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THE EFFECT OF WEIGHT GAIN IN METABOLICALLY NORMAL OR ABNORMAL OBESE SUBJECTS

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13 April 2016

To all those that I truly love

Statement of Originality

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Abstract

Obesity is associated with insulin resistance and increased intrahepatic triglyceride (IHTG) content, both of which are risk factors for diabetes and cardiovascular disease. However, a subset of obese people does not develop these metabolic complications. Weight gain is associated with the development and worsening of nonalcoholic fatty liver disease (NAFLD), but the mechanisms responsible for this association are not know. Here, we tested the hypothesis that people defined by IHTG content and insulin sensitivity as "metabolically normal obese" (MNO), but not those defined as "metabolically abnormal obese" (MAO), are protected from the adverse metabolic effects of weight gain. An increased number of macrophages in adipose tissue is associated with insulin resistance and metabolic dysfunction in obese people. However, little is known about other immune cells in adipose tissue from obese people, and whether they contribute to insulin resistance. In an effort to investigate the underlying differences between MNO and MAO, we investigated the characteristics of T cells in adipose tissue from MAO subjects, MNO subjects, and lean subjects.

Body composition, multiorgan insulin sensitivity, VLDL apolipoprotein B100 (apoB100) kinetics, and global transcriptional profile in adipose tissue were evaluated before and after moderate (~6%) weight gain in MNO (n=12) and MAO (n=8) subjects with a mean BMI of 36 ± 4 kg/m^2 who were matched for BMI and fat mass. Imaging and stable isotope tracers techniques were combined to evaluate the effect of moderate weight gain in 27 obese people on intrahepatic triglyceride (IHTG) content and hepatic lipid metabolism in order to elucidate the mechanisms responsible for weight-gain induced IHTG accumulation. Plasma cytokine concentrations and subcutaneous adipose tissue CD4+ T-cell populations were assessed in 9 lean, 12 MNO, and 13 MAO subjects. Skeletal muscle and liver samples were collected from 19 additional obese patients undergoing bariatric surgery to determine the presence of cytokine receptors

Although the increase in body weight and fat mass was the same in both groups, hepatic, skeletal muscle, and adipose tissue insulin sensitivity deteriorate, and VLDL apoB100 concentrations and secretion rates increased in MAO, but not MNO, subjects. Moreover, biological pathways and genes associated with adipose tissue lipogenesis increased in MNO, but not MAO, subjects. Also, our results demonstrate that weight gain causes an imbalance between hepatic availability and

disposal of fatty acid by increasing *de novo* lipogenesis, reducing fatty acid oxidation, and by inadequately increasing VLDL secretion. Adipose tissue from MAO subjects had 3- to 10-fold increasese in numbers of CD4+ T cells that produce interleukin (IL)-22 and IL-17 (a T-helper [Th] 17 and Th22 phyenotype) compared with MNO and lean subjects. MAO subjects also had increased plasma concentrations of IL-22 and IL-6. Receptors for IL-17 and IL-22 were expressed in human liver and skeletal muscle samples. Il-17 and Il-22 inhibited uptake of glucose in skeletal muscle isolate from rats and reduced insulin sensitivity in cultured human hepatocytes.

These data demonstrate that MNO people are resistant, whereas MAO people are predisposed, to the adverse metabolic effects of moderate weight gain and that increased adipose tissue capacity for lipogenesis might help protect MNO people from weight gain—induced metabolic dysfunction. Weight gain causes and imbalance between hepatic availability and disposal of fatty acid which are likely responsible for increased IHTG accumulation. Adipose tissue from MAO people contains increased numbers of Th17 and Th22 cells, which produce cytokines that cause metabolic dysfunction in liver and muscle in vitro. Additional studies are needed to determine whether such alterations in adipose tissue T cells contribute to the pathogenesis of insulin resistance in obese people.

