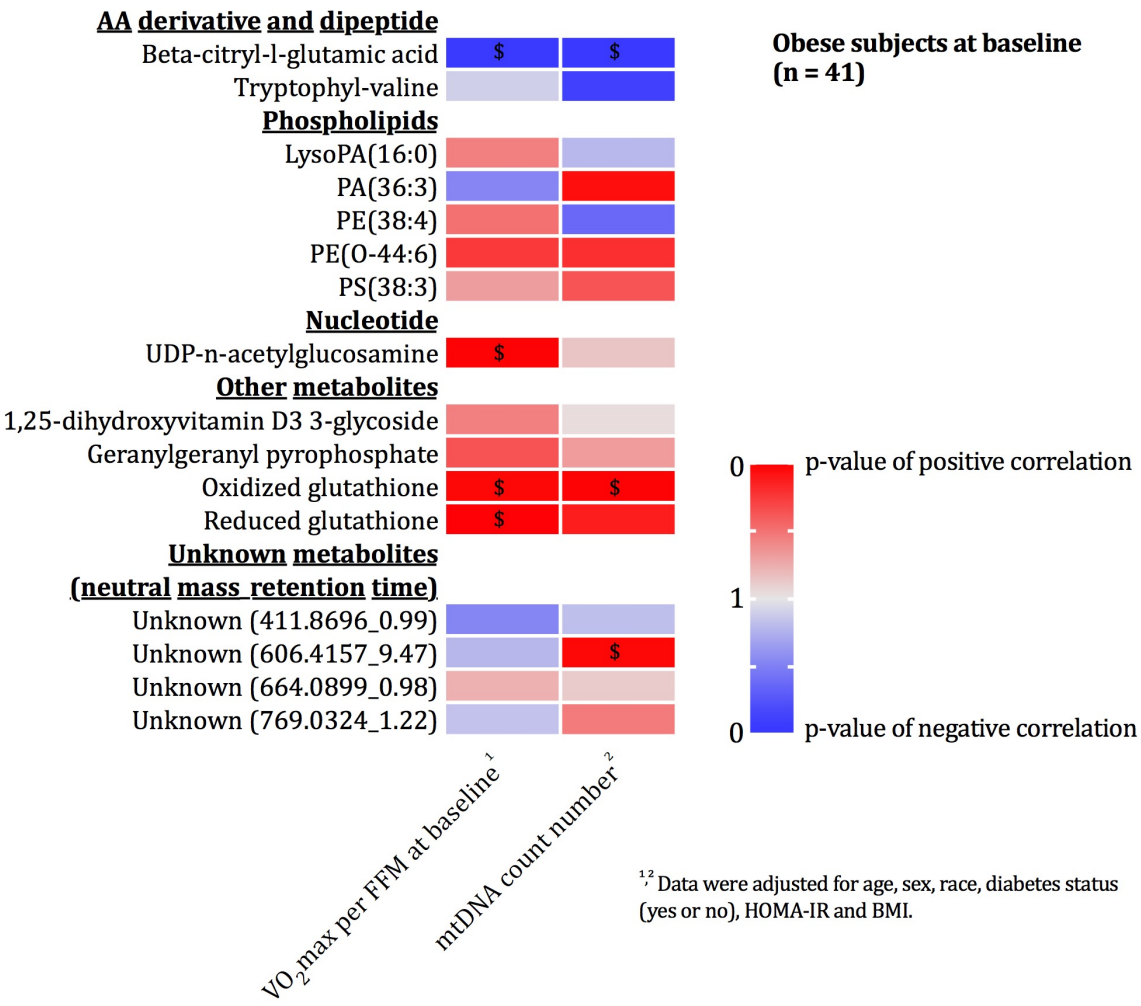


[1,2] Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.

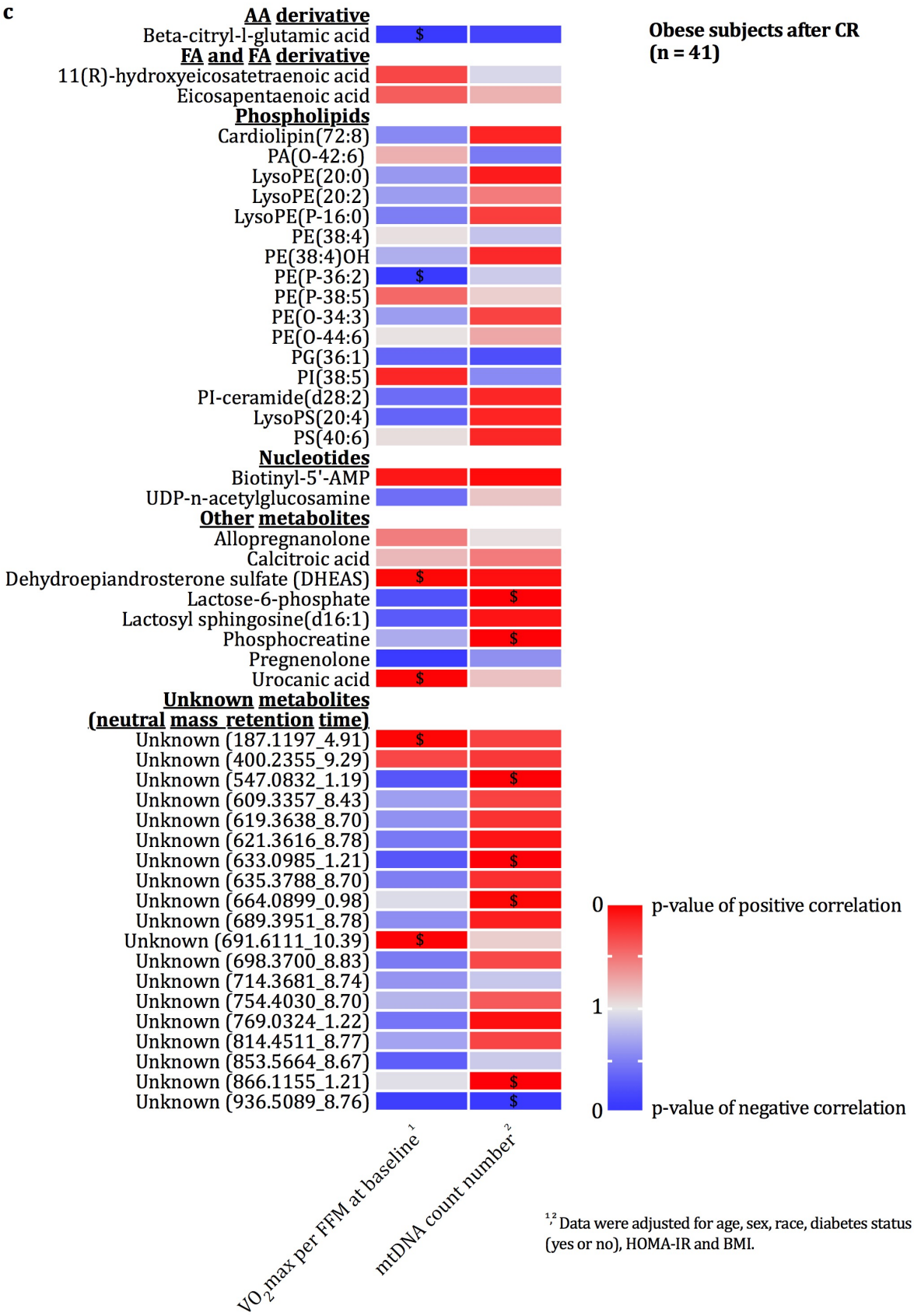
Figure 5.4 Correlations of untargeted metabolites and mtDNA profiles vs. VO_2max per FFM at baseline, as well as untargeted metabolites vs. mtDNA count number. $ = p-value <0.050.



b



[1,2] Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.


c
AA derivative
Beta-citryl-l-glutamic acid
FA and FA derivative
11(R)-hydroxyeicosatetraenoic acid
Eicosapentaenoic acid
Phospholipids
Cardiolipin(72:8)
PA(O-42:6)
LysoPE(20:0)
LysoPE(20:2)
LysoPE(P-16:0)
PE(38:4)
PE(38:4)OH
PE(P-36:2)
PE(P-38:5)
PE(O-34:3)
PE(O-44:6)
PG(36:1)
PI(38:5)
PI-ceramide(d28:2)
LysoPS(20:4)
PS(40:6)
Nucleotides
Biotinyl-5'-AMP
UDP-n-acetylglucosamine
Other metabolites
Allopregnanolone
Calcitroic acid
Dehydroepiandrosterone sulfate (DHEAS)
Lactose-6-phosphate
Lactosyl sphingosine(d16:1)
Phosphocreatine
Pregnenolone
Urocanic acid
Unknown metabolites (neutral mass_retention time)
Unknown (187.1197_4.91)
Unknown (400.2355_9.29)
Unknown (547.0832_1.19)
Unknown (609.3357_8.43)
Unknown (619.3638_8.70)
Unknown (621.3616_8.78)
Unknown (633.0985_1.21)
Unknown (635.3788_8.70)
Unknown (664.0899_0.98)
Unknown (689.3951_8.78)
Unknown (691.6111_10.39)
Unknown (698.3700_8.83)
Unknown (714.3681_8.74)
Unknown (754.4030_8.70)
Unknown (769.0324_1.22)
Unknown (814.4511_8.77)
Unknown (853.5664_8.67)
Unknown (866.1155_1.21)
Unknown (936.5089_8.76)
$
Obese subjects after CR
(n = 41)
0
p-value of positive correlation
1
0
p-value of negative correlation
VO2max per FFM at baseline [1]
mtDNA count number [2]
[1,2] Data were adjusted for age, sex, race, diabetes status (yes or no), HOMA-IR and BMI.

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Chapter 6
A drift correction and batch combination tool for mass spectrometry-based metabolomics data

6.1 Introduction

As metabolomics has matured as a member of the 'omics' sciences, its application to large-scale epidemiological studies of human subjects has undergone substantial growth. Unlike experiments using cell culture or animal models in which carefully controlled conditions allow studies with modest sample numbers to achieve sufficient statistical power, studies of human subjects require analysis of hundreds or thousands of samples to overcome natural variability and detect subtle biological effects. However, due to the extended nature of some protocols in clinical research, metabolomics data may be acquired in multiple batches over the course of weeks, months or years. In mass spectrometry (MS)-based metabolomics studies, both short- and long-term instrument sensitivity drift add variability to the data and may limit the ability to detect biologically significant effects (1). Thus, the difficulty of achieving reproducible quantitation is a great barrier to successful completion of large-scale metabolomics studies.

The need for robust normalization techniques for large sets of MS-based metabolomics data has long been apparent and various approaches have been implemented previously. The simplest option is to normalize data to total metabolite signal intensity or median signal intensity (2, 3). However, this approach assumes that all metabolites experience the same pattern of drift over and that the sum or median metabolite abundance is approximately the same in all samples, which is often not the case (4). A second option is to use internal standards (IS), added at a uniform concentration to all samples, to monitor or correct for quantitative drift. This is most effective when stable isotope-labeled versions of the exact metabolites to be quantitated are used (e.g., isotope

dilution mass spectrometry) (5, 6), but in most cases it is impractical or impossible to include an isotope-labeled IS for all metabolites of interest. To enable more comprehensive metabolomics profiling with IS normalization, computational techniques have been proposed to aid in selection of an internal standard from the available subset that most optimally corrects for drift or matrix effects in each metabolite (4). While superior to ad-hoc internal standard selection, it is not possible to ensure the drift correction is valid for each compound, as the pattern of intensity drift often varies significantly from metabolite to metabolite, even within a class of compounds.

The other major drift correction strategy, which has become the most widely cited approach in recent metabolomics literature, is to use a quality control (QC) sample analyzed once or repeatedly with each batch of samples as the basis for performing drift correction (1, 7-12). This technique requires an ample supply of a suitable QC sample to be prepared in advance for use as long as sample analysis is being performed, and increases the number of samples to be analyzed. However, the strategy allows correction for quantitative drift in each individual metabolite, regardless of whether an appropriate internal standard is available, making it suited for both targeted and untargeted metabolomics studies. One of the most widely cited variants of this strategy was described by Dunn et al., who used locally estimated scatterplot smoothing (LOESS) to correct for peak area drift based upon observed intensity drift in a QC sample (7), although other curve-fitting strategies have also been proposed (13).

Existing tools for metabolomics data analysis such as XCMS Online and MetaboAnalyst offer basic methods for automated normalization of metabolomics data (14, 15), but no currently available software offers an automated workflow for combining batches and performing QC-sample based drift correction with visualization. In this manuscript, we present a Microsoft Excel (2013) based tool which allows data acquired on any platform to be easily visualized and assessed for within and between-batch drift. The tool performs automatic drift correction based on QC sample data using a quadratic regression or LOESS smoothing. We demonstrate the use of the tool on archived data and an original set of LC-MS-based metabolomics data: a study of metabolic response to a mixed-meal tolerance test (MMTT) in a cohort of obese subjects before and after CR, and non-obese controls.

6.2 Materials and methods

Enrollment criteria, CR protocol and mixed meal tolerance test (MMTT) were described in chapter 4.

6.2.1 Plasma extraction and LC-MS metabolomics analysis

Targeted metabolomics was used to quantitate 122 plasma metabolites from a variety of classes; all species measured are listed in Table A6.1 of appendices. To serve as a QC sample, a pooled human plasma sample obtained from the American Red Cross was separated into 50 µl aliquots in microcentrifuge tubes and stored at -80 °C until individual aliquots were thawed and extracted along with each analytical batch. To precipitate proteins and extract metabolites, 200 µl of extraction solvent (1:1:1 methanol: acetonitrile: acetone) containing a mixture of isotope-labeled internal standards (Table A6.2 of appendices) was added to 50 µl of plasma. The samples were vortexed for 10 sec, allowed to rest on ice for 5 min, and then centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was transferred to an autosampler vial for liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent 1200 LC with an Agilent 6220 time-of-flight MS (Santa Clara, CA). All solvents and reagents used were LC-MS grade and were obtained from Sigma-Aldrich (St. Louis, MO). Hydrophilic interaction chromatography (HILIC) in negative ion mode was performed using a method similar to that described previously (16) using a Phenomenex Luna NH2 column, 3 µm particle size, 150 mm x 1 mm inner diameter (i.d.) (Torrance, CA). Mobile phase A was 5 mM ammonium acetate in water adjusted to pH 9.9 using ammonium hydroxide, and mobile phase B was acetonitrile. The gradient consisted of a 15-min linear ramp from 80 to 0% B, 5 min at 0% B, and 15 min of re-equilibration at 80% B. The flow rate was 0.07 ml/min from 0-27min, 0.08 ml/min from 27-32 min and 0.09 ml/min from 32-35 min. MS parameters were as follows: full-scanmode (m/z 50 to 1,200), acquisition rate 1 spectrum/sec, capillary voltage 3500 V, gas temperature 350 °C, drying gas 10 l/min, nebulizer pressure 20 psig, and reference mass correction enabled. Reversed-phase liquid chromatography (RPLC) in both negative and positive ion modes was performed a method similar to that described previously (17) using a Waters Acquity HSS T3column, 1.8 µm particle size, 2.1 x 100 mm i.d., with a flow rate of 0.2 ml/min (Milford, MA). Mobile phase A was 0.1% formic acid and 0.028% of ammonium

hydroxide in water and mobile phase B was 0.1% formic acid and 0.028% ammonium hydroxide in 8:2 isopropanol:methanol. The gradient consisted of 6-min linear ramp from 0 to 100% B, 4 min at 100% B, and 5 min of re-equilibration at 0% B. MS parameters were the same as HILIC except the acquisition rate was 2 spectra/sec and the nebulizer pressure was 40 psig. A total of 418 test samples and 66 QC samples were analyzed in 14 batches using both HILIC and RPLC methods. The QC sample was analyzed approximately every 8th run within each batch. To improve data quality and eliminate the runs prone to the most extreme drift, the QC sample prepared with each batch was injected and analyzed 3 times (for HILIC) or 5 times (for RPLC) before starting analysis of each new batch, and data from all these QC pre-injections were disregarded in subsequent data analysis and drift correction. Targeted metabolomics data analysis was performed using Agilent Masshunter Quantitative Analysis software version B.07.00. Peaks were identified by comparison of accurate mass and retention time with those of authentic standards analyzed using the same method. Peaks were quantitated by peak area using the 'Agile2' peak integrator and these data were exported into a spreadsheet (comma-separated values file). A second set of metabolomics data, generated by the West Coast Metabolomics Core at the University of California, Davis, was obtained from the NIH Common Fund's Data Repository and Coordinating Center (Metabolomics Workbench Project ID: PR000303, accessible at http://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Project&ProjectID=PR000303. These data consisted of 250 test samples and 26 QC samples analyzed by RPLC in both positive and negative ion mode. Based on run timestamp data, positive mode data were acquired in a single batch of consecutive runs; negative ion mode data were broken into two batches. Raw data were confirmed to have no prior drift correction or other normalization applied.

6.2.2 Peak area drift correction, calculations and statistics

To correct for peak area drift within and between each sample batch, we developed a custom macro-enabled Excel spreadsheet called MetaboDrift (attached separately as the Microsoft Excel file). Detailed instructions for using MetaboDrift are provided within the Excel file; the general workflow for the software is as follows. Peak area data are inserted into the worksheet and samples are labeled and sorted by batch number and run order

number. Quality control samples are flagged to enable their use for drift correction. Peak intensity profiles for each metabolite are then automatically visualized for QC and all other samples. Two options are available for correction of within-batch drift: quadratic fit (QUAD) and LOESS. QUAD applies a standard least-squares quadratic regression to the QC sample data. For the purpose of curve fitting, *y*-values are metabolite peak areas from the QC samples and *x*-values are the run number. The output of the regression is an equation in the format of $y=ax^2+bx+c$; the polynomial coefficients are used to estimate a predicted peak area (*y*) on the quadratic curve given any run number (*x*) (Figure 6.1). LOESS uses a locally-estimated polynomial smoothing procedure, as described by Dunn et al., to generate a predicted peak area at each point along the smoothed curve (7). Our implementation of LOESS drift correction in Excel uses a publically available Excel add-on function, accessible at (http://peltiertech.com/loess-smoothing-in-excel). It accepts the same *x* and *y* inputs as QUAD, plus a parameter (n) for the number of points to use in the moving smoothing function. Optimal setting for this parameter can be adjusted to optimize LOESS fit as described previously (7). After predicted peak areas are calculated for all samples in a data set, peak area correction is applied to each data point in the raw data using the following formula:

$$\text{Peak area}_{\text{corrected}} = \text{Peak area}_{\text{raw}} \times (\text{Peak area}_{\text{first run in data set}} / \text{Peak area}_{\text{predicted}})$$

This normalization has the effect of setting the peak area in the first QC sample of each batch to the same value, and applying individual drift correction to each batch. The data resulting from quadratic or LOESS drift-correction are placed in new worksheets, which allows comparison of the drift-corrected data with the raw data. If the drift-corrected data does not meet acceptable criteria (e.g., <10% RSD for corrected peak area in QC samples), the metabolite can be dropped from further analysis or an alternate drift correction strategy can be considered.

Drift-corrected area under the curve (AUC) values were calculated by summing the trapezoidal areas between the 0, 15, 30, 60, and 120 minute timepoints. Comparison of metabolite levels between non-obese and obese groups were performed using an unpaired-two-tailed student's t test. For the comparison between obese subjects at baseline and after CR, a paired-two-tailed student's t test was used. All data were reported as

average±standard error of mean (SEM); p-value <0.050 were considered statistically significant.

6.3 Results and discussion

6.3.1 Participant characteristics and non-metabolite parameters

Participant characteristics were listed in Table 6.1. Samples were obtained from 35 obese (20 males and 15 females) and 16 non-obese (6 males and 10 females) subjects. Age was not significantly different between groups. Obese individuals were heavier (39.8±0.5 vs. 23.1±0.8 kg/m^2). Body weight of obese individuals was decreased by 17.7±1.3% with CR (range 4.1-35.8%). The obese group had higher fasting plasma glucose and insulin, as well as HOMA-IR. All of these parameters altered significantly after CR and values became closer to those of non-obese subjects.

6.3.2 Peak area drift within and between batches

Substantial within-batch metabolite peak area drift was observed in the raw MMTT and Fiehn metabolomics data (Figure 6.1a-c). IS compounds also showed similar patterns of drift (Figure 6.1d). For many metabolites, the trend was for declining peak area over the course of the runs; however, for some metabolites, drift was random or the peak area increased. Between-batch intensity shifts were also prominent, reflected as abrupt changes in peak area in the QC sample data (Figure 6.3a,c). In contrast to the observed intensity drift, there was little retention time drift nor alteration in peak shape for the vast majority of metabolites (average retention time RSDs were 0.28%, 0.26% and 1.22% for RPLC+, RPLC-, and HILIC-, respectively), suggesting that deterioration in chromatographic performance was not responsible for the observed intensity drift. Possible causes for peak area drift include fouling of the instrument ion source, charging of ion optics, and degradation or interconversion of labile metabolites in solution, but detailed assessment of the origin of the drift was beyond the scope of this manuscript. Peak area drift is a common occurrence (7, 8) and frequently interferes with metabolite quantification. Although cleaning the source and ion optics of the mass spectrometer can sometimes restore sensitivity and reduce the impact of drift, in our experience it is often not possible or

practical to perform such maintenance frequently enough to eliminate its impact on the data. Therefore, unless a validated internal standard is available for each metabolite to be quantitated, it is essential to correct large metabolomics data sets for peak area drift.

6.3.3 Peak area drift correction using MetaboDrift

We evaluated two curve fitting approaches to model peak area drift: QUAD and LOESS (Figure 6.2). In many cases, both methods fit within-batch drift nearly equally well, particularly when the drift had a trend toward higher or lower abundance. QUAD has the advantage of using a simple curve which is unlikely to suffer from issues of 'overfitting', in which random variation in pooled sample peak caused by measurement error may have an undue influence on curve shape (18). This may be particularly relevant when batch size and the number of QC samples per batch is small, as was the case in the MMTT data (Figure 6.2a-c). LOESS was unable to fit data from one sample batch in which only two QC samples were used. On the other hand, LOESS has advantages over QUAD in that, as a non-parametric fitting method, it is not constrained to a specific curve shape. In the case of metabolomics data with large batch size and a more random trend in peak area drift, such as the Fiehn lab data, LOESS produced a superior curve fit and resulted in lower peak area RSD values (Figure 6.2d-f). We also evaluated the use of higher-order polynomials for drift correction, but found these methods to be more prone to overfitting than QUAD, while showing no clear advantages over LOESS for large batches or irregular peak drift patterns. Therefore, no polynomial drift correction options other than QUAD were included in MetaboDrift.