Gemma Fraterrigo

Table of Contents

Gemma Fraterrigo

Gemma Fraterrigo

Gemma Fraterrigo

Gemma Fraterrigo

Gemma Fraterrigo

Gemma Fraterrigo

List of Figures

Chapter 1

Chapter 2

Gemma Fraterrigo

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreviations and Units

Abbreviations	
IHTG	intrahepatic triglyceride
BMI	body mass index
NAFLD	nonalcoholic fatty liver disease
VLDL	very low-density lipoprotein
TG	triglyceride
NIH	National Institute of Health
WHO	World Health Organization
CHD	coronary heart disease
HDL-C	high-density lipoprotein cholesterol
NHANES	National Health and Nutrition Examination Survey
HOMA-IR	homeostatic model assessment of insulin resistance
CRP	C-reactive protein
T2DM	Type 2 Diabetes Mellitus
Kcal	kilocalorie
Trp	tryptophan
Arg	arginine
PC1/PCSK1	prohormone convertase 1
POMC	pro-opiomelanocortin

MSH	melanocortin-stimulating hormone
ACTH	adenocorticotropic hormone
MC4R	melanocortin 4 receptor
TrkB	tyrosine receptor kinase B
NTRK2	neurotrophic tyrosine kinase receptor, type 2
SIM1	single-minded homolog 1
DNA	deoxyribonucleic acid
FTO	fat mass and obesity-associated protein
SNP	single nucleotide polymorphism
mRNA	messenger ribonucleic acid
TEE	total daily energy expenditure
REE	resting energy expenditure
TEF	thermic effect of food
LPL	lipoprotein lipase
TNF	tumor necrosis factor
HSL	hormone-sensitive lipase
IL	interleukin
MCP	monocyte chemotactic protein
P450	cytochrome P450
FFM	fat-free mass
BAT	brown adipose tissue

UCP1	uncoupling protein 1
LDL	low-density lipoprotein
PAI1	plasminogen activator inhibitor 1
PCO_2	partial pressure of carbon dioxide
IIH	idiopathic intracranial hypertension
VLCD	very-low-calorie-diet
DGAT	diacylglycerol acyltransferase
CVD	cardiovascular disease
MAO	metabolically abnormal obesity
MNO	metabolically normal obesity
FFA	free fatty acid
DAG	diacylglycerol
mTOR	mammalian target of rapamycin
Akt	protein kinase B
GLUT-4	glucose transporter type 4
IκB	nuclear factor kappa B
PKC	protein kinase C
IRS1	insulin receptor substrate 1
IKK-β	inhibitor of nuclear factor kappa-B kinase subunit
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
CD36	cluster of differentiation 36 or fatty acid translocase

Gemma Fraterrigo

PPARγ	peroxisome proliferator-activated receptor gamma
RXR	retinoid x receptor
LXR	liver x receptor
JNK	c-Jun N-terminal kinase
p-38	p38 mitogen-activated protein kinase
CD4	cluster of differentiation 4
T_H1	T helper 1 cell
T_H2	T helper 2 cell
Treg	regulatory T cells
CD39	cluster of differentiation 39
ATP	adenosine triphosphate
AMP	adenosine monophosphate
P1	purinoreceptor 1
P2	purinoreceptor 2
STAT3	signal transducer and activator of transcription 3
CCR2	C-C chemokine receptor type 2
EMR1	EGF-like module-containing mucin-like hormone receptor-like 1
ITGAM	integrin alpha M
ITGAX	integrin, alpha X (complement component 3 receptor 4 subunit)
CD68	cluster of differentiation 68
MRS	magnetic resonance spectroscopy

CRU	Clinical Research Unit
DNL	*de novo* lipogenesis
FM	fat mass
FFM	fat-free mass
DXA	dual energy x-ray absorptiometry
VAT	visceral adipose tissue
SAT	subcutaneous abdominal adipose tissue
L4-L5	lumbar vertebra 4 – lumbar vertebra 5
MRI	magnetic resonance imaging
3T	3 Tesla
MRE	magnetic resonance elastography
^{1}H	hydrogen atom
T_1	first order rate constant
T_2	second order rate constant
1.5T	1.5 Tesla
NMR	nuclear magnetic resonance
H_2O	dihydrogen monoxide (water)
CH_{2n}	methylene groups
[6,6-$^{2}H_2$]glucose	$^{2}H_2$ stable isotope labeled *D* glucose
[2,4-$^{13}C_2$] β-hydroxybutyrate	$^{13}C_2$ stable isotope labeled sodium *D-3* hydroxybutyrate
[U-^{13}C]palmitate	U-^{13}C stable isotope labeled palmitate

Gemma Fraterrigo

[$^{2}H_5$]glycerol	$^{2}H_5$ stable isotope labeled glycerol
[2-^{3}H]glycerol	^{3}H stable isotope labeled glycerol
[$^{2}H_3$]leucine	$^{2}H_3$ stable isotope labeled leucine
[^{13}C]leucine	^{3}C stable isotope labeled leucine
[1,2-$^{13}C_2$]acetate	$^{13}C_2$ stable isotope labeled acetate
Ra	rate of appearance
Rd	rate of disappearance
TTR	tracer-to-tracee ratio
apoB	apolipoprotein B
PCR	polymerase chain reaction
CD11c	integrin alpha X chain protein
CD11b	cluster of differentiation molecule 11B
CD14	cluster of differentiation 14
CD16	cluster of differentiation 16
CD1a	cluster of differentiation 1a
CD123	interleukin-3 receptor
BDCA-2	plasmacytoid dendritic cell-specific antigen
MHC	major histocompatibility complex
CD163	cluster of differentiation 163
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (CD209)

CD-Lamp	cluster of differentiation lysosome-associated membrane glycoprotein
ER	endoplasmic reticulum
Grp78	78 kDa glucose-regulated protein
XBP-1	X-box binding protein 1
eIF2α	eukaryotic initiation factor 2 alpha
FAS	apoptosis antigen part of the tumor necrosis factor and nerve growth factor family (Apo-1 or CD95)
LPL	lipoprotein lipase
RNA	ribonucleic acid
PBS	phosphate buffered saline
ALT	alanine aminotransferase
AST	aspartate aminotransferase
DSMB	data and safety monitoring board
CMP	comprehensive metabolic panel
IDL	intermediate-density lipoprotein
NaCl	sodium chloride
KBr	potassium bromide
GC/MS	gas chromatography-mass spectrometry
FTR	fractional turnover rate
FCR	fractional catabolic rate
FSR	fractional secretion rate

i.e./e.g.	for example
CoA	coenzyme A
MPE	molar percent excess
EF	enrichment factor
SMX	sulfamethoxazole
TLC	thin-layer chromatography
PTFE	politetrafluoroetilene
ESI/MS	electrospray ionization-mass spectrometry
LCQ	licence controller qual
LiOH	lithium oxide
N	nitrogen
Cer	ceramide
HCl	hydrogen chloride
DC	dendritic cell
CD83	cluster of differentiation 83
RPMI	Roswell Park Memorial Institute
Triton-X-100	$C_{14}H_{22}O(C_2H_4O)_n$
EDT	ethylenediaminetetraacetic acid
NaF	Sodium fluoride
SDS	sodium dodecyl sulphate
TBST	Tris (hydroxymethyl) aminomethane buffered saline and polysorbate 20