We selected QUAD for further examination of the MMTT data and applied the peak area drift correction procedure to all metabolites as described in the methods section. Variability between metabolite peak area before and after drift correction were assessed by percent relative standard deviation (%RSD). As expected, drift correction compensated for most of the peak area drift in the QC samples, as illustrated in Figure 6.3. Average %RSD for all metabolites in the QC samples of the MMTT data was 50.7% and 6.6% before and after QUAD drift correction, respectively. Internal standard compounds, which were added at the same concentration to all samples, also showed improved reproducibility in both QC samples (Figure 6.3c,d) and biological samples (Figure 6.3e,f). To validate the performance

of the drift correction strategy, metabolites with an exact-matching internal standard were quantitated by isotope dilution MS. After QC sample drift correction, the correlation between peak area vs. concentration increased (Figure 6.4), indicating that the corrected peak areas more accurately reflect true metabolite concentrations than the uncorrected data. Further comparison between the raw and drift-corrected data are included as Supporting Information as copies of the MetaboDrift worksheet pre-loaded with the data sets from this study.

6.3.4 Drift-correction enhances interpretation of MMTT data

An important measure of the utility of drift correction is whether it enables interpretation of biological phenomena in data sets which experienced substantial instrumental drift over the course of data acquisition. To this end, we used the entire drift-corrected data set described above to examine the dynamics of metabolite levels the MMTT in non-obese and obese individuals. The time-resolved profiles of select metabolites are highlighted in Figure 6.5, and all metabolites are visualized as a heatmap in Figure 6.6. Plasma glucose and most amino acids (AAs) increased and reached a maximum at 30 and 60 min postprandial, respectively, consistent with the typical timecourse of appearance and clearance of these nutrients from the bloodstream following a meal (Figure 6.5a,b) (19, 20). Lactate, the downstream product of non-oxidative glucose metabolism, peaked at 60 min (Figure 6.5c) and was elevated in obese subjects throughout the timecourse. Fatty acids (FAs) and their metabolic byproducts, medium-chain acylcarnitines, decreased postprandially and reached a minimum at 60 min, suggesting insulin-induced suppression of lipolysis (Figure 6.5d,e). Small to no changes were observed in triglycerides, phospholipids, acetyl-CoA or TCA cycle metabolites during the 2-hour time course, which is expected given the relatively short duration and moderate caloric load of the MMTT. The bile acid chenodeoxyglycholic acid, which facilitates lipid digestion in the intestine, increased over the time course of the MMTT, consistent with the metabolic response to food consumption (Figure 6.5f).

To investigate differences in metabolite clearance post-meal between the groups, we calculated AUC values as described in the methods. Glucose AUC was significantly higher in obese subjects as compared to non-obese subjects, and this level decreased

significantly after CR (Figure 6.7a). Isoleucine+leucine AUC, which have been described as metabolic markers of insulin resistance (21, 22), were significantly higher in obese subjects and weight loss decreased their levels (Figure 6.7b). Free fatty acid (FFA) AUC values were significantly lower in non-obese subjects, and were reduced by weight loss in the obese group (Figure 6.7c). AUC of certain medium-chain (C6 to C14) acylcarnitines, which are metabolic intermediates of incomplete lipid oxidation, were significantly higher in obese subjects and were reduced by weight loss, suggesting incomplete fatty acid oxidation (FAO) associated with obesity (Figure 6.7d) (23-25). Likewise, the postprandial reduction in FFAs and acylcarnitines (expressed as change in drift-corrected peak area from baseline to 60 min) was significantly smaller in obese than non-obese subjects (Figure 6.7e and 6.7f), suggesting lesser reduction in lipolysis in response to food consumption. To evaluate whether drift correction was essential to interpretation of results from the MMTT, we attempted the comparisons described above using uncorrected data. Greater variability in the uncorrected data resulted in the loss of statistical significance of most of the comparisons described above (Figure 6.8), confirming that drift correction allowed improved biological insight into this multi-batch metabolomics data.

6.4 Summary

Our strategy allows metabolomics data drift correction and batch combination to be implemented with a minimum of prior data manipulation using a simple Excel worksheet. Selection of Excel as the platform for MetaboDrift has distinct advantages and disadvantages. On one hand, Excel is a familiar data analysis environment with a small learning curve, and thus should be accessible to novice users interested in evaluating and correcting intensity drift in metabolomics data obtained from a core facility or downloaded from a publically-accessible data repository. Additionally, the spreadsheet-based environment with integrated graphics allows rapid visualization of the entire data set, allowing trends to be observed and/or specific abnormalities in individual metabolites to be detected and flagged for further assessment. However, Excel is generally less computationally efficient than environments such as R or Matlab, which may increase analysis time for large data sets, and may not be as versatile for automation or incorporation into a larger data-analysis workflow scheme. Although experienced

statisticians and bioinformaticians may already be comfortable with LOESS or other drift-correction techniques and may not require software tools such as MetaboDrift, as the metabolomics community continues to expand to a wider diversity of researchers, approachable tools for data analysis become increasingly necessary, and solutions for common challenges such as intensity drift have an important role to play in maximizing accessibility of the data.

Table 6.1 Participants' characteristics. Data are shown as average (minimum-maximum). $ = p-value <0.050; NA = not applicable

Characteristics	Non-obese	Obese at baseline	Obese after CR	p-value of non-obese vs. obese at baseline	p-value of non-obese vs. obese after CR	p-value of obese at baseline vs. obese after CR
Number (n) of subjects	16	35		NA	NA	NA
Male/female (n)	6/10	20/15		NA	NA	NA
Age (years)	48 (27-60)	50 (30-63)		0.412	0.412	NA
BMI (kg/m^2)	23.1 (19.2-26.0)	39.8 (30.5-50.6)	32.6 (26.0-41.9)	<0.001$	<0.001$	<0.001$
% Weight loss after CR	NA	NA	17.7 (4.1-35.8)	NA	NA	NA
Fasting plasma glucose (mg/dl)	88 (75-106)	104 (73-146)	95 (69-114)	<0.001$	0.024$	0.003$
Fasting plasma insulin (mIU/l)	9.2 (5.1-14.5)	23.3 (10.4-53.2)	14.9 (6.3-27.0)	<0.001$	<0.001$	<0.001$
HOMA-IR ((fasting plasma glucose x fasting plasma insulin) /405)	2.0 (1.0-3.4)	6.1 (2.1-13.4)	3.5 (1.3-7.0)	<0.001$	<0.001$	<0.001$

Figure 6.1 Scatter plots of QC and internal standard metabolite peak areas vs. run number. Figure 6.1a, 6.1b and 6.1d are derived from the MMTT data, whereas Figure 6.1c was derived from Fiehn lab data.

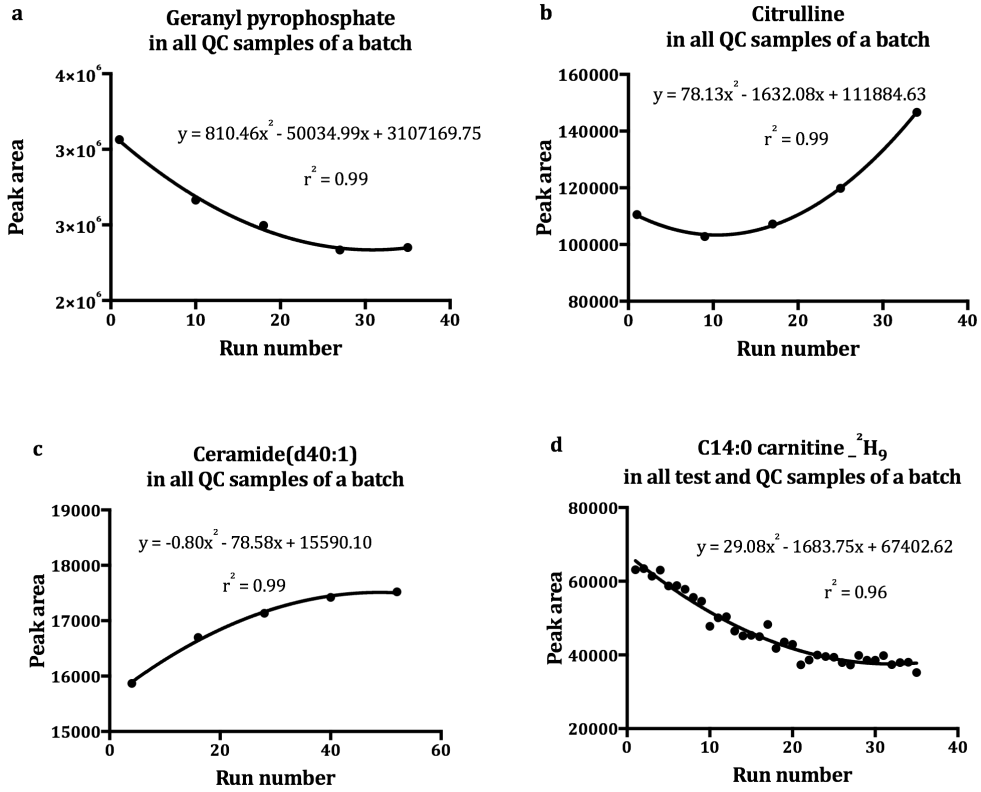


Figure 6.2 Comparison between quadratic and LOESS drift-correction for LC-MS metabolomics data. Figure 6.2a-c were derived from the MMTT data; Figure 6.2d-f were derived from the Fiehn lab data set.

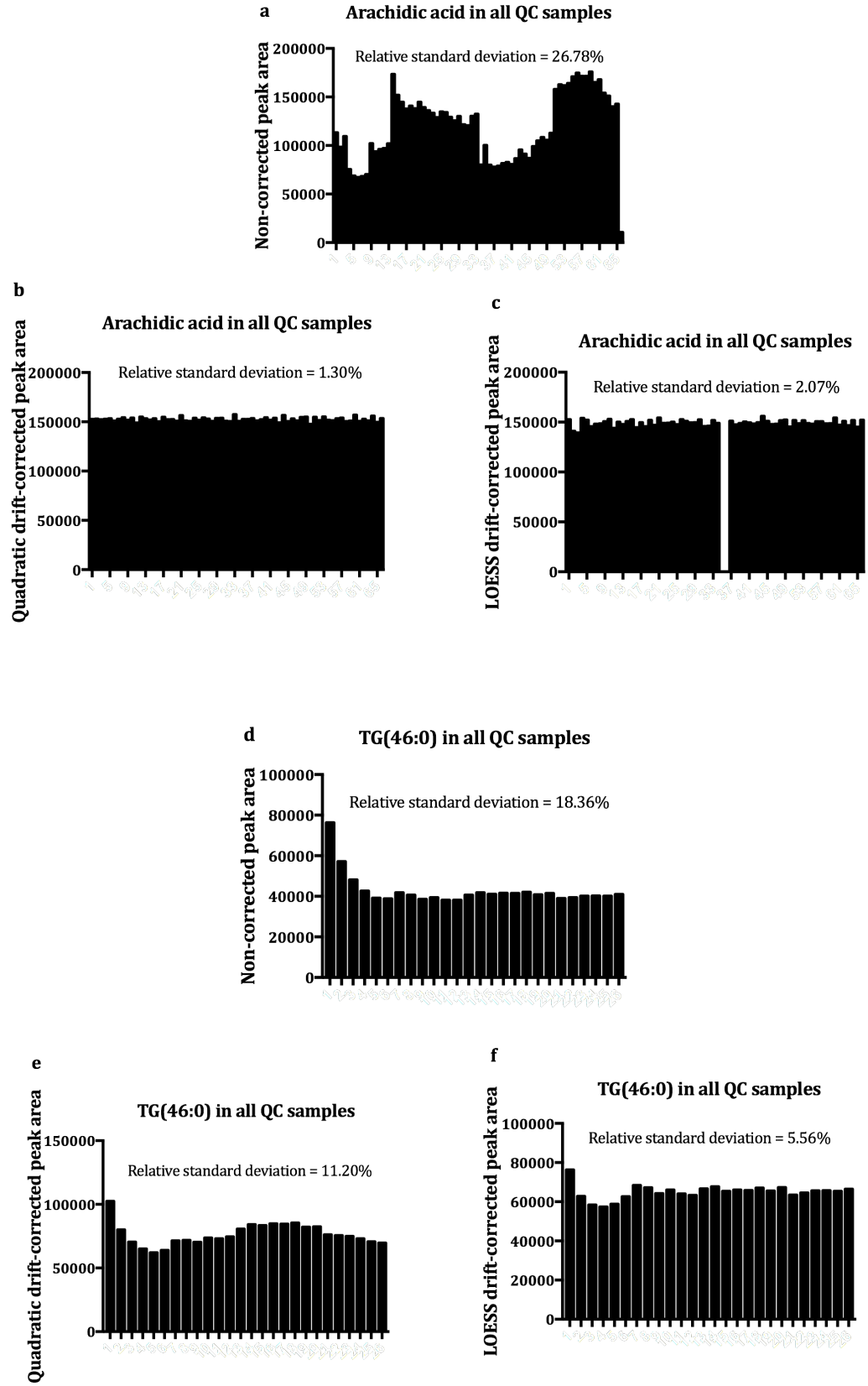


Figure 6.3 Peak area of metabolites and internal standards before (Figure 6.3a, 6.3c, 6.3e and 6.3g) vs. after (Figure 6.3b, 6.3d, 6.3f and 6.3h) quadratic drift-correction. Figure 6.3a-f were generated from MMTT data; Figure 6.3g and 6.3h were generated from the Fiehn database.

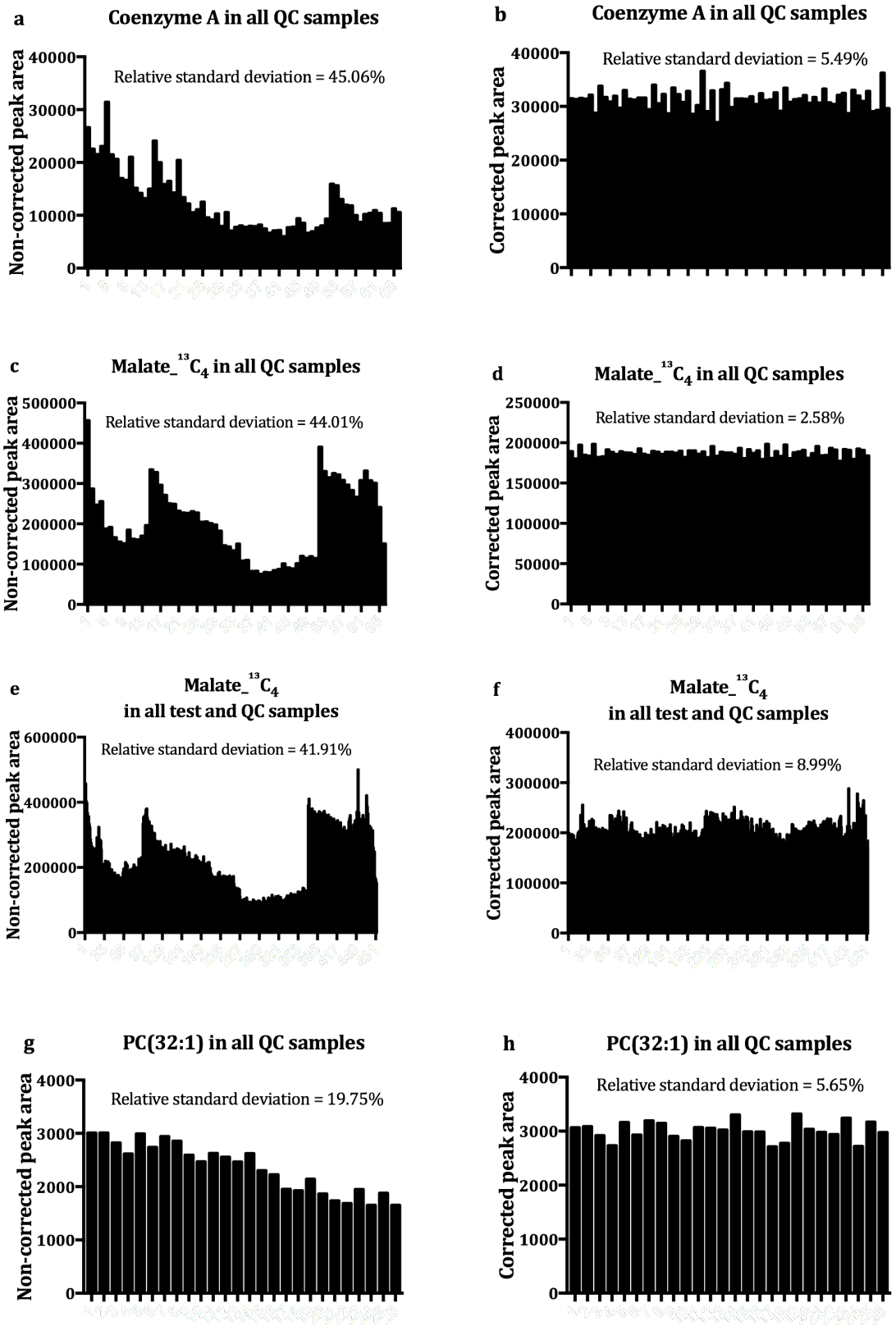


Figure 6.4 Peak area vs. concentrations of metabolites in all test and QC samples before (Figure 6.4a and 6.4c) and after (Figure 6.4b and 6.4d) quadratic drift-correction.

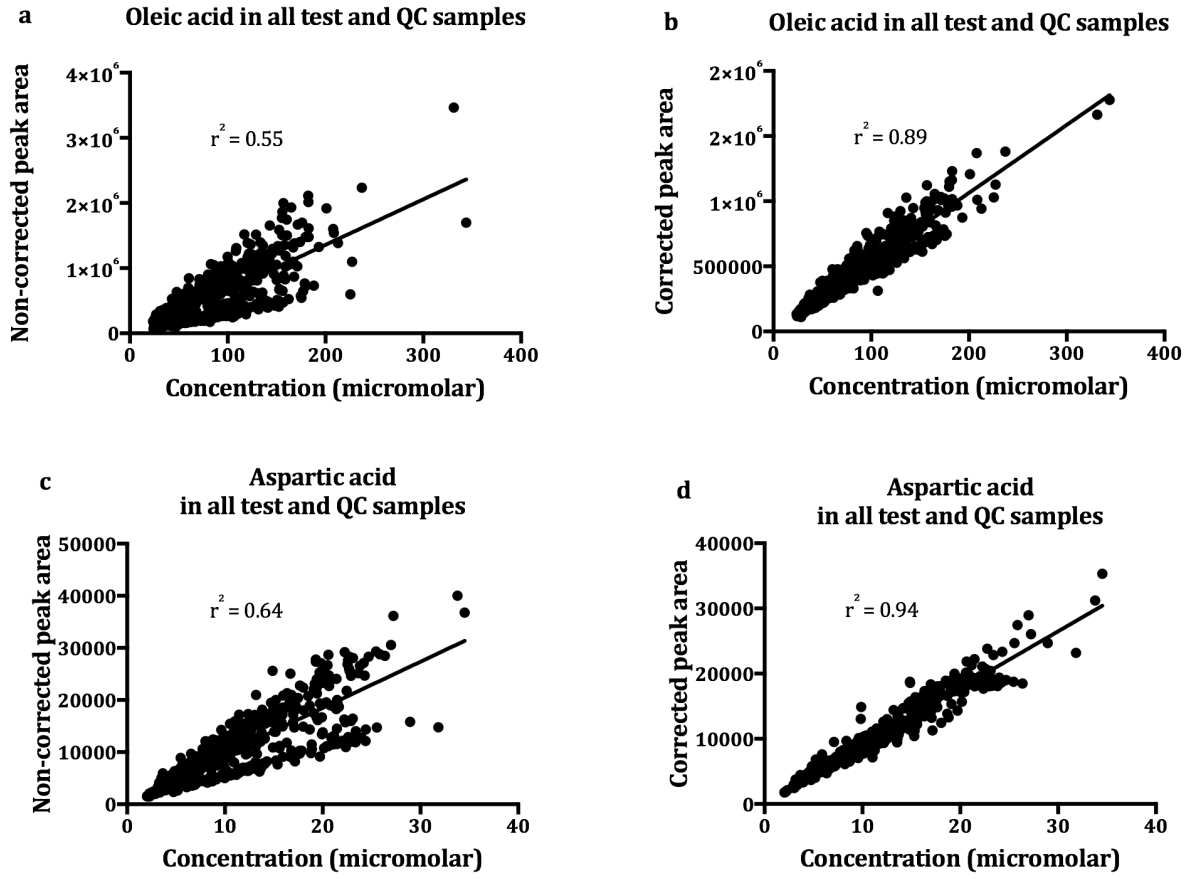


Figure 6.5 Corrected peak areas of selected metabolites during MMTT. Data are shown as average±SEM. Non-OB = non-obese, OB-pre = obese at baseline, OB-post = obese after CR.

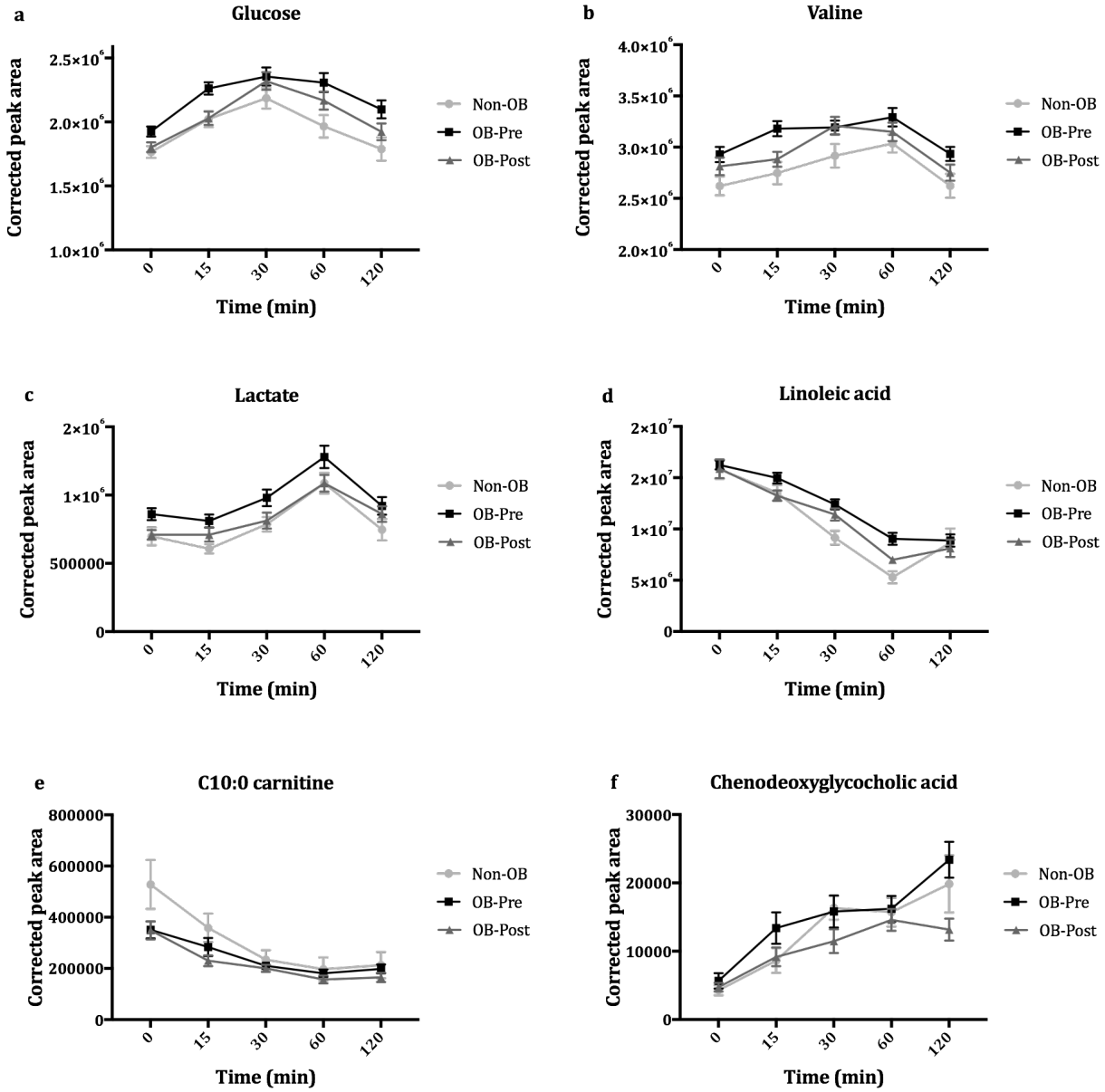


Figure 6.6 Heatmap of average metabolite fold-change vs. fasting (0 min) over MMTT timecourse.