Tween 20	polysorbate 20
BSA	bovine serum albumin
IgG	immunoglobulin G
H_2SO_4	sulfuric acid
cDNA	complementary deoxyribonucleic acid
IL-17RA	interleukin 17 receptor A
IL-17RC	interleukin 17 receptor C
IL-22RA	interleukin 22 receptor A
CCL5	chemokine (C-C motif) ligand 5
SYBR	asymmetrical cyanine dye used as nucleic acid stain
ELISA	enzyme-linked immunosorbent assay
HIRI	hepatic insulin resistance index
HISI	hepatic insulin sensitivity index
Na_3VO_4	sodium orthovanadate
$Na_4P_2O_7$	sodium pyrophosphate tetrabasic
^3H_2O	tritium radioisotope labeled water
[5-^{3}H]glucose	tritium radioisotope labeled *D* glucose
DG	deoxyglucose
3-MG	3-O-methyl-*D*-glucose
2-DG-6-P	2-deoxyglucose-6-phosphate
^3H	tritium, radioactive isotope of hydrogen

^{14}C	radiocarbon, radioactive isotope of carbon
BCAA	branched-chain amino acid
PAGE	parametric analysis of gene set enrichment
FADS	fatty acid desaturase
ELOVL6	fatty acid elongase 6
ANOVA	analysis of variance
IAAT	intra-abdominal adipose tissue
Ser473	second phosphorylation site of protein kinase B
Ser307	phosphorylation site in insulin receptor substrate-1

Units	
kg	kilogram
kgFFM	kilogram of fat-free-mass
kgFM	kilogram of fat-mass
g	gram
mg	milligram
μg	microgram
pg	picogram
m	meter
cm	centimeter
mm	millimeter
μm	micrometer
%	percentage
lb	pound
mmHg	millimeter of mercury
pH	log [H+]
L	liter
dl	deciliter
ml	milliliter
μl	microliter
ms	milliseconds

min	minute
t	time
h/hr	hour
d	day
wk	week
yr	year
n	number
ppm	parts per million
Hz	Hertz
mmol	millimole
μmol	micromole
nmol	nanomole
M	molar
mM	millimolar
nM	nanomolar
μM	micromolar
mU	milliunit
°C	degrees centigrade
g	gravitational acceleration
m/z	mass-to-charge ratio
kV	kilovolt

eV	electronvolt
mTorr	millitorr
W	watt
µCi	microcurie
p	statistical significance
z score	standard score
±	standard deviation
SD	standard deviation
SEM	standard error of the mean

Published Papers

The following papers have been published or in preparation in support of this thesis.

1. Fabbrini E, Yoshino J, Yoshino M, Magkos F, Tiemann Luecking C, Samovski D, **Fraterrigo G**, Okunade AL, Patterson BW, Klein S. (2015) Metabolically normal obese people are protected from adverse effects following weight gain. *J Clin Invest.* 2015 Feb;125(2):787-95.

2. Fabbrini E, Cella M, McCartney SA, Fuchs A, Abumrad NA, Pietka TA, Chen Z, Finck BN, Han DH, Magkos F, Conte C, Bradley D, **Fraterrigo G**, Eagon JC, Patterson BW, Colonna M, Klein S. (2013) Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. *Gastroenterology.* 2013 Aug;145(2):366-74.e1-3.

3. Fabbrini E, Tiemann Luecking C, Love-Gregory L, Okunade AL, Yoshino M, **Fraterrigo G,** Patterson BW, Klein S. (2015) Physiological Mechanisms of Weight-gain Induced Steatosis in People with Obesity. *Gastroenterology.* 2013 Sep;S0016-5085(15)01309-8.

4. Magkos F, **Fraterrigo G**, Yoshino J, Luecking C, Kirbach K, Kelly SC, De Las Fuentes L, He S, Okunade AL, Patterson BW, Klein S. (2016) Effects of Moderate and Subsequent Progressive Weight Loss on Metabolic Function and Adipose Tissue Biology in Humans with Obesity. *Cell Metabolism*. 2016 Feb 22.pii:S1550-4131(16)30053-5.