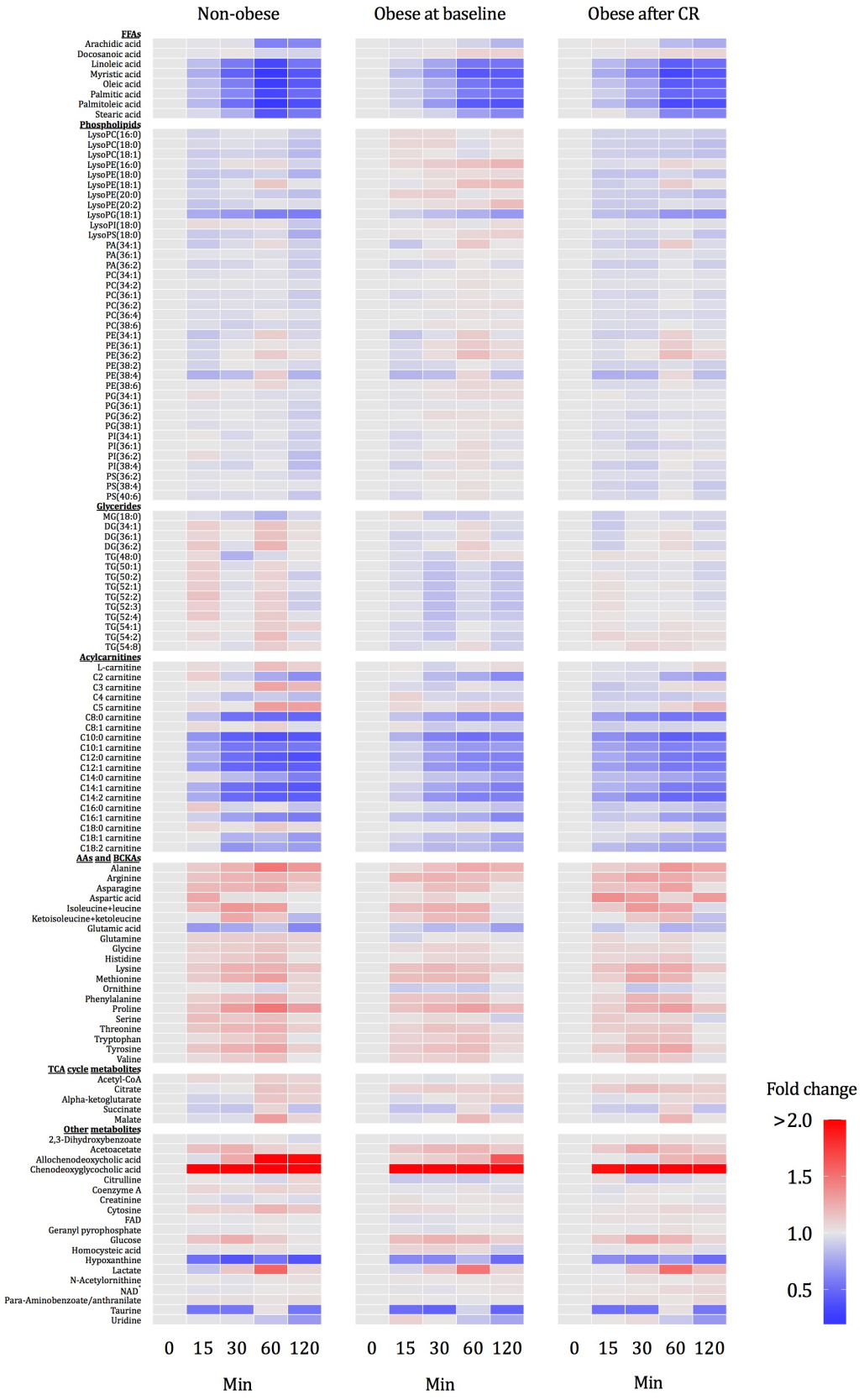


Figure 6.7 Corrected area under the curve (AUC) values for select metabolites during the MMTT (Figure 6.7a-d). Change in corrected peak area from fasting to 60 min by subject group (Figure 6.7e-f). Data are shown as average±SEM. $ = p-value <0.050 vs. non-obese subjects; * = p-value <0.050 vs. obese at baseline. Non-OB = non-obese, OB-pre = obese at baseline, OB-post = obese after CR.

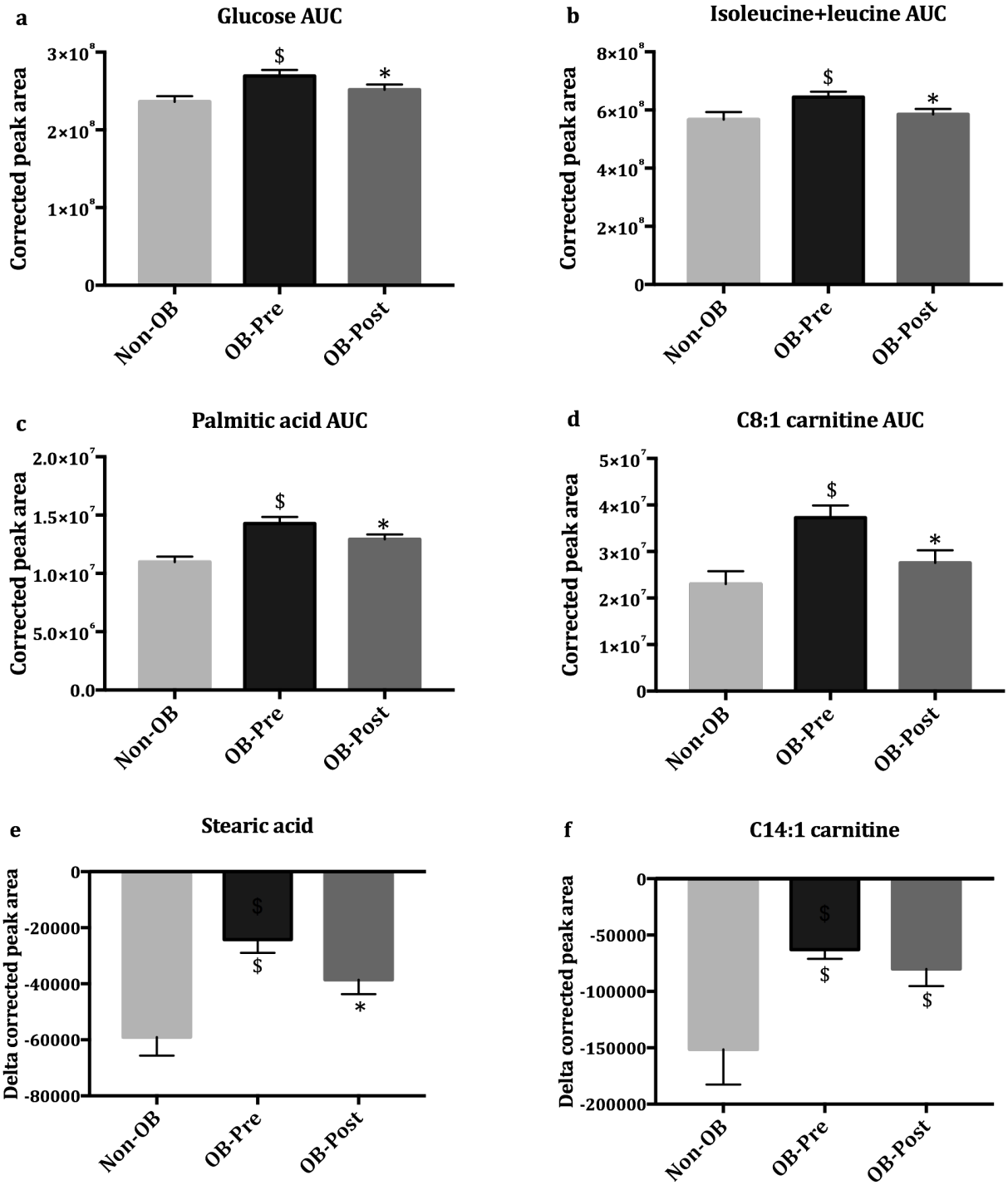
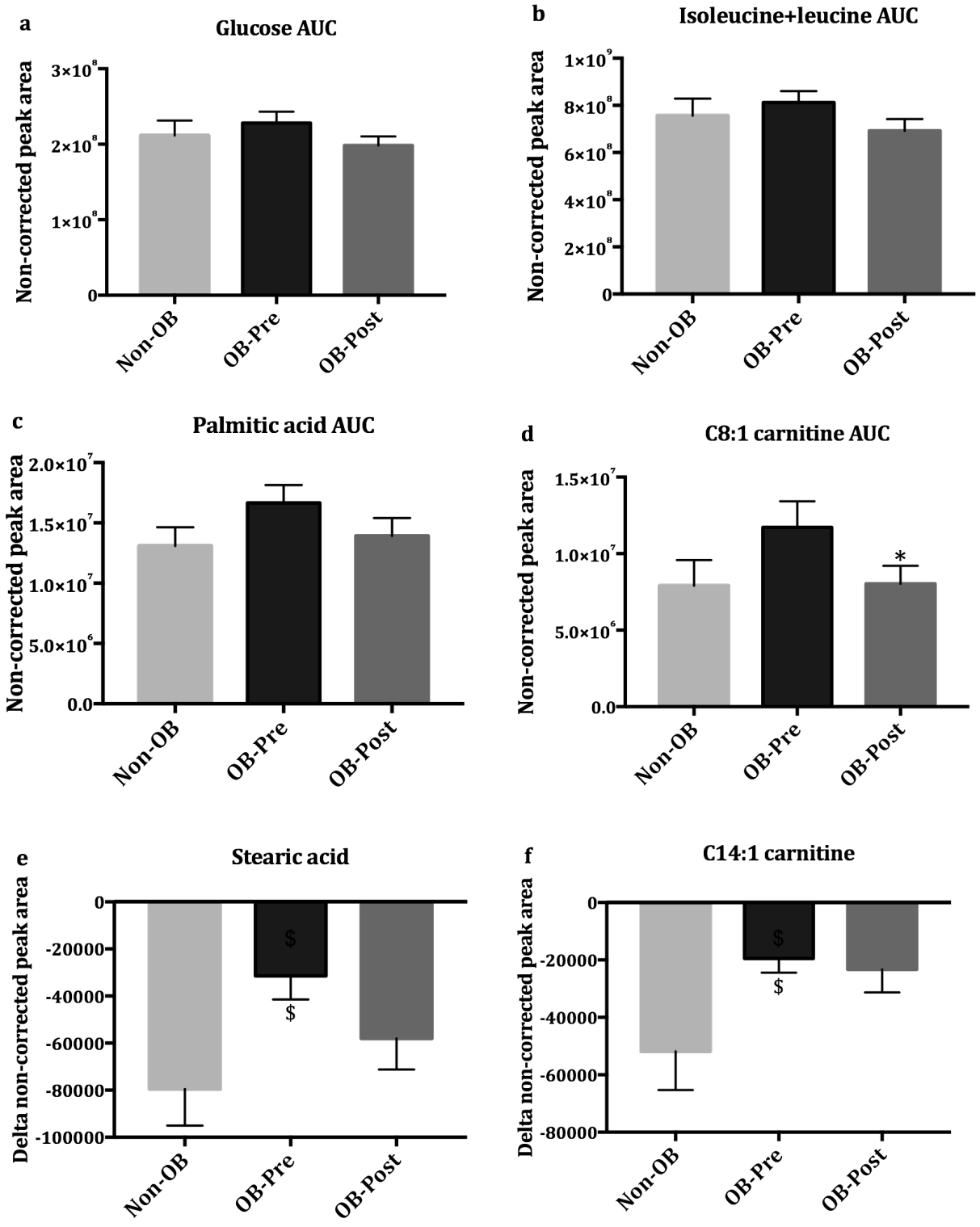


Figure 6.8 Non-corrected area under the curve (AUC) values for select metabolites during the MMTT (Figure 6.8a-d). Change in non-corrected peak area from fasting to 60 min by subject group (Figure 6.8e-f). Data are shown as average±SEM. $ = p-value <0.050 vs. non-obese subjects; * = p-value <0.050 vs. obese at baseline. Non-OB = non-obese, OB-pre = obese at baseline, OB-post = obese after CR.



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Chapter 7
Implications and future directions

7.1 Implications to health and longevity

In fasting plasma (Chapter 4) and postprandial skeletal muscle (Chapter 5), we found that several metabolites change with VO_2max, suggesting that cardiorespiratory fitness (CRF) impacts metabolism. Not surprisingly, the affected metabolites were primarily associated with mitochondrial metabolism and supports the hypothesis that it is through effects on mitochondrial metabolism that CRF is correlated with beneficial effect of on health and longevity. As in previous studies from our laboratory in a rat model, CRF was associated with increased and more complete fatty acid oxidation (FAO). In other words, high CRF was associated with higher mitochondrial capacity to use fat as fuel, but, given its relationship with decreased weight in animals with high CRF, it is associated with reduced mitochondrial efficiency. This hypothesized pathway would be consistent with observational longitudinal studies which indicate that high CRF is protective against incident obesity, weight gain and increased adiposity and risk of type 2 diabetes (Chapter 2).

In postprandial skeletal muscle (Chapter 5), people with higher CRF had lower levels of branched-chain amino acids (BCAAs) and aromatic amino acids (phenylalanine and tyrosine), suggesting that, in parallel with fatty acid oxidation, high CRF is associated with higher capacity for BCAA and aromatic amino acid (AA) catabolism. Given that BCAAs and aromatic AAs are associated with insulin resistance (1-8), increased utilization of those AAs due to higher CRF is consistent with observational longitudinal studies which found that high CRF is protective against impaired fasting glucose (IFG) and diabetes mellitus (DM) as reviewed in Chapter 2. Moreover, since obesity and insulin resistance are also associated with increased risk of cancer (9-16) and cardiovascular diseases (CVD) (17-25),

our findings are consistent with the hypothesis that it is a higher CRF which is providing protection against incident cancer, incident CVD, as well as mortality from these diseases. We also observed that higher CRF was associated with higher skeletal muscle glutathione, which is the most abundant endogenous antioxidant that protects mitochondria against oxidative stress (26). This agrees with previous research which found that high CRF is protective against oxidative stress (Chapter 2). Additionally, we found that high CRF was associated with higher levels of UDP-n-acetyl glucosamine and DHEAs and decreased levels of glycocholic acid (Chapter 5), each reversing the trends of each metabolite in ageing, also suggesting that high CRF is consistent with a 'younger' metabolic state.

Because CRF has a high genetic underpinning, a strategy to target efforts to promote good health would be more impactful in individuals who are born 'unfit'. However, given that there are diminishing returns from increasing exercise in low CRF individuals, and maintaining an elevated CRF is difficult to maintain, alternative strategies are needed. As reviewed in Chapter 3, caloric restriction (CR) can extend life span and attenuate age-related pathology. Our results suggest that those individuals with lower CRF may have a greater impact on their metabolism from CR. Indeed, we found that CR is associated with more complete FAO as well as higher mitochondrial capacity for BCAA catabolism, that is also seen in CRF. In addition, we demonstrated that CR decreased muscle membrane lipid unsaturation, which is associated with decreased susceptibility to age-related lipid peroxidative damage in mitochondria.

Our results suggest that CR can make people who were born 'unfit' attain a metabolism that is more akin to those who were born 'fit'. In contrast, our results also suggest that people who were born 'fit' may not appreciably benefit from CR. We found that CR minimized the beneficial effects of high CRF on improved FAO and mitochondrial DNA (mtDNA) count number that mediates the effects of CRF on some metabolites.

7.2 Future directions

As mentioned in Chapter 1, lifelong CR is rarely possible and obesity is becoming the 'normal state'. And, as mentioned earlier, lifelong aerobic training to maintain a higher CRF is difficult as we age and there are data that suggests that once one adjusts for basal CRF, only those with the lowest levels of CRF attain any health benefit from exercise. There are

emerging strategies that alter mitochondrial metabolism, such as timed eating (27, 28) and intermittent fasting (29, 30), that may have the beneficial effect of caloric restriction but potentially easier to put into clinical use.

Development of drugs or specific diets that would induce a high CRF or CR state obviously would be a boon in the quest to achieve better health and maximize life span. We found that the beneficial effects of high CRF and CR involve multiple metabolic pathways within the mitochondria. Therefore, metabolic pathway-targeted therapy may be helpful. Indeed, the suggestion that the mitochondria of individuals with higher CRF have a capacity for fatty acid oxidation, but may also so a degree of uncoupling, identification of the site of potential energy leak may provide a target for investigation. It will also be important to understand if the mitochondria from heart or other tissues have similar properties that are seen in skeletal muscle. Future 'fluxomic' studies, using heavy isotopes (31) could be employed to identify the rate-limiting steps of metabolic pathways within the mitochondria may provide insights into potentially druggable targets.

These targets would have an advantage in that one would not have to necessarily lose weight if the intervention can induce or mimic the metabolism associated with high CRF. As outlined in the discussions in Chapters 4 and 5, the molecular mechanisms that underlie the beneficial effects of high CRF are still unclear. It is tempting to speculate that the measurable inefficiency of mitochondria with higher oxidative/fatty acid oxidative capacity, seen in human studies (32) as well as the rat model of divergent CRF (31), results in signals to the nucleus to increase cassettes of genes to enhance fatty acid (FA) and BCAA, as the latter is seen in the HCR/LCR rat model and in humans with higher CRG (33). Additional metabolomics studies, focusing on metabolites generated from FA and BCAA metabolism may be fruitful.

An intriguing set of studies in C. elegans may provide a clue to candidate metabolites. In these studies (34-36), diets deficient in B12, which increases flux of l-methylmalonyl-CoA to succinyl-CoA and TCA cycle metabolites, have a life extending effect and are associated with an up-regulation of the cassette of mitochondrial genes involved in FA and BCAA metabolism that parallel that found in HCR rats (37). Treating worms with some metabolites, such as BCAAs, branched-chain FAs, odd-chain FAs, propionic acid and α-ketobutyric acid, activates FA and BCAA catabolizing genes as found in HCR rats (37) and

extends lifespan even in the presence of B12. Thus, if we can identify the pathways and probe metabolites within these pathways, we may be able to find a metabolite, or create a mimetic that could convey the salutary effects of high CRF. The candidate metabolites are downstream metabolites of treated metabolites and upstream metabolites of B12 action, including propionyl-CoA, d-methylmalonyl-CoA and l-methylmalonyl-CoA.

Finally, the above studies could be complemented with genetic studies. Only association studies have been done, but no publications on localization of genes associated with intrinsically high CRF have been published. An ongoing NIH study 'Molecular Transducers of Physical Activity' will be a step towards this goal as it will collect baseline and exercise-trained measures of CRF as well as genetic information in up to 3000 individuals, which may be the first step into dissecting the underlying genes responsible for a highly clinically relevant trait.

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Appendices

Appendices of chapter 4

Table A4.1 All metabolites identified in this study

Identified metabolites	
AAs Alanine Arginine Asparagine Aspartic acid Glutamic acid Glutamine Glycine Histidine Isoleucine+leucine Lysine Methionine Phenylalanine Proline Serine Threonine Tryptophan Tyrosine Valine	Acylcarnitines L-carnitine C2 carnitine C3 carnitine C4 carnitine C5 carnitine C5-DC carnitine C6 carnitine C8:0 carnitine C8:1 carnitine C10:0 carnitine C10:1 carnitine C12:0 carnitine C12:1 carnitine C12-OH carnitine C14:0 carnitine C14:1 carnitine C14:2 carnitine C14-OH carnitine C16:0 carnitine C16:1 carnitine C16-OH carnitine C18:0 carnitine C18:1 carnitine C18:2 carnitine C18:2-OH carnitine C20:0 carnitine C20:1 carnitine C20:2 carnitine C20:3 carnitine C20:4 carnitine
BCKAs Ketosoleucine+ketoleucine Ketovaline	FFAs Oleic acid Palmitic acid

Table A4.2 Internal standards used for quantitation of metabolites in plasma. NSK-B internal standard mix was purchased from Cambridge Isotope (Andover, MA). All other carbon-13 stable isotope internal standards were purchased from Sigma-Aldrich (St. Louis, MO).

Internal Standards	**Internal standard concentration added to extraction solvent**
Algal AA mixture_^{13}C	20 µg/ml
2-keto-3-methylbutyric acid (ketovaline)_$^{13}C_5$, 3-d sodium salt	3 µM
Oleic acid_$^{13}C_{18}$	15 µM
Palmitic acid_$^{13}C_{16}$	15 µM
NSK-B internal standard mix	
$^{2}H_9$-Carnitine (L-carnitine)	510.33 nM
$^{2}H_3$-Acetylcarnitine (C2)	128.33 nM
$^{2}H_3$-Propionylcarnitine (C3)	25.33 nM
$^{2}H_3$-Butyrylcarnitine (C4)	25.33 nM
$^{2}H_9$-Isovalerylcarnitine (C5)	25.33 nM
$^{2}H_3$-Octanoylcarnitine (C8)	25.33 nM
$^{2}H_9$-Myristoylcarnitine (C14)	25.33 nM
$^{2}H_3$-Palmitoylcarnitine (C16)	50.67 nM

Table A4.3 MRM for AA detection. Dwell time of 50 ms was used for all AAs.

Metabolites	Precursor Ion	Product Ion	Fragmentor	Collision Energy
Alanine	90.05	44.1	44	8
Alanine_$^{13}C_3$	93.05	46.1	44	8
Arginine	175.11	70	100	24
Arginine_$^{13}C_6$	181.11	74	100	24
Asparagine	133.05	74.02	86	8
Aspartic acid	134.04	74.02	72	4
Aspartic acid_$^{13}C_4$	138.04	76.02	72	4
Glutamic acid	148.05	84.1	72	12
Glutamic acid_$^{13}C_5$	153.05	88.1	72	12
Glutamine	147.07	84.1	72	16
Glycine	76.03	30	30	4
Glycine_$^{13}C_2$	78.03	31	30	4
Histidine	156.07	110.1	86	16
Histidine_$^{13}C_6$	162.07	115.1	86	16
Isoleucine+leucine	132.09	86.1	72	8
Isoleucine+leucine_$^{13}C_6$	138.09	91.1	72	8
Lysine	147.11	84.1	72	20
Lysine_$^{13}C_6$	153.11	89.1	72	20
Methionine	150.05	55.8	72	16
Methionine_$^{13}C_5$	155.05	58.8	72	16
Phenylalanine	166.08	119.9	72	12
Phenylalanine_$^{13}C_9$	175.08	127.9	72	12
Proline	116.06	70.1	86	20
Proline_$^{13}C_5$	121.06	74.1	86	20
Serine	106.04	60.1	58	8
Serine_$^{13}C_3$	109.04	62.1	58	8
Threonine	120.06	74.06	100	10
Threonine_$^{13}C_4$	124.06	77.06	100	10
Tryptophan	205.09	118.1	72	28
Tyrosine	182.07	91.1	72	32
Tyrosine_$^{13}C_9$	191.07	98.1	72	32
Valine	118.08	72.1	58	8
Valine_$^{13}C_5$	123.08	76.1	58	8

Table A4.4 SIM for BCKA and FFA detection. Dwell time of 200 ms was used for all BCKAs and FFAs.

Metabolites	Precursor Ion	Fragmentor
BCKAs		
Ketoisoleucine+ketoleucine	129.06	70
Ketovaline	115.05	60
Ketovaline_$^{13}C_6$	121.05	60
FFAs		
Oleic acid	281.26	90
Oleic acid _$^{13}C_{18}$	299.26	90
Palmitic acid	255.24	90
Palmitic acid_$^{13}C_{16}$	271.24	90

Table A4.5 Calibration parameters used for quantitation of metabolites in plasma. All calibration curve fits were linear. All metabolite standards were purchased from Sigma-Aldrich.

Metabolites	Calibration curve concentrations (μM)	R^2	IS isotope chosen
Amino acids			
Alanine	0, 7.5, 25, 75, 250, 750	0.9995	Alanine
Arginine	0, 7.5, 25, 75, 250, 750	0.9994	Arginine
Asparagine	0, 7.5, 25, 75, 250, 750	0.9988	Threonine
Aspartic acid	0, 7.5, 25, 75, 250	0.9994	Aspartic acid
Glutamic acid	0, 7.5, 25, 75, 250, 750	0.9996	Glutamic acid
Glutamine	0, 15, 50, 150, 500, 1500	0.9946	Threonine
Glycine	0, 7.5, 25, 75, 250, 750	0.9989	Glycine
Histidine	0, 7.5, 25, 75, 250, 750	0.9993	Histidine
Isoleucine+leucine	0, 7.5, 25, 75, 250, 750	0.9995	Isoleucine+leucine
Lysine	0, 7.5, 25, 75, 250, 750	0.9994	Lysine
Methionine	0, 7.5, 25, 75, 250	0.9955	Methionine
Phenylalanine	0, 7.5, 25, 75, 250, 750	0.9994	Phenylalanine
Proline	0, 7.5, 25, 75, 250, 750	0.9990	Proline
Serine	0, 7.5, 25, 75, 250, 750	0.9995	Serine
Threonine	0, 7.5, 25, 75, 250, 750	0.9997	Threonine
Tryptophan	0, 7.5, 25, 75, 250, 750	0.9992	Tyrosine
Tyrosine	0, 7.5, 25, 75, 250, 750	0.9995	Tyrosine
Valine	0, 7.5, 25, 75, 250, 750	0.9995	Valine
BCKAs			
Ketoisoleucine+ketoleucine	0, 1.5, 5, 15, 50	0.9942	Ketovaline
Ketovaline	0, 1.5, 5, 15, 50	0.9933	Ketovaline
FFAs			
Oleic acid	0, 6, 20, 60, 200, 600	0.9976	Oleic acid
Palmitic acid	0, 6, 20, 60, 200, 600	0.9934	Palmitic acid

Table A4.6 Age and VO_2max per FFM in non-obese vs. obese subjects at baseline whose age 35 years or more. Data were reported as average (minimum-maximum). n = 8 non-obese males, n = 11 non-obese females, n = 54 obese males and n = 63 obese females; $ = p-value <0.050

Characteristics	Non-obese	Obese at baseline	p-value of non-obese vs. obese at baseline
Age (years)			
Male	51 (44-60)	51 (35-65)	0.991
Female	47 (35-59)	51 (35-67)	0.130
All	49 (35-60)	51 (35-67)	0.221
VO_2max per fat-free mass (ml/(kg x min))			
Male	50.04 (38.62-60.93)	41.34 (26.44-60.99)	0.002$
Female	51.18 (42.19-68.28)	39.28 (14.42-54.62)	<0.001$
All	50.70 (38.62-68.28)	40.23 (14.42-60.99)	<0.001$

Table A4.7 Correlations of metabolites and insulin resistance profiles at baseline (non-obese and obese subjects) vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]

Group I	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[a]	Regression coefficient of ANOVA	p-value of ANOVA[b]	Regression coefficient of ANOVA	p-value of ANOVA[c]
Arginine	-1.42×10^{-1}	0.450	-9.71×10^{-2}	0.665	9.14×10^{-3}	0.529
Asparagine	-9.24×10^{-2}	0.061	-2.29×10^{-2}	0.695	-9.26×10^{-5}	0.981
Glutamine	3.17×10^{-1}	0.620	1.87×10^{-1}	0.807	-3.11×10^{-2}	0.529
Glutamine-to-glutamic acid ratio	-5.25×10^{-2}	0.459	-5.24×10^{-2}	0.536	-9.84×10^{-3}	0.071
Glycine	3.77	0.032$	2.76	0.185	-1.83×10^{-1}	0.174
Histidine	-3.03×10^{-1}	0.008$	-1.20×10^{-1}	0.375	4.27×10^{-3}	0.627
Serine	-9.31×10^{-2}	0.612	1.10×10^{-1}	0.614	-1.15×10^{-2}	0.415
Threonine	-5.81×10^{-1}	0.030$	-7.74×10^{-1}	0.016$	7.33×10^{-3}	0.721
C18:0 carnitine	2.72×10^{-4}	0.006$	-7.18×10^{-5}	0.539	7.70×10^{-6}	0.312
C20:0 carnitine	3.03×10^{-5}	<0.001$	-2.50×10^{-6}	0.796	8.00×10^{-7}	0.201
C16:0 carnitine-to-palmitic acid ratio	3.40×10^{-6}	0.130	5.00×10^{-7}	0.861	$<1.00 \times 10^{-7}$	0.825
C18:1 carnitine-to-oleic acid ratio	3.40×10^{-6}	0.048$	-8.00×10^{-7}	0.711	1.00×10^{-7}	0.582
Group II	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[d]	Regression coefficient of ANOVA	p-value of ANOVA[e]	Regression coefficient of ANOVA	p-value of ANOVA[f]
Glucose	3.01×10^{-1}	0.110	-1.00×10^{-1}	0.654	1.20×10^{-2}	0.410
Insulin	-1.34×10^{-1}	0.355	-2.90×10^{-1}	0.094	5.90×10^{-3}	0.598
HOMA-IR	-3.51×10^{-2}	0.452	-9.91×10^{-2}	0.076	2.99×10^{-3}	0.406
Alanine	4.18×10^{-2}	0.957	-9.07×10^{-1}	0.326	-2.87×10^{-2}	0.631
Aspartic acid	8.20×10^{-3}	0.627	2.06×10^{-4}	0.992	1.20×10^{-3}	0.355
Glutamic acid	3.39×10^{-1}	0.474	-3.72×10^{-1}	0.510	2.53×10^{-2}	0.489
Isoleucine+leucine	-6.82×10^{-1}	0.048$	-4.66×10^{-1}	0.256	4.38×10^{-3}	0.869
Lysine	-4.19×10^{-2}	0.886	-2.71×10^{-1}	0.437	4.93×10^{-3}	0.828
Methionine	-2.34×10^{-2}	0.494	1.31×10^{-2}	0.748	-3.21×10^{-3}	0.222

Phenylalanine	-3.90×10^{-3}	0.965	-3.87×10^{-2}	0.716	-4.88×10^{-3}	0.480
Proline	-8.11×10^{-1}	0.090	-2.14	<0.001[$]	-2.08×10^{-2}	0.571
Tryptophan	-5.26×10^{-2}	0.461	1.92×10^{-1}	0.025[$]	-1.09×10^{-3}	0.843
Tyrosine	4.59×10^{-3}	0.724	-5.56×10^{-2}	0.720	9.29×10^{-3}	0.355
Valine	-2.02×10^{-1}	0.612	-2.40×10^{-1}	0.613	1.60×10^{-2}	0.604
Ketoisoleucine+ketoleucine	-3.04×10^{-1}	0.019[$]	-2.00×10^{-1}	0.194	6.88×10^{-4}	0.945
Ketovaline	-2.22×10^{-2}	0.390	-5.17×10^{-2}	0.094	2.10×10^{-4}	0.916
C5-DC carnitine	8.10×10^{-6}	0.948	-7.83×10^{-5}	0.601	-1.07×10^{-5}	0.271
C8:0 carnitine-to-C10:0 carnitine ratio	1.09×10^{-3}	0.019[$]	8.81×10^{-4}	0.110	8.20×10^{-6}	0.819
Group III	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[g]	Regression coefficient of ANOVA	p-value of ANOVA[h]	Regression coefficient of ANOVA	p-value of ANOVA[i]
L-carnitine	3.16×10^{-1}	0.002[$]	-1.04×10^{-1}	0.374	3.60×10^{-3}	0.635
C3 carnitine	2.00×10^{-3}	0.175	-1.46×10^{-3}	0.405	-3.82×10^{-5}	0.737
C4 carnitine	1.74×10^{-3}	0.141	1.75×10^{-4}	0.900	-2.11×10^{-4}	0.020[$]
C5 carnitine	4.38×10^{-4}	0.299	-3.77×10^{-4}	0.452	1.50×10^{-6}	0.963
C8:1 carnitine	2.08×10^{-3}	0.078	-2.59×10^{-3}	0.065	-8.30×10^{-6}	0.927
C3 carnitine-to-ketovaline ratio	9.10×10^{-6}	0.095	-3.10×10^{-6}	0.637	-4.00×10^{-7}	0.375
C3 carnitine-to-valine ratio	2.03×10^{-4}	0.095	2.49×10^{-5}	0.863	-3.90×10^{-6}	0.674
C5 carnitine-to-ketoisoleucine+ketoleucine ratio	4.50×10^{-6}	0.037[$]	-4.00×10^{-7}	0.881	-1.00×10^{-7}	0.677
C5 carnitine-to-isoleucine+leucine ratio	2.11×10^{-5}	0.030[$]	2.00×10^{-7}	0.984	-1.00×10^{-7}	0.938
Group IV	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[j]	Regression coefficient of ANOVA	p-value of ANOVA[k]	Regression coefficient of ANOVA	p-value of ANOVA[l]
Palmitic acid	1.03×10^{-1}	0.901	-9.31×10^{-1}	0.345	-7.09×10^{-2}	0.266
Oleic acid	1.03	0.262	-3.25×10^{-2}	0.976	-1.64×10^{-1}	0.019[$]
C2 carnitine	7.31×10^{-2}	0.051	-9.40×10^{-2}	0.036[$]	-4.81×10^{-3}	0.094

C6 carnitine	8.87×10^{-4}	<0.001$	-6.80×10^{-4}	0.022$	-4.21×10^{-5}	0.026$
C8:0 carnitine	2.73×10^{-3}	0.016$	-1.51×10^{-3}	0.259	-1.27×10^{-4}	0.141
C10:0 carnitine	4.98×10^{-3}	0.048$	-3.87×10^{-3}	0.195	-2.50×10^{-4}	0.195
C10:1 carnitine	3.03×10^{-3}	0.004$	-1.67×10^{-3}	0.184	-1.63×10^{-4}	0.043$
C12:0 carnitine	3.27×10^{-4}	0.303	-7.78×10^{-4}	0.041$	-4.89×10^{-5}	0.045$
C12:1 carnitine	6.66×10^{-4}	0.080	-6.24×10^{-4}	0.168	-7.64×10^{-5}	0.008$
C12-OH carnitine	5.72×10^{-5}	0.232	-7.59×10^{-5}	0.150	-1.02×10^{-5}	0.002$
C14:0 carnitine	1.85×10^{-4}	0.054	-2.78×10^{-4}	0.016$	-1.82×10^{-5}	0.013$
C14:1 carnitine	5.59×10^{-4}	0.220	-9.15×10^{-4}	0.093	-1.06×10^{-4}	0.002$
C14:2 carnitine	3.42×10^{-4}	0.151	-4.39×10^{-4}	0.122	-5.00×10^{-5}	0.006$
C14-OH carnitine	2.56×10^{-5}	0.266	-5.00×10^{-5}	0.069	-4.30×10^{-6}	0.014$
C16:0 carnitine	3.86×10^{-4}	0.067	-2.05×10^{-4}	0.412	-2.29×10^{-5}	0.157
C16:1 carnitine	3.88×10^{-4}	0.009$	-2.02×10^{-4}	0.248	-3.27×10^{-5}	0.003$
C16-OH carnitine	7.40×10^{-6}	0.669	-5.50×10^{-5}	0.009$	-4.00×10^{-6}	0.003$
C18:1 carnitine	1.32×10^{-3}	<0.001$	1.14×10^{-4}	0.791	-6.40×10^{-5}	0.020$
C18:2 carnitine	4.32×10^{-4}	0.001$	4.00×10^{-5}	0.800	-1.63×10^{-5}	0.111
C18:2-OH carnitine	3.77×10^{-5}	0.037$	-3.09×10^{-5}	0.150	-5.30×10^{-6}	<0.001$
C20:1 carnitine	1.08×10^{-4}	<0.001$	3.03×10^{-5}	0.223	-1.80×10^{-6}	0.249
C20:2 carnitine	4.54×10^{-5}	<0.001$	1.43×10^{-5}	0.208	-1.00×10^{-6}	0.156
C20:3 carnitine	2.68×10^{-5}	<0.001$	6.60×10^{-6}	0.375	-2.00×10^{-7}	0.709
C20:4 carnitine	2.78×10^{-5}	<0.001$	8.70×10^{-6}	0.317	-9.00×10^{-7}	0.122

[1]Data were adjusted for sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI and diabetes status (yes or no).
[3]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
$ = p-value <0.050
p-value of MANOVA [a] = 0.003, [b] = 0.203, [c] = 0.668, [d] = 0.018, [e] = 0.030, [f] = 0.935, [g] = 0.012, [h] = 0.477, [i] = 0.410, [j] <0.001, [k] = 0.031, [l] = 0.004

Table A4.8 Correlations of metabolites and insulin resistance profiles in obese subjects at baseline vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]

Group I	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[a]	Regression coefficient of ANOVA	p-value of ANOVA[b]	Regression coefficient of ANOVA	p-value of ANOVA[c]
Arginine	-2.53×10^{-1}	0.261	-6.55×10^{-2}	0.800	-1.27×10^{-2}	0.510
Asparagine	-1.21×10^{-1}	0.023[$]	-3.61×10^{-2}	0.553	-4.71×10^{-3}	0.298
Glutamine	4.88×10^{-1}	0.532	1.31×10^{-1}	0.884	-4.23×10^{-2}	0.530
Glutamine-to-glutamic acid ratio	4.98×10^{-3}	0.939	-9.54×10^{-3}	0.899	-6.98×10^{-3}	0.209
Glycine	5.36	0.016[$]	4.08	0.109	-2.07×10^{-1}	0.270
Histidine	-2.91×10^{-1}	0.026[$]	-1.05×10^{-1}	0.480	-2.79×10^{-3}	0.801
Serine	1.54×10^{-1}	0.442	2.01×10^{-1}	0.386	-4.47×10^{-3}	0.795
Threonine	-6.42×10^{-1}	0.038[$]	-8.13×10^{-1}	0.023[$]	-1.09×10^{-3}	0.967
C18:0 carnitine	1.65×10^{-4}	0.138	-8.92×10^{-5}	0.484	-3.30×10^{-6}	0.730
C20:0 carnitine	2.33×10^{-5}	0.004[$]	-9.00×10^{-7}	0.922	$<1.00 \times 10^{-7}$	0.977
C16:0 carnitine-to-palmitic acid ratio	5.00×10^{-6}	0.036[$]	2.60×10^{-6}	0.332	-1.00×10^{-7}	0.681
C18:1 carnitine-to-oleic acid ratio	3.40×10^{-6}	0.075	2.00×10^{-7}	0.921	-1.00×10^{-7}	0.728
Group II	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[d]	Regression coefficient of ANOVA	p-value of ANOVA[e]	Regression coefficient of ANOVA	p-value of ANOVA[f]
Glucose	3.01×10^{-1}	0.207	-1.73×10^{-1}	0.529	1.76×10^{-2}	0.391
Insulin	-1.91×10^{-1}	0.306	-3.60×10^{-1}	0.096	6.34×10^{-3}	0.693
HOMA-IR	-5.27×10^{-2}	0.378	-1.26×10^{-1}	0.071	4.10×10^{-3}	0.425
Alanine	-2.87×10^{-1}	0.755	-1.22	0.251	-6.55×10^{-2}	0.407
Aspartic acid	5.45×10^{-3}	0.791	-1.73×10^{-2}	0.467	2.62×10^{-3}	0.138
Glutamic acid	3.48×10^{-1}	0.567	-5.27×10^{-1}	0.454	5.59×10^{-2}	0.285
Isoleucine+leucine	-8.93×10^{-1}	0.037[$]	-5.73×10^{-1}	0.244	5.66×10^{-3}	0.877
Lysine	-2.28×10^{-1}	0.515	-3.71×10^{-1}	0.358	-1.82×10^{-2}	0.546
Methionine	-3.17×10^{-2}	0.424	-2.69×10^{-3}	0.953	-5.77×10^{-3}	0.089

Phenylalanine	-7.01×10^{-2}	0.528	-1.28×10^{-1}	0.318	-1.11×10^{-2}	0.243
Proline	-7.50×10^{-1}	0.175	-1.92	0.003[$]	-5.01×10^{-2}	0.290
Tryptophan	-5.32×10^{-2}	0.521	1.82×10^{-1}	0.060	-5.75×10^{-3}	0.420
Tyrosine	-2.24×10^{-2}	0.889	-6.99×10^{-2}	0.705	7.16×10^{-3}	0.603
Valine	-2.51×10^{-1}	0.601	-2.21×10^{-1}	0.690	3.72×10^{-2}	0.367
Ketoisoleucine+ketoleucine	-3.56×10^{-1}	0.024[$]	-2.21×10^{-1}	0.220	4.42×10^{-4}	0.974
Ketovaline	-3.23×10^{-2}	0.301	-5.73×10^{-2}	0.114	6.76×10^{-4}	0.801
C5-DC carnitine	9.00×10^{-6}	0.953	-1.16×10^{-4}	0.515	-1.20×10^{-5}	0.364
C8:0 carnitine-to-C10:0 carnitine ratio	9.48×10^{-4}	0.093	6.35×10^{-4}	0.328	1.01×10^{-5}	0.835
Group III	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[g]	Regression coefficient of ANOVA	p-value of ANOVA[h]	Regression coefficient of ANOVA	p-value of ANOVA[i]
L-carnitine	3.00×10^{-1}	0.011[$]	-1.25×10^{-1}	0.353	6.54×10^{-3}	0.514
C3 carnitine	1.91×10^{-3}	0.271	-1.63×10^{-3}	0.418	-5.73×10^{-5}	0.702
C4 carnitine	2.05×10^{-3}	0.105	-8.04×10^{-4}	0.580	-8.91×10^{-5}	0.410
C5 carnitine	6.36×10^{-4}	0.207	-2.61×10^{-4}	0.652	3.81×10^{-5}	0.377
C8:1 carnitine	2.36×10^{-3}	0.098	-2.77×10^{-3}	0.094	4.77×10^{-5}	0.697
C3 carnitine-to-ketovaline ratio	9.80×10^{-6}	0.120	-3.00×10^{-6}	0.676	-6.00×10^{-7}	0.234
C3 carnitine-to-valine ratio	2.39×10^{-4}	0.075	3.85×10^{-5}	0.803	-5.60×10^{-6}	0.629
C5 carnitine-to-ketoisoleucine+ketoleucine ratio	6.30×10^{-6}	0.015[$]	6.00×10^{-7}	0.834	1.00×10^{-7}	0.690
C5 carnitine-to-isoleucine+leucine ratio	2.83×10^{-5}	0.015[$]	3.10×10^{-6}	0.813	8.00×10^{-7}	0.436
Group IV	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[j]	Regression coefficient of ANOVA	p-value of ANOVA[k]	Regression coefficient of ANOVA	p-value of ANOVA[l]
Palmitic acid	-9.34×10^{-2}	0.916	-1.06	0.304	-5.14×10^{-2}	0.502
Oleic acid	1.68	0.078	-1.89×10^{-1}	0.863	-7.82×10^{-2}	0.338
C2 carnitine	8.57×10^{-2}	0.035[$]	-9.72×10^{-2}	0.039[$]	-2.48×10^{-3}	0.476