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CHAPTER 1 General Introduction

It is well known that obesity is associated with multiple metabolic risk factors for cardiovascular disease, including insulin resistance, diabetes, and dyslipidemia[1,2]. However, about one-third of obese adults do not have obvious metabolic abnormalities [3]. It is not known why weight gain and body fat accumulation causes metabolic abnormalities in some persons but not in others. We have found that excessive intrahepatic triglyceride (IHTG) content is a robust marker of obese persons who have metabolic dysfunction (insulin resistance in liver, muscle and adipose tissue and increased VLDL-triglyceride [VLDL-TG] secretion rate), independent of body mass index (BMI), percent body fat, and visceral fat mass [4]. Conversely, obese persons who have normal IHTG content appear to be resistant to developing obesity-related metabolic complications[5]. The mechanisms responsible for the accumulation of ectopic fat and the development of metabolic disease in some, but not all, obese persons are not known. This thesis will centre on the difference between these two groups, on the difference in susceptibility to metabolic abnormalities between these two groups, which might be due to the differences in the response to overfeeding. Therefore, this thesis will try to the overall goals of provide a better understanding of the metabolic mechanisms responsible for the accumulation of excessive ectopic fat (intrahepatic triglyceride [IHTG]) and metabolic dysfunction in obese persons, and try to determine the specific metabolic adaptations that prevent the adverse metabolic effects of weight gain in obese persons who have normal IHTG content compared with those who have nonalcoholic fatty liver disease (NAFLD).

1.1 OBESITY

Obesity is a chronic disease and a worldwide epidemic with significant repercussions on not only health but also the economy. In the United States alone, obesity related diseases lead to an estimated 300,000 deaths per year [6]. Also, medical expenses and cost of lost productivity due to obesity are more than $100 billion per year [7].

1.1.1 Definition and Classification

Body Mass Index (BMI) is calculated by dividing a person's weight in kilograms by height measured in meters squared. It has been shown that there is a curvilinear relation between BMI and percent body fat mass [8] but for practical purposes, the current definition of obesity is

Gemma Fraterrigo

based on the relationship between BMI and health outcome rather than BMI and body composition [9].

The way to classify obesity based on BMI, proposed by the major national and international health organizations is shown in the Table 1.1 [10-12]. Many studies have shown that there is an inverse relationship between BMI and mortality for BMI values of ≥25 kg/m^2 [12,13]. It is a known fact that obese people have a greater risk for adverse health events (Figures 1.1, 1.2) and the cutoff values to determine overweight and obese have been selected based on the information about BMI and mortality.

TABLE 1.1. Weight Classification by Body Mass Index

Weight Classification	Obesity Class	BMI (kg/m^2)	Risk of Disease
Underweight		<18.5	Increased
Normal		18.5-24.9	Normal
Overweight		25.0-29.9	Increased
Obesity	I	30.0-34.9	High
	II	35.0-39.9	Very High
Extreme Obesity	III	≥40.0	Extremely High

BMI, body mass index.
Adapted from the National Institutes of Health, National Heart, Lung, and Blood Institute. Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults—The Evidence Report. Obes Res 1998;6(suppl 2):51S-209S.