C6 carnitine	8.26×10^{-4}	0.004$	-8.33×10^{-4}	0.011$	-5.46×10^{-5}	0.021$
C8:0 carnitine	2.19×10^{-3}	0.071	-1.83×10^{-3}	0.190	-2.27×10^{-4}	0.027$
C10:0 carnitine	3.78×10^{-3}	0.159	-4.30×10^{-3}	0.165	-4.76×10^{-4}	0.037$
C10:1 carnitine	2.47×10^{-3}	0.039$	-2.19×10^{-3}	0.112	-2.59×10^{-4}	0.010$
C12:0 carnitine	2.54×10^{-4}	0.458	-8.60×10^{-4}	0.031$	-6.65×10^{-5}	0.023$
C12:1 carnitine	6.93×10^{-4}	0.115	-7.59×10^{-4}	0.135	-9.15×10^{-5}	0.014$
C12-OH carnitine	3.37×10^{-5}	0.486	-1.30×10^{-4}	0.021$	-1.04×10^{-5}	0.012$
C14:0 carnitine	2.08×10^{-4}	0.049$	-2.85×10^{-4}	0.021$	-2.13×10^{-5}	0.018$
C14:1 carnitine	7.37×10^{-4}	0.144	-1.07×10^{-3}	0.068	-9.87×10^{-5}	0.021$
C14:2 carnitine	3.46×10^{-4}	0.198	-5.12×10^{-4}	0.101	-5.46×10^{-5}	0.017$
C14-OH carnitine	2.34×10^{-5}	0.364	-7.49×10^{-5}	0.013$	-3.80×10^{-6}	0.085
C16:0 carnitine	3.42×10^{-4}	0.162	-2.51×10^{-4}	0.373	-3.82×10^{-5}	0.066
C16:1 carnitine	4.70×10^{-4}	0.005$	-2.11×10^{-4}	0.263	-3.31×10^{-5}	0.017$
C16-OH carnitine	1.39×10^{-5}	0.485	-6.48×10^{-5}	0.006$	-3.70×10^{-6}	0.028$
C18:1 carnitine	1.45×10^{-3}	<0.001$	-2.25×10^{-5}	0.960	-5.85×10^{-5}	0.082
C18:2 carnitine	4.22×10^{-4}	0.005$	1.54×10^{-5}	0.929	-1.40×10^{-5}	0.276
C18:2-OH carnitine	5.02×10^{-5}	0.014$	-3.77×10^{-5}	0.106	-5.00×10^{-6}	0.004$
C20:1 carnitine	1.12×10^{-4}	<0.001$	2.29×10^{-5}	0.411	-2.00×10^{-6}	0.347
C20:2 carnitine	4.90×10^{-5}	<0.001$	1.48×10^{-5}	0.253	-1.10×10^{-6}	0.236
C20:3 carnitine	2.47×10^{-5}	0.001$	4.20×10^{-6}	0.621	-5.00×10^{-7}	0.408
C20:4 carnitine	2.36×10^{-5}	0.007$	4.10×10^{-6}	0.679	-1.50×10^{-6}	0.036$

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no).
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
$ = p-value <0.050
p-value of MANOVA [a] <0.001 , [b] = 0.117, [c] = 0.968, [d] = 0.161, [e] = 0.190, [f] = 0.611, [g] = 0.036, [h] = 0.348, [i] = 0.639, [j] = 0.005, [k] = 0.018, [l] = 0.116

Table A4.9 Correlations of metabolites and insulin resistance profiles in obese subjects after CR vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline]

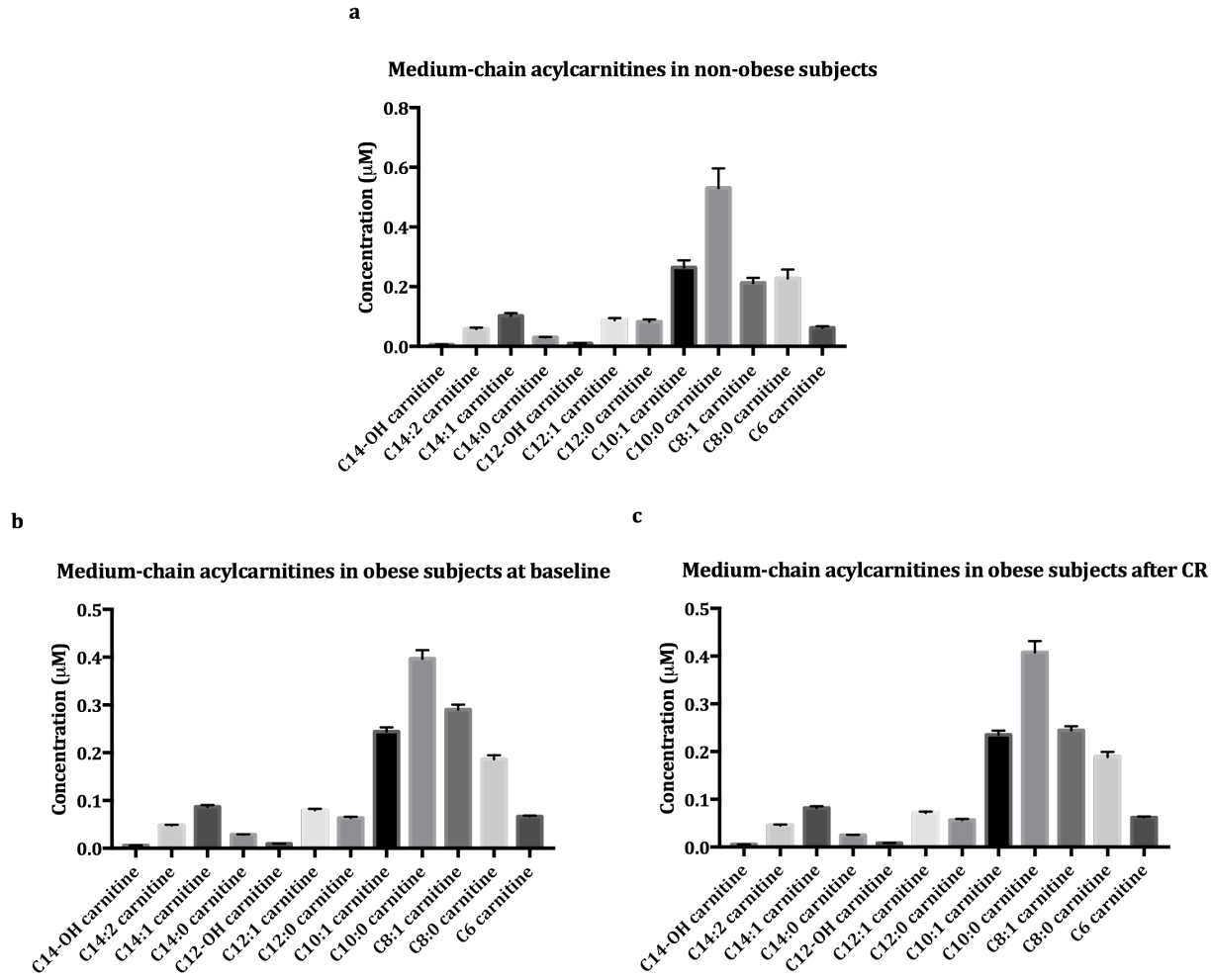
Group I	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[a]	Regression coefficient of ANOVA	p-value of ANOVA[b]	Regression coefficient of ANOVA	p-value of ANOVA[c]
Arginine	1.70×10^{-1}	0.452	1.37×10^{-1}	0.602	-1.04×10^{-2}	0.595
Asparagine	-3.28×10^{-2}	0.599	1.02×10^{-1}	0.162	-2.09×10^{-3}	0.698
Glutamine	2.07	0.003$	1.37	0.082	-1.02×10^{-2}	0.862
Glutamine-to-glutamic acid ratio	1.28×10^{-1}	0.192	1.03×10^{-2}	0.928	-1.44×10^{-2}	0.086
Glycine	6.07	0.036$	3.20	0.340	-3.87×10^{-1}	0.118
Histidine	-8.72×10^{-2}	0.549	1.50×10^{-1}	0.376	8.58×10^{-3}	0.495
Serine	2.17×10^{-1}	0.376	7.21×10^{-1}	0.012$	-2.80×10^{-4}	0.990
Threonine	4.40×10^{-1}	0.185	4.61×10^{-1}	0.233	1.41×10^{-4}	0.961
C18:0 carnitine	2.22×10^{-4}	0.073	1.12×10^{-5}	0.937	-8.00×10^{-6}	0.449
C20:0 carnitine	2.97×10^{-5}	0.002$	6.70×10^{-6}	0.545	-1.10×10^{-6}	0.186
C16:0 carnitine-to-palmitic acid ratio	3.90×10^{-6}	0.160	-4.00×10^{-7}	0.897	3.00×10^{-7}	0.239
C18:1 carnitine-to-oleic acid ratio	2.10×10^{-6}	0.406	-3.00×10^{-7}	0.918	1.00×10^{-7}	0.658
Group II	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[d]	Regression coefficient of ANOVA	p-value of ANOVA[e]	Regression coefficient of ANOVA	p-value of ANOVA[f]
Glucose	4.06×10^{-1}	0.035$	3.49×10^{-2}	0.875	5.88×10^{-3}	0.722
Insulin	8.18×10^{-3}	0.892	-1.31×10^{-1}	0.063	1.70×10^{-3}	0.743
HOMA-IR	1.41×10^{-2}	0.440	-3.56×10^{-2}	0.095	4.87×10^{-4}	0.757
Alanine	7.02×10^{-1}	0.463	-2.28×10^{-1}	0.837	1.05×10^{-2}	0.899
Aspartic acid	1.76×10^{-2}	0.301	-2.51×10^{-3}	0.899	-2.81×10^{-4}	0.849
Glutamic acid	3.87×10^{-1}	0.383	-4.21×10^{-2}	0.935	3.81×10^{-2}	0.320
Isoleucine+leucine	-9.30×10^{-1}	0.017$	-2.84×10^{-1}	0.526	-4.42×10^{-3}	0.894
Lysine	2.84×10^{-1}	0.501	7.22×10^{-1}	0.144	-3.75×10^{-2}	0.304
Methionine	3.86×10^{-3}	0.934	8.82×10^{-2}	0.104	-6.20×10^{-3}	0.121

Phenylalanine	-1.64×10^{-2}	0.899	-1.33×10^{-3}	0.993	-2.27×10^{-2}	0.041[$]
Proline	9.21×10^{-2}	0.866	-6.27×10^{-1}	0.324	4.41×10^{-2}	0.350
Tryptophan	-9.23×10^{-2}	0.278	7.92×10^{-2}	0.423	-1.93×10^{-3}	0.794
Tyrosine	2.61×10^{-1}	0.102	2.08×10^{-1}	0.260	3.92×10^{-3}	0.775
Valine	2.30×10^{-2}	0.963	3.09×10^{-1}	0.592	-2.15×10^{-3}	0.960
Ketoisoleucine+ketoleucine	-3.77×10^{-1}	0.003[$]	-2.43×10^{-1}	0.096	6.53×10^{-3}	0.545
Ketovaline	-1.62×10^{-2}	0.534	-4.52×10^{-2}	0.137	9.09×10^{-4}	0.686
C5-DC carnitine	-6.06×10^{-5}	0.684	-4.58×10^{-5}	0.792	-1.98×10^{-5}	0.122
C8:0 carnitine-to-C10:0 carnitine ratio	7.83×10^{-5}	0.126	-2.55×10^{-4}	0.668	-2.70×10^{-6}	0.951
Group III	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[g]	Regression coefficient of ANOVA	p-value of ANOVA[h]	Regression coefficient of ANOVA	p-value of ANOVA[i]
L-carnitine	2.18×10^{-1}	0.056	-1.79×10^{-1}	0.178	2.06×10^{-3}	0.834
C3 carnitine	3.11×10^{-3}	0.044[$]	-2.26×10^{-3}	0.206	-1.77×10^{-4}	0.181
C4 carnitine	2.76×10^{-3}	0.019[$]	-1.18×10^{-4}	0.931	-1.28×10^{-4}	0.203
C5 carnitine	4.29×10^{-4}	0.330	-5.76×10^{-4}	0.262	-5.94×10^{-5}	0.117
C8:1 carnitine	7.48×10^{-4}	0.543	-1.68×10^{-3}	0.242	1.13×10^{-5}	0.916
C3 carnitine-to-ketovaline ratio	1.53×10^{-5}	0.006[$]	-8.00×10^{-6}	0.212	-1.00×10^{-6}	0.027[$]
C3 carnitine-to-valine ratio	3.09×10^{-4}	0.012[$]	3.70×10^{-6}	0.979	-1.93×10^{-5}	0.062
C5 carnitine-to-ketoisoleucine+ketoleucine ratio	6.00×10^{-6}	0.007[$]	-1.90×10^{-6}	0.459	-4.00×10^{-7}	0.019[$]
C5 carnitine-to-isoleucine+leucine ratio	3.05×10^{-5}	0.002[$]	-6.00×10^{-7}	0.955	-1.90×10^{-6}	0.018[$]
Group IV	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]	
	Regression coefficient of ANOVA	p-value of ANOVA[j]	Regression coefficient of ANOVA	p-value of ANOVA[k]	Regression coefficient of ANOVA	p-value of ANOVA[l]
Palmitic acid	4.85×10^{-2}	0.963	1.37	0.266	-1.53×10^{-1}	0.091
Oleic acid	2.41	0.064	1.63	0.278	-1.20×10^{-1}	0.282
C2 carnitine	8.78×10^{-2}	0.047[$]	-5.34×10^{-2}	0.296	-4.90×10^{-3}	0.195

C6 carnitine	1.13×10^{-3}	<0.001$	-2.19×10^{-4}	0.542	-4.76×10^{-5}	0.072
C8:0 carnitine	3.97×10^{-3}	0.012$	2.16×10^{-5}	0.991	-1.59×10^{-4}	0.236
C10:0 carnitine	8.36×10^{-3}	0.017$	3.03×10^{-4}	0.940	-3.22×10^{-4}	0.283
C10:1 carnitine	3.87×10^{-3}	0.002$	2.56×10^{-5}	0.986	-2.06×10^{-4}	0.056
C12:0 carnitine	8.90×10^{-4}	0.019$	6.78×10^{-5}	0.877	-4.19×10^{-5}	0.197
C12:1 carnitine	1.08×10^{-3}	0.011$	1.86×10^{-4}	0.701	-5.84×10^{-5}	0.103
C12-OH carnitine	8.57×10^{-5}	0.052	-2.00×10^{-7}	0.998	-8.40×10^{-6}	0.025$
C14:0 carnitine	3.25×10^{-4}	0.003$	4.15×10^{-5}	0.743	-1.43×10^{-5}	0.127
C14:1 carnitine	1.40×10^{-3}	0.010$	2.85×10^{-4}	0.645	-6.22×10^{-5}	0.174
C14:2 carnitine	6.18×10^{-4}	0.037$	1.55×10^{-4}	0.652	-3.90×10^{-5}	0.124
C14-OH carnitine	4.25×10^{-5}	0.101	-5.30×10^{-6}	0.860	-4.60×10^{-6}	0.037$
C16:0 carnitine	5.12×10^{-4}	0.068	2.36×10^{-4}	0.466	-3.51×10^{-5}	0.144
C16:1 carnitine	6.21×10^{-4}	<0.001$	2.17×10^{-4}	0.255	-2.46×10^{-5}	0.080
C16-OH carnitine	1.61×10^{-5}	0.464	-3.14×10^{-5}	0.221	-2.10×10^{-6}	0.277
C18:1 carnitine	1.82×10^{-3}	<0.001$	7.77×10^{-4}	0.176	-4.91×10^{-5}	0.247
C18:2 carnitine	5.43×10^{-4}	0.002$	2.53×10^{-4}	0.213	-1.56×10^{-5}	0.298
C18:2-OH carnitine	6.59×10^{-5}	0.005$	1.68×10^{-5}	0.531	-3.50×10^{-6}	0.075
C20:1 carnitine	1.72×10^{-4}	<0.001$	4.80×10^{-5}	0.371	-5.10×10^{-6}	0.202
C20:2 carnitine	6.83×10^{-5}	<0.001$	1.83×10^{-5}	0.399	-1.60×10^{-6}	0.321
C20:3 carnitine	2.94×10^{-5}	0.001$	3.40×10^{-6}	0.742	-7.00×10^{-7}	0.333
C20:4 carnitine	2.42×10^{-5}	0.020$	-4.10×10^{-6}	0.731	-1.30×10^{-6}	0.159

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no).
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and VO_2max per FFM at baseline.
$ = p-value <0.050
p-value of MANOVA [a] = 0.001, [b] = 0.683, [c] = 0.822, [d] = 0.004, [e] = 0.095, [f] = 0.795, [g] <0.001, [h] = 0.150, [i] = 0.089, [j] <0.001, [k] = 0.545, [l] = 0.494

Figure A4.1 Medium-chain acylcarnitines in non-obese (a) and obese (b,c) subjects. Data were reported as average±SEM.



Appendices of chapter 5

Method of external calibration

When exact-matching stable isotope internal standards were not available, metabolite concentrations were estimated by external calibration in the presence of sample matrix as follows. A pooled sample was generated by combining aliquots of extracted human subject samples. The pooled sample was divided into 4 aliquots, which were spiked with the concentrations of metabolite standards listed in table A5.4, such that the volume ratio of pooled sample-to-spiked standard was 9:1. The slopes of the resulting calibration curves were used to calculate metabolite concentrations from measured peak areas (the y-intercept was set as the average peak area from replicate runs of a blank sample).

Table A5.1 Internal standards used for quantitation of targeted metabolites. NSK-B internal standard mix was purchased from Cambridge Isotope (Andover, MA). All other carbon-13 stable isotope internal standards were purchased from Sigma-Aldrich (St. Louis, MO).

Internal Standards	Internal standard concentration added to extraction solvent
Algal AA mixture_^{13}C	10 μg/ml
AMP_$^{13}C_{10}$_$^{15}N_5$	800 nM
ATP_$^{13}C_{10}$_$^{15}N_5$	20 μM
Citrate_$^{13}C_6$	2 μM
Fructose 1,6-bisphosphate_$^{13}C_6$	16 μM
Frucose-6-phosphate_$^{13}C_6$	20 μM
Glutamine_$^{13}C_5$	4 μM
Glucose_$^{13}C_6$	40 μM
Lactate_$^{13}C_3$	40 μM
Malate_$^{13}C_4$	4 μM
NSK-B internal standard mix	
$^2H_9$-Carnitine (L-carnitine)	510.33 nM
$^2H_3$-Acetylcarnitine (C2)	128.33 nM
$^2H_3$-Propionylcarnitine (C3)	25.33 nM
$^2H_3$-Butyrylcarnitine (C4)	25.33 nM
$^2H_9$-Isovalerylcarnitine (C5)	25.33 nM
$^2H_3$-Octanoylcarnitine (C8)	25.33 nM
$^2H_9$-Myristoylcarnitine (C14)	25.33 nM
$^2H_3$-Palmitoylcarnitine (C16)	50.67 nM
Oleic acid_$^{13}C_{18}$	10 μM
Succinate-$^{13}C_4$	4 μM

Table A5.2 All targeted metabolites identified in this study

Identified targeted metabolites	
AAs	Acylcarnitines
Alanine	L-carnitine
Arginine	C2 carnitine
Asparagine	C3 carnitine
Aspartic acid	C4 carnitine
Glutamic acid	C5 carnitine
Glutamine	C5-DC carnitine
Glycine	C6 carnitine
Histidine	C8:0 carnitine
Isoleucine+leucine	C8:1 carnitine
Lysine	C10:0 carnitine
Methionine	C10:1 carnitine
Phenylalanine	C12:0 carnitine
Proline	C12:1 carnitine
Serine	C12-OH carnitine
Threonine	C14:0 carnitine
Tryptophan	C14:1 carnitine
Tyrosine	C14:2 carnitine
Valine	C14-OH carnitine
Glycolysis metabolites	C16:0 carnitine
Glucose	C16:1 carnitine
Glucose-6-phosphate+fructose-6-phosphate	C16-OH carnitine
Fructose 1,6-bisphosphate	C18:0 carnitine
Dihydroxyacetone phosphate	C18:1 carnitine
2-phosphoglyceric acid+3-phosphoglyceric acid	C18:2 carnitine
Glycerol-3-phosphate	C18:2-OH carnitine
Lactate	C20:0 carnitine
FFA	C20:1 carnitine
Oleic acid	C20:2 carnitine
	C20:3 carnitine
	C20:4 carnitine
TCA cycle metabolites	Nucleotides
Citrate	AMP
Succinate	ADP
Malate	ATP
	NAD
	NADH
	NADP
	FAD

Table A5.3 MRM for detection of AAs, oleic acids, glycolysis metabolites, TCA metabolites and nucleotides. Dwell time of 10 ms was used for all metabolites.

Metabolites	Precursor Ion	Product Ion	Fragmentor	Collision Energy
2-phosphoglyceric acid+ 3-phosphoglyceric acid	185	79	100	45
ADP	426	79	70	55
Alanine	88.05	88.05	50	0
Alanine_$^{13}C_3$	91.05	91.05	50	0
AMP	346.1	79	155	60
AMP_$^{13}C_{10}$_$^{15}N_5$	361.1	79	155	60
Arginine	173.11	131.1	110	10
Arginine_$^{13}C_6$	179.11	136.1	110	10
Asparagine	131.05	113	80	6
Aspartic acid	132	88	60	7
Aspartic acid_$^{13}C_4$	136	91	60	7
ATP	506	79	90	100
ATP_$^{13}C_{10}$_$^{15}N_5$	521	79	90	100
Citrate	191	87	95	13
Citrate_$^{13}C_6$	197	116.1	80	7
Dihydroxyacetone phosphate	169	79	50	34
FAD	784	79	170	165
Fructose 1,6-bisphosphate	339	97	85	18
Fructose 1,6-bisphosphate_$^{13}C_6$	345	79	85	18
Glucose	179.1	59	80	15
Glucose_$^{13}C_6$	185.1	61	80	15
Glucose-6-phosphate+ fructose-6-phosphate	259	97	75	10
Fructose-6-phosphate_$^{13}C_6$	265	79	75	10
Glutamic acid	146	102	60	7
Glutamic acid_$^{13}C_5$	151	106	60	7
Glutamine	145.07	127.1	80	6
Glutamine_$^{13}C_5$	150.07	132.1	80	6
Glyceraldehyde-3-phosphate	171.01	79	80	30
Glycine	74.03	74.03	30	0
Glycine_$^{13}C_2$	76.03	76.03	30	0
Histidine	154.07	93	80	14
Isoleucine+leucine	130.09	130.09	72	0
Isoleucine+leucine_$^{13}C_6$	136.09	136.09	72	0
Lactate	89	43	65	9
Lactate_$^{13}C_3$	92	45	65	9
Lysine	145.11	145.11	72	0

Malate	133	115	50	5
Malate_$^{13}C_4$	137	119	50	5
Methionine	148.05	47.1	80	10
Methionine_$^{13}C_5$	153.05	48.1	80	10
NAD	662	540	150	15
NADH	664	408	140	40
NADP	742	620	140	20
Oleic acid	281.26	281.26	50	0
Oleic acid_$^{13}C_{18}$	299.26	299.26	50	0
Phenylalanine	164.08	147.1	80	10
Phenylalanine_$^{13}C_9$	173.08	156.1	80	10
Proline	114.06	114.06	86	0
Proline_$^{13}C_5$	119.06	119.06	86	0
Serine	104.04	74.1	80	6
Serine_$^{13}C_3$	107.04	76.1	80	6
Succinate	117	73	55	7
Succinate_$^{13}C_4$	121	76	55	7
Threonine	118.06	74.1	80	6
Threonine_$^{13}C_4$	122.06	76.1	80	6
Tryptophan	203.09	159.1	80	6
Tyrosine	180.07	163	80	10
Tyrosine_$^{13}C_9$	189.07	172	80	10
Valine	116.08	116.08	58	0
Valine_$^{13}C_5$	121.08	121.08	58	0

Table A5.4 Calibration parameters used for quantitation of targeted metabolites. All calibration curve fits were linear. All metabolite standards were purchased from Sigma-Aldrich.