Gemma Fraterrigo

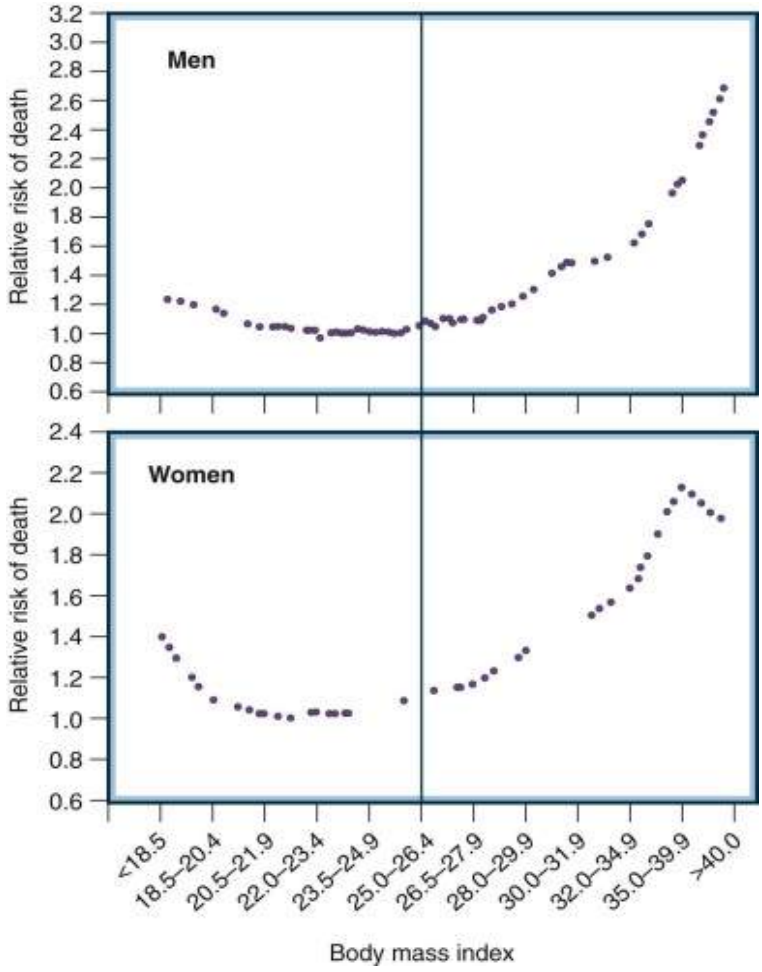

FIGURE 1.1. Relationship Between Body Mass Index and Cardiovascular Mortality in Men and Women in the United States Who Never Smoked and Had No Preexisting Illness. The vertical line separates underweight and lean subjects *(left side)* from overweight and obese subjects *(right side)*.

(Adapted from Calle EE, Thun MJ, Petrelli JM, et al. Body-mass index and mortality in a prospective cohort of U.S. adults. N Engl J Med. 1999;341:1097-1105.)

Gemma Fraterrigo

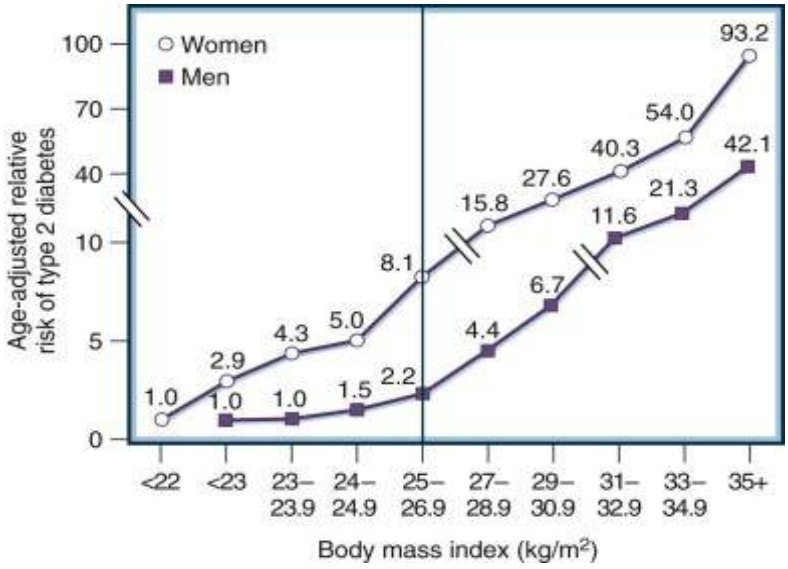

FIGURE 1.2. Relationship Between Body Mass Index and Type 2 Diabetes in Men and Women in the United States. The vertical line separates underweight and lean subjects *(left side)* from overweight and obese subjects *(right side)*. The data demonstrate that the risk of diabetes begins to increase at the upper end of the lean body mass index category.
(Adapted from Colditz GA, Willett WC, Rotnitzky A, et al. Weight gain as a risk factor for clinical diabetes mellitus in women. Ann Intern Med. 1995;122:481-486; and Chan JM, Rimm EB, Colditz GA, et al. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care. 1994;17:961-969.)