Metabolites	Calibration curve concentrations (µM)	R^2
Metabolites with exact-matching stable isotope internal standards		
AAs		
Alanine	0, 25, 83.33, 250, 833.33, 2500	0.9997
Arginine	0, 5, 16.67, 50, 166.67, 500	0.9988
Aspartic acid	0, 5, 16.67, 50, 166.67, 500	0.9999
Glutamic acid	0, 25, 83.33, 250, 833.33, 2500	0.9973
Glutamine	0, 75, 250, 750, 2500, 7500	0.9999
Glycine	0, 5, 16.67, 50, 166.67, 500	0.9998
Isoleucine+leucine	0, 10, 33.33, 100, 333.33	0.9998
Methionine	0, 5, 16.67, 50, 166.67, 500	0.9992
Phenylalanine	0, 5, 16.67, 50, 166.67, 500	0.9998
Proline	0, 5, 16.67, 50, 166.67, 500	0.9999
Serine	0, 5, 16.67, 50, 166.67, 500	0.9999
Threonine	0, 5, 16.67, 50, 166.67, 500	0.9998
Tyrosine	0, 5, 16.67, 50, 166.67, 500	0.9990
Valine	0, 5, 16.67, 50, 166.67, 500	0.9981
Glycolysis metabolites		
Glucose	0, 200, 666.67, 2000, 6666.67, 20000	0.9985
Glucose-6-phosphate+fructose-6-phosphate	0, 45, 150, 450, 1500, 4500	0.9995
Fructose 1,6-bisphosphate	0, 45, 150, 450, 1500, 4500	0.9999
Lactate	0, 480, 1600, 4800, 16000, 48000	0.9998
FFA		
Oleic acid	0, 2, 6.67, 20, 66.67, 200	0.9998
TCA cycle metabolites		
Citrate	0, 5, 16.67, 50, 166.67, 500	0.9997
Succinate	0, 10, 33.33, 100, 333.33, 1000	0.9996
Malate	0, 10, 33.33, 100, 333.33, 1000	0.9998
Nucleotides		
AMP	0, 10, 33.33, 100, 333.33	0.9998
ATP	0, 10, 33.33, 100, 333.33, 1000	0.9996
Metabolites without exact-matching stable isotope internal standards		
AAs		
Asparagine	0, 9.6, 32, 96	0.9970
Histidine	0, 21, 70, 210	0.9963
Lysine	0, 50, 166.67, 500	0.9988
Tryptophan	0, 0.9, 3, 9	0.9750
Glycolysis metabolites		
Dihydroxyacetone phosphate	0, 6, 20, 60	0.9973
2-phosphoglyceric acid+3-phosphoglyceric acid	0, 6.9, 23, 69	0.9911
Glycerol-3-phosphate	0, 30, 100, 300	0.9986
Nucleotides		
ADP	0, 18, 60, 180	0.9956
NAD	0, 10.05, 33.5, 100.5	0.9786
NADH	0, 1, 3.33, 10	0.9207
NADP	0, 1.05, 3.5, 10.5	0.9985
FAD	0, 0.6, 2, 6	0.9986

Table A5.5 Primers used for DNA analysis. All primers were purchased from Invitrogen.

Primers	Binding site position	Sequence (5′-3′)
mtDNA at the major arc (Forward sequence)	mt 10,912 – 10,931	CTGTTCCCCAACCTTTTCCT
mtDNA at the major arc (Reverse sequence)	mt 10,975 – 10,994	CCATGATTGTGAGGGGTAGG
mtDNA at the minor arc (Forward sequence)	mt 16,528 – 16,548	CTAAATAGCCCACACGTTCCC
mtDNA at the minor arc (Reverse sequence)	mt 23 – 42	AGAGCTCCCGTGAGTGGTTA
DNA at β2M gene of nucleus (Forward sequence)	Chr15 15,798,932 – 15,798,958	GCTGGGTAGCTCTAAACAATGTATTCA
DNA at β2M gene of nucleus (Reverse sequence)	Chr15 15,798,999 – 15,799,026	CCATGTACTAACAAATGTCTAAAATGGT

Table A5.6 All 212 selected untargeted metabolites for final analysis

Neutral mass (Da)	Retention time (min)	Annotation
115.9962	0.91	Fumarate
136.0318	1.15	
138.0308	4.62	Urocanic acid
138.0313	6.03	
140.0130	9.50	
152.0442	5.71	Xanthine
168.0724	1.78	
172.0082	1.21	
176.0308	1.17	
177.0778	4.82	
180.0512	1.16	
187.0619	4.06	2-keto-6-acetamidocaproate
187.1197	4.91	Unknown
188.0487	1.24	
193.0250	1.17	5,6-dihydroxyindole-2-carboxylic acid
202.1188	6.20	
215.1509	5.90	
217.0934	2.83	Alanyl-glutamine
219.1122	3.89	Pantothenic acid
222.0883	6.79	Glycyl-phenylalanine
226.1024	1.06	Carnosine
232.0022	4.71	Vanillin 4-sulfate
240.1015	6.92	
243.1385	6.59	
244.2029	8.15	
246.1450	5.69	3-hydroxydodecanedioic acid
254.1507	6.85	N-(4-aminobutyryl)-l-histidine
254.2238	9.01	
258.2186	8.37	3-hydroxypentadecanoic acid
259.9227	1.02	
270.2158	8.58	Ketopalmitic acid
280.2383	9.09	
287.9841	1.07	Unknown
298.1644	8.90	
300.2090	9.26	
300.2569	9.05	
302.2231	9.25	Eicosapentanoic acid
303.1412	4.17	Tryptophyl-valine
304.2378	9.25	
308.2030	8.61	Ketooleic acid
316.2047	8.06	Pregnenolone
321.0681	2.59	Beta-citryl-glutamic acid
329.0572	1.69	
336.0495	1.18	Nicotinate beta-d-ribonucleotide
342.1288	6.30	Unknown
351.1184	1.27	
352.2276	8.76	
361.1471	4.02	Unknown
368.1663	6.34	Dehydroepiandrosterone (DHEA)
374.2597	9.28	Calcitroic acid
377.9507	1.31	
378.2780	9.13	Allopregnanolone
379.9179	0.98	Unknown

381.2653	8.34	
382.1083	1.21	
393.9162	0.89	
394.2826	9.24	11(R)-hydroxyeicosatetranoic acid
396.2357	8.79	
400.2355	9.29	Unknown
408.3204	9.13	
410.2419	8.73	LysoPA(16:0)
411.8696	0.99	Unknown
432.0969	1.69	Reduced glutathione
433.1873	5.54	
434.2439	8.54	LysoPA(18:2)
434.3319	9.24	Unknown
436.1828	8.77	
438.2734	9.11	LysoPA(18:0)
444.3021	9.26	Steryl citrate
446.0355	0.97	
446.3331	9.05	Arachidonic acid
450.1803	7.34	Geranylgeranyl pysophosphate
450.2626	8.11	
450.3600	9.27	
460.0205	1.22	Lactose-6-phosphate
468.3664	9.02	Glycocholic acid
474.1936	0.95	Unknown
477.0836	1.20	Phosphocreatine
481.3144	8.43	LysoPE(18:0)
488.3158	9.04	
496.3743	9.06	
496.3938	9.22	
501.2856	8.25	LysoPE(20:4)
513.3107	8.00	
520.0430	1.20	
521.2764	8.21	LysoPS(18:1)
529.3160	8.53	LysoPE(22:4)
534.3481	9.32	
540.4328	9.03	
542.3785	9.06	
545.2778	8.24	LysoPS(20:4)
545.9797	1.01	
547.0832	1.19	Unknown
549.3422	9.03	
553.9799	1.04	
561.3344	8.80	LysoPE(20:0)
565.3374	8.18	LysoPE(20:2)
567.3513	8.44	LysoPE(20:1)
573.1171	1.20	Biotinyl-5-‘AMP
578.3776	9.31	1,25-dihydroxyvitamin D_3 3-glycoside
591.3519	8.33	Unknown
593.3368	8.32	Lactosyl sphingosine(d16:2)
595.3681	8.76	Lactosyl sphingosine(d16:1)
598.3691	8.93	Unknown
601.7907	1.02	
605.3634	8.88	LysoPC(20:3)
606.4157	9.47	Unknown
607.0696	1.49	UDP-n-acetylglucosamine

609.3357	8.43	Unknown
612.1097	1.18	Oxidized glutatione
612.1507	2.60	
617.3690	8.48	Neuromedin N
619.3638	8.70	Unknown
621.3616	8.78	Unknown
624.3997	8.90	
626.4273	9.06	
628.4136	9.52	
633.0985	1.21	Unknown
635.3788	8.70	Unknown
635.4111	8.97	LysoPC(22:2)
638.0621	1.23	
653.3852	8.66	
664.0899	0.98	Unknown
665.4161	8.51	
667.6105	10.59	Ceramide(t42:0)
670.3112	8.57	
672.0854	1.20	Unknown
676.0198	1.43	
681.6250	10.71	Ceramide(d40:1)
689.3951	8.78	Unknown
691.6111	10.39	Unknown
693.0396	1.22	Unknown
693.4124	8.74	PI-ceramide(d28:2)
695.4084	8.62	
695.9773	0.96	
698.3700	8.83	Unknown
698.4830	9.30	PA(36:3)
699.5203	9.93	PE(P-34:2)
703.4040	8.68	
706.5164	9.48	
713.4994	6.97	
714.3681	8.74	Unknown
717.5310	9.95	PE(34:1)
718.5823	9.40	PA(36:1)OH
721.4498	8.68	LysoPE(P-16:0)
723.5166	9.88	PE(P-36:4)
725.5296	9.95	PE(P-36:3)
727.5425	10.10	PE(P-36:2)
731.4928	9.32	Cardiolipin(72:8)
737.4261	8.77	PE(36:5)
737.5373	9.98	PC(28:0)
739.5151	9.70	PE(36:4)
740.4112	8.92	
741.5288	9.82	PE(36:3)
743.0656	1.51	Unknown
745.5555	10.14	PE(36:1)
746.5209	10.21	
748.5262	10.54	PG(34:1)
749.5365	9.91	PE(P-38:5)
754.4030	8.70	Unknown
759.5267	9.46	PE(O-34:3)
761.0813	1.20	
761.0840	1.66	

761.5324	10.04	
762.5422	10.77	PA(O-42:6)
763.5656	10.32	Unknown
769.0324	1.22	Unknown
770.5218	10.02	PE(38:4)
771.5340	9.57	PS(O-36:3)
772.5292	10.21	
774.5334	10.04	
774.5429	10.70	
774.5602	9.84	
775.5370	9.69	
776.5577	10.98	PG(36:1)
776.6040	10.03	PE(P-40:6)
779.5770	10.20	
779.9435	1.10	
783.5330	9.50	PE(38:4)OH
787.5561	10.04	PE(38:2)OH
795.5713	10.21	
797.8691	0.90	
801.5529	9.73	Unknown
803.5684	9.87	PE(36:2)
813.5573	10.15	PS(38:3)
814.4511	8.77	Unknown
817.5797	10.01	
822.4115	8.73	
823.5687	8.79	Unknown
825.5562	10.13	
827.5675	9.76	Unknown
829.5778	9.82	Unknown
831.5981	10.18	Unknown
832.6560	10.69	
833.6155	10.46	PE(O-44:6)
835.5422	9.22	PS(40:6)
837.4364	8.78	
841.5952	10.24	PC(36:4)
848.4519	10.55	PG(P-36:5)
851.5363	8.97	PE(40:6)
853.5664	8.67	Unknown
860.6974	11.09	
862.5579	10.56	
866.1155	1.21	Unknown
866.5786	10.89	
884.5422	10.32	PI(38:5)
886.5584	10.43	PI(38:4)
936.5089	8.76	Unknown
950.5388	9.28	
976.5048	8.71	
994.0692	1.20	
995.7851	1.02	

Table A5.7 Correlations of targeted metabolites and mtDNA profiles at baseline (non-obese and all obese subjects) vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites at baseline vs. mtDNA count number at baseline

AAs	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Alanine	2.85	0.961	47.85	0.545	7.61	0.142	-6.21×10^{-1}	0.345
Arginine	7.30×10^{-1}	0.934	-20.81	0.083	1.16×10^{-1}	0.883	-1.31×10^{-2}	0.896
Asparagine	-44.99	0.066	-67.68	0.041$	2.74	0.203	-5.83×10^{-1}	0.035$
Aspartic acid	2.07	0.836	-29.32	0.032$	-6.61×10^{-1}	0.457	1.28×10^{-1}	0.265
Glutamic acid	7.91×10^{-1}	0.982	87.58	0.072	-4.37	0.168	1.16	0.004$
Glutamine	36.08	0.775	-1.70×10^{2}	0.322	11.12	0.323	-1.38×10^{-1}	0.923
Glutamine-to-glutamic acid ratio	-3.00×10^{-3}	0.865	-6.44×10^{-2}	0.045$	2.43×10^{-3}	0.246	-7.48×10^{-4}	0.005$
Glycine	-31.88	0.042$	-61.75	0.004$	1.52	0.272	-2.87×10^{-1}	0.112
Histidine	-45.91	0.120	-37.89	0.340	-2.58	0.323	1.14×10^{-1}	0.731
Isoleucine+leucine	-2.16×10^{-1}	0.075	-4.33×10^{-1}	0.009$	3.36×10^{-4}	0.975	-6.58×10^{-4}	0.638
Lysine	1.24×10^{2}	0.573	-3.14×10^{2}	0.294	-4.93	0.802	-3.28	0.188
Methionine	-4.75×10^{-1}	0.296	-1.03	0.093	2.48×10^{-2}	0.538	-5.93×10^{-3}	0.249
Phenylalanine	-5.96×10^{-2}	0.925	-4.86×10^{-1}	0.572	5.10×10^{-3}	0.928	-6.44×10^{-3}	0.368
Proline	-18.67	0.082	-52.22	<0.001$	-2.34×10^{-1}	0.805	-1.83×10^{-1}	0.148
Serine	-8.45	0.041$	-7.55	0.174	1.78×10^{-1}	0.624	-5.43×10^{-2}	0.240
Threonine	-14.81	0.001$	-16.87	0.005$	5.05×10^{-1}	0.195	-7.01×10^{-2}	0.170
Tryptophan	-6.51	0.010$	-6.15	0.070	1.44×10^{-1}	0.516	2.95×10^{-2}	0.299
Tyrosine	9.85×10^{-2}	0.910	-1.74×10^{-2}	0.883	7.84×10^{-2}	0.314	-1.28×10^{-2}	0.191
Valine	-2.27	0.260	-5.49	0.045$	-1.20×10^{-3}	0.995	-6.09×10^{-3}	0.791
Glycolysis metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Glucose	-55.87	0.174	-45.57	0.412	-6.75×10^{-1}	0.850	-5.02×10^{-1}	0.272
Glucose-6-phosphate+fructose-6-phosphate	-2.30×10^{2}	0.201	1.42×10^{2}	0.558	7.17	0.654	-3.62	0.071
Fructose 1,6-bisphosphate	1.04×10^{2}	0.441	-1.11×10^{2}	0.540	-5.10	0.670	-1.13	0.457
Glyceraldehyde-3-phosphate	-25.93	0.930	3.21×10^{2}	0.423	41.50	0.114	-3.11	0.352
Dihydroxyacetone	-37.45	0.434	-22.80	0.724	-8.09×10^{-1}	0.849	-1.21	0.022$

phosphate								
2-phosphoglyceric acid+ 3-phosphoglyceric acid	-13.72	0.381	22.55	0.290	9.13×10^{-1}	0.512	-2.67×10^{-1}	0.132
Lactate	-50.46	0.965	1.09×10^{3}	0.479	62.97	0.534	-22.34	0.079
Fructose 1,6-bisphosphate-to-glucose-6-phosphate+fructose -6-phosphate ratio	3.44×10^{-2}	0.594	5.38×10^{-3}	0.951	3.55×10^{-3}	0.536	2.18×10^{-3}	0.002$
FFA	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Oleic acid	-2.81×10^{-1}	0.965	-4.30	0.621	-3.32×10^{-1}	0.562	3.33×10^{-2}	0.646
Acylcarnitines	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
L-carnitine	57.32	0.344	-32.36	0.693	5.93	0.269	5.81×10^{-2}	0.932
C2 carnitine	-40.32	0.033$	48.19	0.059	-8.48	<0.001$	2.14×10^{-1}	0.316
C3 carnitine	1.09×10^{-1}	0.629	-7.62×10^{-1}	0.014$	-9.39×10^{-3}	0.640	-2.28×10^{-3}	0.381
C4 carnitine	-4.26×10^{-1}	0. 321	4.15×10^{-1}	0.474	-1.15×10^{-1}	0.002$	1.12×10^{-3}	0.816
C5 carnitine	-5.90×10^{-2}	0.700	-7.24×10^{-2}	0.726	7.45×10^{-3}	0.584	-3.29×10^{-4}	0.848
C5-DC carnitine	-7.45×10^{-3}	0.671	3.40×10^{-2}	0.153	-4.91×10^{-3}	0.001$	1.56×10^{-4}	0.431
C6 carnitine	-1.88×10^{-1}	0.099	9.69×10^{-2}	0.527	-1.24×10^{-2}	0.217	-4.63×10^{-4}	0.717
C8:0 carnitine	-5.30×10^{-2}	0.159	5.37×10^{-2}	0.290	-2.35×10^{-3}	0. 481	-5.04×10^{-4}	0.232
C8:1 carnitine	1.11×10^{-2}	0.488	7.81×10^{-3}	0.719	4.23×10^{-4}	0.767	3.43×10^{-3}	0.056
C10:0 carnitine	-4.72×10^{-2}	0.190	6.17×10^{-2}	0.205	-2.67×10^{-3}	0.404	-5.43×10^{-4}	0.181
C10:1 carnitine	-1.39×10^{-2}	0.265	1.73×10^{-2}	0.305	-3.74×10^{-4}	0.737	-1.21×10^{-4}	0.392
C12:0 carnitine	-9.91×10^{-2}	0.374	1.09×10^{-1}	0.470	6.13×10^{-4}	0.951	-1.82×10^{-3}	0.144
C12:1 carnitine	-9.29×10^{-3}	0.437	3.30×10^{-2}	0.042$	-3.53×10^{-4}	0.740	-1.12×10^{-4}	0.412
C12-OH carnitine	-5.12×10^{-2}	0.155	1.11×10^{-2}	0.818	-4.48×10^{-4}	0.889	-5.06×10^{-4}	0.208
C14:0 carnitine	2.46×10^{-2}	0.872	3.34×10^{-1}	0.107	2.94×10^{-3}	0.829	-1.53×10^{-3}	0.377
C14:1 carnitine	3.28×10^{-2}	0. 748	3.23×10^{-1}	0.021$	3.66×10^{-3}	0.687	-6.02×10^{-4}	0.609
C14:2 carnitine	1.13×10^{-2}	0.719	9.97×10^{-2}	0.021$	2.76×10^{-3}	0.325	-2.11×10^{-4}	0.561
C14-OH carnitine	-7.13×10^{-3}	0.190	9.42×10^{-3}	0.200	-4.71×10^{-4}	0.328	-5.79×10^{-5}	0.345
C16:0 carnitine	3.63×10^{-1}	0.549	7.72×10^{-1}	0.347	-5.94×10^{-3}	0.912	-4.94×10^{-3}	0.470

C16:1 carnitine	4.01×10^{-1}	0.384	9.63×10^{-1}	0.123	7.49×10^{-3}	0.855	-1.83×10^{-3}	0.727
C16-OH carnitine	-1.77×10^{-2}	0.454	2.44×10^{-2}	0.444	2.46×10^{-3}	0.907	-2.59×10^{-4}	0.329
C18:0 carnitine	3.28×10^{-1}	0.492	7.24×10^{-1}	0.263	1.83×10^{-2}	0.667	-2.65×10^{-3}	0.624
C18:1 carnitine	1.84	0.333	2.32	0.367	-7.48×10^{-3}	0.965	-1.18×10^{-2}	0.581
C18:2 carnitine	6.21×10^{-1}	0.283	9.34×10^{-1}	0.233	1.24×10^{-2}	0.809	-1.88×10^{-3}	0.774
C20:0 carnitine	5.05×10^{-3}	0.178	9.17×10^{-3}	0.072	2.42×10^{-5}	0.942	1.83×10^{-5}	0.669
C20:1 carnitine	3.52×10^{-2}	0.356	6.20×10^{-2}	0.230	3.07×10^{-4}	0.928	-1.44×10^{-4}	0.738
C20:2 carnitine	1.26×10^{-2}	0.343	1.86×10^{-2}	0.300	5.82×10^{-5}	0.961	-4.30×10^{-5}	0.742
C20:3 carnitine	1.30×10^{-2}	0.386	1.63×10^{-2}	0.420	8.36×10^{-5}	0.950	-9.26×10^{-5}	0.582
C20:4 carnitine	1.02×10^{-2}	0.758	2.92×10^{-2}	0.515	4.11×10^{-4}	0.890	-3.51×10^{-4}	0.346
TCA cycle metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Citrate	-6.48×10^{-1}	0.748	3.82	0.163	-3.65×10^{-1}	0.040[$]	7.97×10^{-2}	<0.001[$]
Succinate	2.28	0.468	6.63	0.121	-6.87×10^{-2}	0.806	8.07×10^{-2}	0.022[$]
Malate	11.94	0.422	27.53	0.172	1.45	0.272	-9.15×10^{-2}	0.587
Nucleotides	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
AMP	2.82×10^{-1}	0.297	3.94×10^{-1}	0.281	1.89×10^{-2}	0.430	-8.43×10^{-3}	0.782
ADP	7.30	0.857	-62.06	0.257	2.15	0.550	-8.97×10^{-1}	0.048[$]
ATP	-34.02	0.545	-13.75	0.856	1.93	0.700	1.03	0.103
NAD^+	-1.07×10^{2}	0.134	63.06	0.513	3.30	0.602	3.53×10^{-1}	0.660
NADH	-6.42	0.563	-12.44	0.417	-1.38×10^{-1}	0.890	1.27×10^{-1}	0.310
NAD^+-to-NADH ratio	-2.79×10^{-1}	0.955	6.07	0.376	-4.56×10^{-1}	0.306	-5.52×10^{-2}	0.322
NADP	-2.03	0.403	6.26	0.058	-2.79×10^{-2}	0.897	1.37×10^{-1}	<0.001[$]
FAD	-6.35×10^{-1}	0.357	1.32	0.159	-6.37×10^{-2}	0.298	1.88×10^{-3}	0.810
Long-chain acylcarnitines-to-FFA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
C18:1 carnitine-to-oleic acid ratio	6.59×10^{-3}	0.493	2.28×10^{-2}	0.081	1.61×10^{-3}	0.058	-1.73×10^{-4}	0.111
C3 and C5 carnitine-to-BCAA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value

C3 carnitine-to-valine ratio	1.67×10^{-4}	0.525	-7.86×10^{-4}	0.029$	-4.88×10^{-6}	0.835	-3.55×10^{-6}	0.238
C5 carnitine-to-isoleucine+leucine ratio	-9.96×10^{-4}	0.742	-9.13×10^{-4}	0.823	1.42×10^{-4}	0.599	-8.38×10^{-6}	0.805

mtDNA profiles	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
mtDNA deletion ratio	2.77×10^{-1}	0.197	2.18×10^{-2}	0.940	1.15×10^{-2}	0.547	NA	NA
mtDNA count number	-8.55	0.318	25.21	0.031$	1.37×10^{-1}	0.857	NA	NA

[1]Data were adjusted for sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no) and HOMA-IR.
$ = p-value <0.050
NA = not applicable

Table A5.8 Correlations of targeted metabolites and mtDNA profiles at baseline of 41 obese subjects who received muscle biopsy at both baseline and after CR vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites at baseline vs. mtDNA count number at baseline

AAs	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Alanine	21.04	0.804	2.30×10^{2}	0.101	-3.14	0.787	-4.35×10^{-4}	0.999
Arginine	-2.83	0.836	3.76	0.865	-6.57×10^{-1}	0.727	3.53×10^{-1}	0.085
Asparagine	-58.57	0.114	-86.96	0.146	-6.91	0.161	3.07×10^{-2}	0.957
Aspartic acid	31.98	0.122	-19.55	0.553	-2.55	0.360	-6.06×10^{-2}	0.846
Glutamic acid	1.11	0.984	87.13	0.346	-1.44	0.854	1.01	0.247
Glutamine	-1.40×10^{2}	0.521	-46.87	0.894	-32.58	0.273	-4.15×10^{-1}	0.901
Glutamine-to-glutamic acid ratio	-4.94×10^{-3}	0.901	-2.32×10^{-2}	0.718	-1.80×10^{-3}	0.741	-5.15×10^{-4}	0.394
Glycine	-49.86	0.057	-37.28	0.369	-3.10	0.374	-1.05×10^{-1}	0.789
Histidine	-39.88	0.489	6.29	0.946	-15.10	0.049[$]	-2.81×10^{-1}	0.749
Isoleucine+leucine	-1.53×10^{-1}	0.452	-2.21×10^{-1}	0.502	-1.74×10^{-2}	0.531	-6.62×10^{-4}	0.382
Lysine	-146×10^{2}	0.710	-65.12	0.919	-1.04×10^{2}	0.048[$]	3.64	0.544
Methionine	-9.48×10^{-1}	0.133	-3.50×10^{-1}	0.728	-2.68×10^{-2}	0.753	-2.36×10^{-3}	0.803
Phenylalanine	-6.08×10^{-1}	0.546	6.77×10^{-1}	0.678	-2.39×10^{-1}	0.077	7.01×10^{-3}	0.648
Proline	-16.10	0.436	-80.46	0.021[$]	-8.48	0.001[$]	-2.86×10^{-1}	0.401
Serine	-4.93	0.442	7.98	0.442	-6.56×10^{-1}	0.454	-9.40×10^{-2}	0.336
Threonine	-23.29	0.001[$]	-18.74	0.084	-2.04	0.018[$]	1.83×10^{-2}	0.861
Tryptophan	-4.37	0.282	-11.16	0.095	-2.61×10^{-1}	0.638	-1.64×10^{-2}	0.799
Tyrosine	-5.81×10^{-1}	0.651	2.90	0.168	-1.23×10^{-1}	0.485	2.51×10^{-2}	0.209
Valine	-1.80	0.597	1.48	0.788	-6.30×10^{-1}	0.173	-2.16×10^{-2}	0.678
Glycolysis metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Glucose	-1.33	0.983	-8.46	0.932	7.09	0.396	-3.78×10^{-2}	0.968
Glucose-6-phosphate+fructose-6-phosphate	21.93	0.951	6.94×10^{2}	0.232	12.90	0.792	-5.40×10^{-1}	0.922
Fructose 1,6-bisphosphate	13.07	0.882	1.43×10^{2}	0.320	11.07	0.357	9.96×10^{-2}	0.942
Glyceraldehyde-3-phosphate	60.91	0.908	1.45×10^{2}	0.864	91.54	0.199	1.69	0.833

Dihydroxyacetone phosphate	-26.78	0.645	1.13×10^{2}	0.236	8.59×10^{-1}	0.915	-1.53×10^{-1}	0.866
2-phosphoglyceric acid+ 3-phosphoglyceric acid	-13.44	0.600	45.67	0.286	1.45	0.681	-2.19×10^{-1}	0.594
Lactate	81.27	0.958	3.30×10^{3}	0.192	61.28	0.773	-14.34	0.552
Fructose 1,6-bisphosphate-to-glucose-6-phosphate+fructose -6-phosphate ratio	5.48×10^{-2}	0.489	3.31×10^{-2}	0.808	1.83×10^{-2}	0.084	3.27×10^{-3}	0.004[$]
FFA	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Oleic acid	4.24×10^{-1}	0.908	3.89	0.514	5.86×10^{-1}	0.242	3.97×10^{-2}	0.481
Acylcarnitines	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
L-carnitine	36.77	0.725	89.60	0.598	-23.56	0.095	7.23×10^{-1}	0.652
C2 carnitine	18.84	0.373	73.52	0.037[$]	-9.80×10^{-1}	0.735	-2.71×10^{-1}	0.429
C3 carnitine	2.21×10^{-1}	0.511	8.91×10^{-2}	0.870	-5.90×10^{-2}	0.196	1.81×10^{-3}	0.723
C4 carnitine	6.88×10^{-2}	0.933	5.25×10^{-1}	0.692	1.92×10^{-2}	0.864	-1.03×10^{-2}	0.407
C5 carnitine	-1.43×10^{-3}	0.993	-5.94×10^{-2}	0.822	-9.78×10^{-3}	0.662	-6.22×10^{-4}	0.803
C5-DC carnitine	4.30×10^{-2}	0.137	3.74×10^{-2}	0.418	-5.39×10^{-3}	0.161	-3.67×10^{-5}	0.933
C6 carnitine	-6.28×10^{-2}	0.825	9.74×10^{-4}	0.998	-2.37×10^{-2}	0.497	-2.67×10^{-3}	0.491
C8:0 carnitine	-7.58×10^{-3}	0.926	1.18×10^{-2}	0.928	-8.81×10^{-3}	0.429	-1.14×10^{-3}	0.356
C8:1 carnitine	5.35×10^{-2}	0.037[$]	5.62×10^{-2}	0.168	-6.82×10^{-4}	0.842	1.62×10^{-4}	0.678
C10:0 carnitine	1.79×10^{-2}	0.752	5.41×10^{-2}	0.557	-9.15×10^{-3}	0.237	-3.21×10^{-4}	0.713
C10:1 carnitine	-4.60×10^{-4}	0.984	1.93×10^{-2}	0.602	-2.13×10^{-3}	0.496	-2.19×10^{-4}	0.530
C12:0 carnitine	7.95×10^{-2}	0.297	2.36×10^{-1}	0.061	-1.15×10^{-2}	0.264	6.40×10^{-4}	0.599
C12:1 carnitine	-6.57×10^{-3}	0.694	4.60×10^{-2}	0.096	-1.37×10^{-3}	0.550	-8.41×10^{-5}	0.753
C12-OH carnitine	5.84×10^{-3}	0.730	3.33×10^{-2}	0.230	6.06×10^{-4}	0.795	-2.84×10^{-5}	0.914
C14:0 carnitine	4.36×10^{-2}	0.849	5.62×10^{-1}	0.137	2.22×10^{-3}	0.944	-8.76×10^{-4}	0.809
C14:1 carnitine	-4.59×10^{-3}	0.983	4.81×10^{-1}	0.175	4.01×10^{-3}	0.893	-3.93×10^{-4}	0.908
C14:2 carnitine	4.88×10^{-3}	0.946	1.61×10^{-1}	0.171	2.53×10^{-3}	0.798	-2.93×10^{-5}	0.979
C14-OH carnitine	3.29×10^{-3}	0.712	1.61×10^{-2}	0.268	5.31×10^{-4}	0.665	-3.77×10^{-5}	0.786

C16:0 carnitine	2.72×10^{-1}	0.743	2.01	0.142	3.75×10^{-2}	0.743	-7.93×10^{-3}	0.545
C16:1 carnitine	1.23×10^{-1}	0.869	1.77	0.149	2.55×10^{-2}	0.804	-2.01×10^{-3}	0.865
C16-OH carnitine	1.11×10^{-2}	0.786	7.52×10^{-2}	0.264	2.77×10^{-3}	0.625	-6.07×10^{-5}	0.925
C18:0 carnitine	2.79×10^{-1}	0.687	1.67	0.143	5.09×10^{-2}	0.593	-5.77×10^{-3}	0.597
C18:1 carnitine	7.87×10^{-1}	0.757	6.36	0.129	1.31×10^{-1}	0.707	-2.63×10^{-2}	0.513
C18:2 carnitine	2.27×10^{-1}	0.764	1.85	0.138	4.71×10^{-2}	0.650	-6.07×10^{-3}	0.610
C20:0 carnitine	2.21×10^{-3}	0.679	1.24×10^{-2}	0.158	4.39×10^{-4}	0.550	-2.68×10^{-5}	0.750
C20:1 carnitine	2.92×10^{-2}	0.695	1.74×10^{-1}	0.156	4.99×10^{-3}	0.625	-4.37×10^{-4}	0.709
C20:2 carnitine	6.64×10^{-3}	0.692	4.52×10^{-2}	0.103	9.11×10^{-4}	0.693	-1.62×10^{-4}	0.543
C20:3 carnitine	5.30×10^{-3}	0.746	4.04×10^{-2}	0.135	8.47×10^{-4}	0.707	-1.55×10^{-4}	0.549
C20:4 carnitine	1.06×10^{-2}	0.776	7.59×10^{-2}	0.213	2.74×10^{-3}	0.592	-2.78×10^{-4}	0.632
TCA cycle metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Citrate	4.05	0.058	3.44	0.310	-1.64×10^{-2}	0.954	1.73×10^{-2}	0.591
Succinate	3.28	0.455	9.27	0.197	1.08	0.066	1.33×10^{-1}	0.046[$]
Malate	10.86	0.692	56.36	0.209	2.15	0.569	8.81×10^{-3}	0.984
Nucleotides	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
AMP	3.58×10^{-1}	0.514	9.52×10^{-1}	0.287	4.11×10^{-2}	0.585	4.75×10^{-3}	0.576
ADP	29.41	0.641	-1.28×10^{2}	0.217	-4.02	0.643	-1.02	0.295
ATP	-92.23	0.269	-1.52×10^{2}	0.261	-7.82	0.491	-7.51×10^{-3}	0.995
NAD^+	-1.28×10^{2}	0.174	-28.77	0.849	-7.84	0.539	5.82×10^{-1}	0.682
NADH	9.03	0.660	-33.65	0.315	4.66	0.092	-2.60×10^{-2}	0.935
NAD^+-to-NADH ratio	-1.99	0.814	20.59	0.138	-2.54	0.023[$]	5.96×10^{-2}	0.654
NADP	-1.80	0.604	12.17	0.036[$]	-2.77×10^{-1}	0.561	9.82×10^{-2}	0.076
FAD	-1.09×10^{-1}	0.936	2.08	0.347	-8.31×10^{-3}	0.965	2.25×10^{-2}	0.282
Long-chain acylcarnitines-to-FFA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
C18:1 carnitine-to-oleic acid ratio	-1.52×10^{-3}	0.927	5.04×10^{-2}	0.069	-2.87×10^{-3}	0.207	-3.55×10^{-4}	0.180
C3 and C5 carnitine-to-BCAA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression	p-value	Regression	p-value	Regression	p-value	Regression	p-value

	coefficient		coefficient		coefficient		coefficient	
C3 carnitine-to-valine ratio	3.13×10^{-4}	0.377	6.65×10^{-5}	0.907	-3.89×10^{-5}	0.421	2.90×10^{-6}	0.590
C5 carnitine-to-isoleucine+leucine ratio	-4.84×10^{-5}	0.987	-6.25×10^{-4}	0.900	-1.25×10^{-4}	0.769	-1.22×10^{-5}	0.796
mtDNA profiles	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
mtDNA deletion ratio	-1.04×10^{-1}	0.722	1.82×10^{-2}	0.969	-2.46×10^{-2}	0.538	NA	NA
mtDNA count number	10.88	0.321	33.91	0.061	1.07	0.475	NA	NA

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and HOMA-IR.
[$] = p-value <0.050
NA = not applicable

Table A5.9 Correlations of targeted metabolites and mtDNA profiles of 41 obese subjects after CR vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites after CR vs. mtDNA count number after CR

AAs	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Alanine	57.50	0.705	3.82×10^{2}	0.132	24.93	0.209	-3.82	0.087
Arginine	-27.17	0.046[$]	-15.23	0.483	-3.77	0.025[$]	2.66×10^{-1}	0.163
Asparagine	-13.18	0.775	-28.30	0.708	-11.58	0.051	1.04×10^{-1}	0.877
Aspartic acid	32.44	0.024[$]	-16.80	0.462	-2.01×10^{-1}	0.913	-1.66×10^{-2}	0.935
Glutamic acid	-6.34	0.947	-2.39×10^{2}	0.135	-8.67	0.492	2.42	0.086
Glutamine	-1.97×10^{2}	0.423	-7.13×10^{2}	0.083	-28.87	0.370	1.00	0.788
Glutamine-to-glutamic acid ratio	-2.26×10^{-2}	0.759	1.22×10^{-1}	0.316	9.22×10^{-3}	0.341	-1.89×10^{-3}	0.076
Glycine	-28.35	0.362	-26.50	0.603	-2.44	0.550	-1.12×10^{-1}	0.805
Histidine	-87.62	0.296	-1.09×10^{2}	0.427	-14.87	0.171	1.04	0.391
Isoleucine+leucine	-1.88×10^{-1}	0.465	-2.76×10^{-1}	0.514	1.52×10^{-3}	0.964	-5.91×10^{-3}	0.109
Lysine	-6.53×10^{2}	0.056	-3.02×10^{2}	0.581	-62.48	0.148	-2.67	0.582
Methionine	-3.77×10^{-1}	0.776	2.12	0.334	2.47×10^{-2}	0.888	-3.07×10^{-2}	0.111
Phenylalanine	-1.63	0.399	1.49	0.637	-3.05×10^{-1}	0.226	-2.19×10^{-2}	0.433
Proline	-18.46	0.353	-32.71	0.317	-3.86	0.133	2.38×10^{-1}	0.414
Serine	-9.52	0.267	16.32	0.247	-3.12×10^{-1}	0.781	-1.85×10^{-1}	0.136
Threonine	-7.35	0.469	5.85	0.725	-7.37×10^{-1}	0.581	1.85×10^{-2}	0.900
Tryptophan	-1.15	0.796	-9.82	0.185	-1.06	0.065	9.52×10^{-2}	0.146
Tyrosine	-3.66×10^{-1}	0.844	1.89	0.539	7.73×10^{-2}	0.754	-4.10×10^{-2}	0.126
Valine	-2.03	0.614	-8.15	0.224	-3.54×10^{-1}	0.506	-8.36×10^{-2}	0.158
Glycolysis metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Glucose	-1.07×10^{2}	0.391	1.77×10^{2}	0.376	18.00	0.308	-3.27	0.093
Glucose-6-phosphate+fructose-6-phosphate	76.88	0.776	7.91×10^{2}	0.082	62.04	0.076	-8.96	0.024[$]
Fructose 1,6-bisphosphate	-2.99×10^{2}	0.210	2.56×10^{2}	0.511	18.04	0.563	-4.97	0.144
Glyceraldehyde-3-phosphate	-95.23	0.856	-3.45×10^{2}	0.688	59.76	0.386	1.83	0.810
Dihydroxyacetone	-1.53×10^{2}	0.375	91.40	0.746	18.43	0.415	-4.46	0.068

phosphate 2-phosphoglyceric acid+ 3-phosphoglyceric acid	8.71	0.824	72.11	0.266	7.74	0.128	-9.95×10^{-1}	0.080
Lactate	-1.07×10^{3}	0.731	6.40×10^{3}	0.216	6.14×10^{2}	0.129	-93.91	0.037[$]
Fructose 1,6-bisphosphate-to-glucose-6-phosphate+fructose-6-phosphate ratio	3.20×10^{-2}	0.356	8.60×10^{-2}	0.135	4.64×10^{-2}	0.306	7.90×10^{-4}	0.121
FFA	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Oleic acid	-23.54	0.342	20.91	0.606	1.88	0.564	-4.33×10^{-1}	0.224
Acylcarnitines	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
L-carnitine	35.27	0.786	-1.42×10^{2}	0.508	-32.23	0.054	1.49	0.431
C2 carnitine	-19.72	0.653	-13.82	0.848	2.45	0.673	-0.37×10^{-1}	0.564
C3 carnitine	-2.32×10^{-2}	0.953	5.29×10^{-3}	0.994	3.40×10^{-2}	0.516	-1.22×10^{-2}	0.029[$]
C4 carnitine	2.60×10^{-1}	0.209	4.62×10^{-1}	0.175	1.85×10^{-2}	0.494	-3.43×10^{-3}	0.259
C5 carnitine	-5.19×10^{-3}	0.930	-6.23×10^{-2}	0.521	2.52×10^{-3}	0.747	-8.89×10^{-4}	0.300
C5-DC carnitine	5.23×10^{-2}	0.193	2.68×10^{-2}	0.681	-3.48×10^{-3}	0.506	1.47×10^{-4}	0.800
C6 carnitine	5.54×10^{-1}	0.056	4.41×10^{-1}	0.344	-1.39×10^{-2}	0.710	-2.03×10^{-3}	0.625
C8:0 carnitine	2.11×10^{-1}	0.036[$]	1.54×10^{-1}	0.340	-8.28×10^{-4}	0.949	-7.03×10^{-4}	0.625
C8:1 carnitine	2.90×10^{-2}	0.321	3.30×10^{-2}	0.490	-7.77×10^{-3}	0.036[$]	5.07×10^{-4}	0.228
C10:0 carnitine	1.84×10^{-1}	0.062	1.37×10^{-1}	0.388	7.62×10^{-3}	0.549	-1.49×10^{-3}	0.290
C10:1 carnitine	7.29×10^{-2}	0.017[$]	9.50×10^{-2}	0.055	4.28×10^{-3}	0.266	-5.48×10^{-4}	0.220
C12:0 carnitine	1.88×10^{-1}	0.349	1.59×10^{-1}	0.629	3.53×10^{-2}	0.176	-4.73×10^{-3}	0.098
C12:1 carnitine	5.20×10^{-2}	0.042[$]	8.49×10^{-2}	0.044[$]	4.38×10^{-3}	0.176	-5.86×10^{-4}	0.121
C12-OH carnitine	3.06×10^{-2}	0.717	1.53×10^{-1}	0.273	1.65×10^{-2}	0.132	-2.05×10^{-3}	0.094
C14:0 carnitine	2.70×10^{-1}	0.457	1.13	0.063	7.90×10^{-2}	0.092	-1.10×10^{-2}	0.042[$]
C14:1 carnitine	2.90×10^{-1}	0.185	7.62×10^{-1}	0.037[$]	4.55×10^{-2}	0.103	-6.27×10^{-3}	0.055
C14:2 carnitine	1.08×10^{-1}	0.121	2.51×10^{-1}	0.032[$]	1.52×10^{-2}	0.088	-1.90×10^{-3}	0.069
C14-OH carnitine	9.69×10^{-3}	0.755	7.99×10^{-2}	0.124	6.98×10^{-3}	0.082	-8.17×10^{-4}	0.074
C16:0 carnitine	2.28×10^{-1}	0.893	4.97	0.081	3.22×10^{-1}	0.144	-4.43×10^{-2}	0.079

C16:1 carnitine	7.27×10^{-1}	0.497	3.40	0.059	2.32×10^{-1}	0.094	-2.68×10^{-2}	0.095
C16-OH carnitine	-3.44×10^{-3}	0.825	3.63×10^{-1}	0.163	2.83×10^{-2}	0.164	-3.68×10^{-3}	0.109
C18:0 carnitine	-4.03×10^{-3}	0.977	4.40	0.065	2.93×10^{-1}	0.111	-3.81×10^{-2}	0.072
C18:1 carnitine	1.29	0.788	13.09	0.103	9.34×10^{-1}	0.134	-1.18×10^{-1}	0.097
C18:2 carnitine	6.49×10^{-1}	0.691	4.54	0.098	3.26×10^{-1}	0.125	-3.70×10^{-2}	0.129
C20:0 carnitine	1.02×10^{-2}	0.602	6.09×10^{-2}	0.063	4.18×10^{-3}	0.097	-4.93×10^{-4}	0.092
C20:1 carnitine	1.59×10^{-2}	0.903	3.81×10^{-1}	0.083	2.54×10^{-2}	0.135	-3.42×10^{-3}	0.079
C20:2 carnitine	2.68×10^{-3}	0.961	1.52×10^{-1}	0.094	9.99×10^{-3}	0.138	-1.44×10^{-3}	0.073
C20:3 carnitine	1.42×10^{-2}	0.785	1.38×10^{-1}	0.115	9.65×10^{-3}	0.157	-1.29×10^{-3}	0.097
C20:4 carnitine	1.90×10^{-2}	0.889	3.59×10^{-1}	0.116	2.63×10^{-2}	0.138	-2.50×10^{-3}	0.220
TCA cycle metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Citrate	2.50	0.720	17.38	0.135	1.78	0.046[$]	-1.11×10^{-1}	0.269
Succinate	5.55	0.174	6.86	0.303	9.24×10^{-1}	0.076	-1.42×10^{-2}	0.811
Malate	1.47	0.969	79.16	0.208	8.03	0.101	-8.09×10^{-1}	0.145
Nucleotides	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
AMP	6.21×10^{-1}	0.404	-5.93×10^{-1}	0.626	8.72×10^{-2}	0.371	-1.90×10^{-2}	0.072
ADP	90.47	0.274	28.43	0.833	7.04	0.516	3.15×10^{-1}	0.792
ATP	17.82	0.888	-2.37×10^{2}	0.259	-11.80	0.480	4.30	0.017[$]
NAD^+	-54.36	0.714	-4.60×10^{2}	0.066	-21.34	0.273	3.86	0.083
NADH	-5.36	0.798	-12.43	0.720	3.15	0.258	-1.27×10^{-1}	0.683
NAD^+-to-NADH ratio	9.88×10^{-1}	0.466	-8.58×10^{-1}	0.702	-4.42×10^{-1}	0.010[$]	1.40×10^{-2}	0.486
NADP	-2.51	0.498	3.59	0.537	-1.80×10^{-1}	0.702	1.17×10^{-1}	0.018[$]
FAD	1.04	0.443	-5.96×10^{-1}	0.789	2.58×10^{-1}	0.144	3.06×10^{-2}	0.114
Long-chain acylcarnitines-to-FFA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
C18:1 carnitine-to-oleic acid ratio	3.40×10^{-2}	0.055	3.14×10^{-2}	0.272	-2.47×10^{-3}	0.275	1.62×10^{-4}	0.525
C3 and C5 carnitine-to-BCAA ratio	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value

C3 carnitine-to-valine ratio	1.98×10^{-4}	0.594	5.58×10^{-4}	0.363	5.77×10^{-5}	0.235	-9.77×10^{-6}	0.067
C5 carnitine-to-isoleucine+leucine ratio	1.32×10^{-4}	0.897	-7.55×10^{-4}	0.654	-2.61×10^{-5}	0.847	-7.16×10^{-6}	0.632
mtDNA profiles	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
mtDNA deletion ratio	-2.22×10^{-1}	0.373	-4.76×10^{-2}	0.907	3.62×10^{-2}	0.267	NA	NA
mtDNA count number	7.76	0.520	2.37×10^{-1}	0.990	-3.95	0.009[$]	NA	NA

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and HOMA-IR.
[$] = p-value <0.050
NA = not applicable

Table A5.10 Correlations of untargeted metabolites and mtDNA profiles at baseline (non-obese and all obese subjects) vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites at baseline vs. mtDNA count number at baseline

FA	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
3-hydroxypentadecanoic acid	6.45×10^3	0.847	-9.30×10^4	0.041$	3.24×10^2	0.913	-9.71×10^2	0.010$
Phospholipids	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
PA(O-42-6)	4.26×10^2	0.893	1.04×10^4	0.017$	-2.49×10^2	0.379	19.64	0.593
PC(28:0)	-8.09×10^2	0.954	-4.24×10^4	0.028$	-1.24×10^2	0.921	-5.93×10^2	<0.001$
LysoPE(22:4)	-5.53×10^4	0.049$	-4.29×10^4	0.257	4.04×10^3	0.103	25.67	0.935
PE(34:1)	-4.33×10^2	0.968	-3.29×10^4	0.028$	-1.07×10^3	0.272	-1.09×10^2	0.837
PE(36:1)	-2.14×10^4	0.408	-8.40×10^4	0.018$	-1.64×10^3	0.475	-5.29×10^2	0.074
PE(36:2)	-1.07×10^6	0.051	-2.01×10^6	0.007$	-9.70×10^3	0.841	-1.23×10^4	0.049$
PE(36:5)	-8.14×10^4	0.122	-4.72×10^4	0.505	9.74×10^3	0.035$	-2.68×10^2	0.650
PE(40:6)	-1.11×10^5	0.127	8.73×10^4	0.373	-1.42×10^4	0.026$	-3.47×10^2	0.671
PE(38:2)OH	-5.40×10^5	0.024$	-4.68×10^5	0.144	2.40×10^4	0.252	1.44×10^3	0.590
PE(P-36:2)	-3.71×10^5	0.006$	-4.76×10^5	0.009$	-4.09×10^3	0.728	-4.16×10^2	0.786
PE(P-36:3)	-2.46×10^5	0.065	-4.21×10^5	0.020$	4.05×10^3	0.731	-2.49×10^3	0.101
PE(P-40:6)	-3.64×10^4	0.443	-1.97×10^5	0.003$	-6.02×10^2	0.886	-1.75×10^3	0.001$
PG(34:1)	-9.19×10^4	0.405	4.16×10^5	0.006$	1.11×10^3	0.910	4.36×10^2	0.734
PG(36:1)	-8.04×10^4	0.042$	-2.97×10^4	0.574	2.15×10^3	0.537	-5.54×10^2	0.206
PG(P-36:5)	-4.77×10^3	0.031$	4.52×10^3	0.128	1.13	0.995	-43.26	0.080
PI(38:4)	1.16×10^6	0.419	4.57×10^6	0.020$	2.22×10^5	0.081	2.73×10^4	0.098
LysoPS(18:1)	-2.37×10^4	0.035$	-4.42×10^4	0.004$	3.02×10^2	0.761	1.11×10^2	0.394
LysoPS(20:4)	-8.01×10^4	0.012$	-5.61×10^4	0.189	1.51×10^3	0.590	9.01×10^2	0.010$
PS(38:3)	-2.60×10^5	0.003	-5.52×10^4	0.633	1.08×10^4	0.152	1.46×10^3	0.128
TCA cycle metabolite derivative	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Stearyl citrate	1.17×10^4	0.102	-1.23×10^3	0.898	1.41×10^3	0.024$	27.30	0.732
Other metabolites	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression	p-value	Regression	p-value	Regression	p-value	Regression	p-value

	coefficient		coefficient		coefficient		coefficient	
Dehydroxyepiandrosterone sulfate (DHEAS)	-1.29 x 10^4	0.036$	2.08 x 10^3	0.801	-1.89 x 10^2	0.728	1.96 x 10^2	0.004$
Reduced glutathione	2.38 x 10^4	0.114	4.93 x 10^4	0.016$	1.10 x 10^3	0.411	5.51 x 10^2	0.001$
Xanthine	-3.66 x 10^5	0.026$	-3.09 x 10^3	0.989	-1.17 x 10^4	0.417	-7.21 x 10^2	0.693
Unknown metabolites (neutral mass_ retention time)	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Unknown (287.9841_1.07)	4.98 x 10^4	0.039$	4.51 x 10^4	0.165	-8.50 x 10^2	0.690	8.76 x 10^2	<0.001$
Unknown (342.1288_6.30)	-1.93 x 10^3	0.426	-6.67 x 10^3	0.044$	-80.49	0.710	33.61	0.226
Unknown (664.0899_0.98)	-2.50 x 10^4	0.012$	-3.33 x 10^3	0.802	1.33 x 10^3	0.127	65.56	0.552
Unknown (672.0854_1.20)	-1.25 x 10^4	0.362	4.54 x 10^3	0.806	-2.49 x 10^3	0.038$	3.12 x 10^2	0.040$
Unknown (743.0656_1.51)	-1.78 x 10^4	0.212	4.06 x 10^4	0.036$	3.66 x 10^2	0.772	5.36 x 10^2	<0.001
Unknown (827.5675_9.76)	8.38 x 10^4	0.470	-7.29 x 10^4	0.641	2.13 x 10^4	0.037$	-1.10 x 10^3	0.399
Unknown (866.1155_10.43)	-2.10 x 10^4	0.160	-4.56 x 10^4	0.025$	4.01 x 10^2	0.762	1.57 x 10^2	0.360

[1]Data were adjusted for sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, group of subjects (non-obese or obese), BMI, diabetes status (yes or no) and HOMA-IR.
$ = p-value <0.050
NA = not applicable

Table A5.11 Correlations of untargeted metabolites and mtDNA profiles at baseline of 41 obese subjects who received muscle biopsy at both baseline and after CR vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites at baseline vs. mtDNA count number at baseline

AA derivative and dipeptide	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Beta-citryl-l-glutamic acid	1.08×10^6	0.537	-6.89×10^6	0.019$	-3.89×10^5	0.097	-8.20×10^4	0.002$
Tryptophyl-valine	-5.22×10^5	0.046$	-5.25×10^3	0.898	-4.86×10^3	0.158	-5.98×10^2	0.117
Phospholipids	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
LysoPA(16:0)	4.36×10^4	0.030$	1.84×10^4	0.559	2.26×10^3	0.395	-80.02	0.788
PA(36:3)	-2.25×10^4	0.047$	-1.12×10^4	0.531	1.52×10^3	0.312	2.99×10^2	0.069
PE(38:4)	1.39×10^5	0.026$	6.70×10^4	0.492	2.07×10^3	0.897	-8.26×10^2	0.368
PE(O-44:6)	4.27×10^5	0.026$	3.44×10^5	0.255	4.15×10^4	0.096	3.61×10^3	0.205
PS(83:3)	-4.95×10^5	<0.001$	8.52×10^4	0.690	95.17	0.996	1.80×10^3	0.370
Nucleotide	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
UDP-n-acetylglucosamine	-9.13×10^4	0.030$	1.63×10^5	0.017$	-4.64×10^3	0.403	1.18×10^2	0.861
Other metabolites	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
1,25-dihydroxy vitamin D3 3-glycoside	5.77×10^3	0.396	6.42×10^3	0.559	1.83×10^3	0.043$	5.40	0.959
Geranylgeranyl pyrophosphate	7.03×10^4	0.315	1.03×10^5	0.362	1.83×10^4	0.049$	4.43×10^2	0.679
Oxidized glutathione	-3.41×10^3	0.995	2.09×10^5	0.033$	-9.68×10^3	0.225	2.19×10^3	0.017$
Reduced glutathione	2.21×10^4	0.227	8.02×10^4	0.009$	-2.00×10^3	0.419	4.56×10^2	0.130
Unknown metabolites (neutral mass_ retention time)	**Correlations with age[1]**		**Correlations with VO_2max per FFM at baseline[2]**		**Correlations with [age x VO_2max per FFM at baseline][3]**		**Correlations with mtDNA count number[4]**	
	Regression	p-value	Regression	p-value	Regression	p-value	Regression	p-value

	coefficient		coefficient		coefficient		coefficient	
Unknown (411.8986_0.99)	-3.11×10^4	0.023$	-1.36×10^4	0.524	-2.24×10^3	0.209	-44.83	0.824
Unknown (606.4157_9.47)	4.09×10^4	0.379	-2.09×10^4	0.780	1.27×10^4	0.039$	1.47×10^3	0.031$
Unknown (664.0899_0.98)	-3.40×10^4	0.056	8.15×10^3	0.771	-4.76×10^3	0.039$	38.66	0.884
Unknown (769.0324_1.22)	-2.40×10^4	0.046$	-4.04×10^3	0.831	-2.64×10^3	0.094	1.10×10^2	0.535

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and HOMA-IR.
$ = p-value <0.050
NA = not applicable

Table A5.12 Correlations of untargeted metabolites and mtDNA profiles of 41 obese subjects after CR vs. age, VO_2max per FFM at baseline and [age x VO_2max per FFM at baseline], as well as correlations of their metabolites after CR vs. mtDNA count number after CR

AA derivative	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Beta-citryl-l-glutamic acid	4.44×10^5	0.916	-1.59×10^7	0.027$	-5.34×10^4	0.924	-9.18×10^4	0.160
FA and FA derivative	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
11(R)-hydroxyeicosatetraenoic acid	1.62×10^5	0.031$	1.22×10^5	0.311	7.22×10^3	0.452	-88.62	0.935
Eicosapentaenoic acid	6.67×10^5	0.009$	3.28×10^5	0.410	2.89×10^4	0.363	1.01×10^3	0.776
Phospholipids	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Cardiolipin(72:8)	-6.50×10^4	0.028$	-2.73×10^4	0.559	56.85	0.988	5.75×10^2	0.161
PA(O-42:6)	1.85×10^3	0.841	4.49×10^3	0.767	2.68×10^3	0.023$	-94.65	0.481
LysoPE(20:0)	-8.27×10^4	0.030$	-2.90×10^4	0.632	-2.15×10^3	0.658	8.06×10^2	0.127
LysoPE(20:2)	-1.90×10^6	0.034$	-6.52×10^5	0.648	-1.93×10^4	0.866	7.73×10^3	0.541
LysoPE(P-16:0)	-6.67×10^4	0.026$	-3.26×10^4	0.494	-1.70×10^3	0.657	4.58×10^2	0.276
PE(38:4)	1.94×10^5	0.007$	3.49×10^3	0.975	-5.43×10^3	0.547	-2.01×10^2	0.840
PE(38:4)OH	-1.91×10^5	0.020$	-4.24×10^4	0.743	-1.02×10^3	0.922	1.51×10^3	0.183
PE(P-36:2)	-1.52×10^5	0.443	-6.57×10^5	0.049$	6.86×10^3	0.793	-4.66×10^2	0.879
PE(P-38:5)	4.60×10^5	0.044$	2.72×10^5	0.456	-1.03×10^4	0.726	4.01×10^2	0.902
PE(O-34:3)	-3.15×10^5	0.017$	-9.17×10^4	0.658	-8.23×10^3	0.621	1.90×10^3	0.297
PE(O-44:6)	4.73×10^5	0.057	8.30×10^3	0.983	6.82×10^4	0.027$	1.21×10^3	0.731
PG(36:1)	-1.02×10^5	0.182	-1.14×10^5	0.361	2.27×10^4	0.017$	-1.33×10^3	0.225
PI(38:5)	1.34×10^5	0.011$	1.15×10^5	0.172	9.51×10^3	0.148	-4.35×10^2	0.564
PI-ceramide(d28:2)	-8.61×10^4	0.031$	-5.38×10^4	0.397	-2.68×10^3	0.599	7.67×10^2	0.170
LysoPS(20:4)	-1.15×10^5	0.037$	-8.09×10^4	0.359	-4.08×10^3	0.563	1.09×10^3	0.161
PS(40:6)	-1.03×10^6	0.025$	3.00×10^4	0.967	-8.96×10^4	0.121	8.68×10^3	0.174
Nucleotides	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	

	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Biotinyl-5'-AMP	3.68×10^4	0.201	7.65×10^4	0.109	-7.28×10^3	0.046$	8.08×10^2	0.054
UDP-n-acetylglucosamine	-6.43×10^4	0.172	-6.28×10^4	0.412	-1.42×10^4	0.016$	1.16×10^2	0.865
Other metabolites	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Allopregnanolone	6.87×10^5	0.023$	2.85×10^5	0.552	1.72×10^4	0.656	1.29×10^2	0.976
Calcitroic acid	1.76×10^4	0.048$	3.54×10^3	0.803	1.70×10^2	0.882	76.90	0.540
Dehydroxyepiandrosterone sulfate (DHEAS)	-9.13×10^3	0.041$	1.49×10^4	0.042$	-1.01×10^2	0.860	1.16×10^2	0.077
Lactose-6-phosphate	-7.84×10^4	0.132	-9.84×10^4	0.246	-1.42×10^4	0.029$	1.77×10^3	0.015$
Lactosyl sphingosine(d16:1)	-1.10×10^5	0.013$	-7.31×10^4	0.299	-1.16×10^3	0.837	1.03×10^3	0.096
Phosphocreatine	-8.54×10^4	0.444	-6.37×10^4	0.727	-3.24×10^4	0.022$	4.77×10^3	0.002$
Pregnenolone	-8.03×10^4	0.035$	-1.11×10^5	0.073	1.28×10^3	0.794	-3.02×10^2	0.593
Urocanic acid	2.08×10^4	<0.001$	1.80×10^4	0.026$	-8.32×10^2	0.179	15.02	0.840
Unknown metabolites (neutral mass_ retention time)	Correlations with age[1]		Correlations with VO_2max per FFM at baseline[2]		Correlations with [age x VO_2max per FFM at baseline][3]		Correlations with mtDNA count number[4]	
	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value	Regression coefficient	p-value
Unknown (187.1197_4.91)	-4.77×10^3	0.353	1.83×10^4	0.035$	7.51×10^2	0.262	84.07	0.287
Unknown (400.2355_9.29)	7.44×10^4	0.046$	6.00×10^4	0.315	-1.18×10^4	0.009$	6.03×10^2	0.255
Unknown (547.0832_1.19)	-9.24×10^4	0.466	-2.26×10^5	0.280	-3.88×10^4	0.015$	5.04×10^3	0.004$
Unknown (609.3357_8.43)	-2.83×10^5	0.021$	-8.23×10^4	0.671	-6.71×10^2	0.966	1.79×10^3	0.295
Unknown (619.3638_8.70)	-1.02×10^5	0.019$	-3.61×10^4	0.599	-1.87×10^3	0.735	7.34×10^2	0.224
Unknown (621.3616_8.78)	-2.55×10^5	0.022$	-1.27×10^5	0.472	-1.13×10^3	0.936	2.59×10^3	0.092
Unknown (633.0985_1.21)	-2.75×10^5	0.238	-4.11×10^5	0.283	-7.04×10^4	0.016$	8.74×10^3	0.007$

Unknown (635.3788_8.70)	-1.46×10^5	0.025$	-6.90×10^4	0.505	-4.90×10^3	0.555	1.14×10^3	0.212
Unknown (664.0899_0.98)	1.01×10^4	0.566	-1.64×10^3	0.955	-5.43×10^3	0.015$	5.05×10^2	0.042$
Unknown (689.3951_8.78)	-5.51×10^4	0.033$	-2.28×10^4	0.579	-1.70×10^3	0.607	5.29×10^2	0.141
Unknown (691.6111_10.39)	7.15×10^3	0.535	4.38×10^4	0.026$	-29.86	0.984	24.00	0.894
Unknown (698.3700_8.83)	-2.62×10^5	0.019$	-1.23×10^5	0.484	-1.08×10^4	0.444	1.55×10^3	0.321
Unknown (714.3681_8.74)	-1.62×10^5	0.045$	-6.52×10^4	0.612	-7.42×10^3	0.472	-2.07×10^2	0.857
Unknown (754.4030_8.70)	-1.66×10^5	0.035$	-3.57×10^4	0.776	-8.78×10^3	0.383	9.36×10^2	0.399
Unknown (769.0324_1.22)	-2.14×10^4	0.229	-2.21×10^4	0.446	-5.29×10^3	0.018$	4.61×10^2	0.068
Unknown (814.4511_8.77)	-7.82×10^4	0.018$	-2.12×10^4	0.685	-3.30×10^3	0.430	4.77×10^2	0.299
Unknown (853.5664_8.67)	-3.40×10^5	0.035$	-2.56×10^5	0.320	-2.78×10^3	0.893	-3.61×10^2	0.876
Unknown (866.1155_1.21)	-2.57×10^4	0.216	-1.40×10^3	0.967	-6.59×10^3	0.011$	7.57×10^2	0.008$
Unknown (936.5089_8.76)	2.19×10^5	0.038$	-2.75×10^5	0.109	6.10×10^3	0.652	-3.50×10^3	0.018$

[1]Data were adjusted for sex, race, BMI, diabetes status (yes or no), HOMA-IR, and VO_2max per FFM at baseline.
[2]Data were adjusted for age, sex, race, BMI and diabetes status (yes or no) and HOMA IR.
[3]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no), HOMA-IR and VO_2max per FFM at baseline.
[4]Data were adjusted for age, sex, race, BMI, diabetes status (yes or no) and HOMA-IR.
$ = p-value <0.050
NA = not applicable

Appendices of chapter 6

Table A6.1 All targeted metabolites identified in this study

Identified targeted metabolites	
FFAs	AAs and BCKAs
Arachidic acid	Alanine
Docosanoic acid	Arginine
Linoleic acid	Asparagine
Myristic acid	Aspartic acid
Oleic acid	Glutamic acid
Palmitic acid	Glutamine
Palmitoleic acid	Glycine
Stearic acid	Histidine
Phospholipids	Isoleucine+leucine
LysoPC(16:0)	Ketoisoleucine+ketoleucine
LysoPC(18:0)	Lysine
LysoPC(18:1)	Methionine
LysoPC(18:2)	Ornithine
LysoPE(16:0)	Phenylalanine
LysoPE(18:0)	Proline
LysoPE(18:1)	Serine
LysoPE(20:0)	Threonine
LysoPE(20:2)	Tryptophan
LysoPG(18:1)	Tyrosine
LysoPI(18:0)	Valine
LysoPS(18:0)	Acylcarnitines
PA(34:1)	L-carnitine
PA(36:1)	C2 carnitine
PA(36:2)	C3 carnitine
PC(34:1)	C4 carnitine
PC(34:2)	C5 carnitine
PC(36:1)	C6 carnitine
PC(36:2)	C8:0 carnitine
PC(36:4)	C8:1 carnitine
PC(38:6)	C10:0 carnitine
PE(34:1)	C10:1 carnitine
PE(36:1)	C12:0 carnitine
PE(36:2)	C12:1 carnitine
PE(36:3)	C14:0 carnitine
PE(38:2)	C14:1 carnitine
PE(38:4)	C14:2 carnitine
PE(38:6)	C16:0 carnitine
PG(34:1)	C16:1 carnitine
PG(36:1)	C18:0 carnitine
PG(36:2)	C18:1 carnitine
PG(38:1)	C18:2 carnitine

PI(34:1) PI(36:1) PI(36:2) PI(38:4) PS(36:2) PS(38:4) PS(40:6)	
<u>Glycerides</u> MG(18:0) DG(34:1) DG(36:1) DG(36:2) TG(48:0) TG(50:1) TG(50:2) TG(52:1) TG(52:2) TG(52:3) TG(52:4) TG(54:1) TG(54:2) TG(54:8)	<u>TCA cycle metabolites</u> Acetyl-CoA Citrate Alpha-ketoglutarate Succinate Malate <u>Other metabolites</u> 2,3-Dihydroxybenzoate Acetoacetate Citrulline Creatinine Coenzyme A Cytosine FAD Geranyl pyrophosphate Glucose Homocysteic acid Hypoxanthine Lactate N-Acetylornithine NAD^+ Para-Aminobenzoate/anthranilate Taurine Uridine

Table A6.2 Internal standards used for quantitation of targeted metabolites**.** NSK-B internal standard mix was purchased from Cambridge Isotope (Andover, MA). All other carbon-13 stable isotope internal standards were purchased from Sigma-Aldrich (St. Louis, MO).

Internal Standards	**Internal standard concentration in extraction solvent**
Algal amino acid mixture_^{13}C (Sigma 426199)	20 µg/ml
Alpha-ketoglutarate_$^{13}C_4$	4 µM
Citrate_$^{13}C_6$	20 µM
Lactate_$^{13}C_3$	400 µM
Malate_$^{13}C_4$	8 µM
NSK-B acylcarnitine internal standard mix	
$^{2}H_9$-Carnitine (L-carnitine)	510.33 nM
$^{2}H_3$-Acetylcarnitine (C2)	128.33 nM
$^{2}H_3$-Propionylcarnitine (C3)	25.33 nM
$^{2}H_3$-Butyrylcarnitine (C4)	25.33 nM
$^{2}H_9$-Isovalerylcarnitine (C5)	25.33 nM
$^{2}H_3$-Octanoylcarnitine (C8)	25.33 nM
$^{2}H_9$-Myristoylcarnitine (C14)	25.33 nM
$^{2}H_3$-Palmitoylcarnitine (C16)	50.67 nM
Oleic acid_$^{13}C_{18}$	5 µM
Palmitic acid_$^{13}C_{16}$	5 µM
Succinate_$^{13}C_4$	40 µM