Excess abdominal fat has been associated with a higher risk for diabetes, hypertension, dyslipidemia, and ischemic heart disease [14] and those that have a greater amount of lower body fat mass seem to be protected from metabolic complications [15-17]. Waist circumference is highly correlated with abdominal fat mass and therefore is used as a practical but important marker for abdominal obesity. It is an important predictor of health outcomes in adult men and woman of all age groups and ethnicities [9]. The relationship between waist circumference and risk for diabetes is the strongest, being a stronger predictor for diabetes than BMI [10,18]. The NIH's recommended waist circumference cutoff values are 102 cm (40 inches) in men and 88 cm (35 inches) for women [10] and such cutoff values have been derived from wait circumference values that correlated with a BMI ≥30 kg/m^2 [19]. However we need to keep in mind that these are based on populations of European origin and therefore cannot be generalized to non-Europeans. Asian populations have a different classification system based on BMI because they tend to have a higher percentage of body fat for the same BMI value, along with an increased prevalence for cardiovascular risk factors at lower BMI values than Caucasians [20-22]. The relative risk of

Gemma Fraterrigo

mortality is higher at any given waist circumference in Asians compared to African Americans or Europeans [23,24] and therefore the World Health Organization (WHO) indicated that the waist circumference cutoff values in the Asian population be 90 cm for men and 80 cm for women [24,25]. Different cutoff levels have been proposed in Japan and China, and also in India [25].

In people with certain health problems, being overweight and obese is associated with lower mortality rates compared to those that have the same health problems but have a normal BMI. This is the case for 1) cardiovascular disease such as myocardial infarction [26,27], congestive heart failure [27], hypertension and coronary heart disease (CHD) [28], coronary artery bypass graft surgery [29,30], heart transplant [30]; 2) renal disease such as end-stage renal disease [31]; 3) hip fractures [32]; 4) rheumatoid arthritis [33]; and 5) tuberculosis [34].

However, we need to take into consideration other factors that are associated with increased risk factors in overweight and obese people. For example, weight gain of 5 kg or more during adulthood (ages 18 to 20 years), in both men and women increases the risk of developing diabetes, hypertension, and CHD, and this risk increases with the amount of weight gained [35-40]. Another factor is aerobic fitness: incidence of diabetes [41] and cardiovascular mortality [42] is lower in those that are aerobically fit (definite by maximal ability to consume oxygen during exercise), compared to those who were unfit across a range of body adiposity. Ethnicity also seems to play a role since the risk of diabetes is greater in Southeast Asian populations than in whites when matched on BMI [43].

1.1.1.1 Metabolically Normal and Abnormal Obesity

Obesity is commonly associated with alterations in metabolic function, such as insulin resistance, diabetes, dyslipidemia (increased triglyceride and decreased serum high-density lipoprotein-cholesterol [HDL-C]), and increased blood pressure [44]. However, about 25% of obese adults are "metabolically normal" based on insulin sensitivity as measured by the hyperinsulinemic euglycemic clamp technique [45,46]. Also, NHANES data covering 1994 to 2004 showed that 32% of obese adults were metabolically normal, defined as having no more than one cardiometabolic abnormality (i.e., blood pressure, a homeostatic model assessment of insulin resistance [HOMA-IR] value, and plasma glucose, triglyceride, HDL-C, and C-reactive protein levels) [47].

The recognition that a subset of obese people are resistant to the typical metabolic complications of obesity has led to several studies that attempted to characterize the

distinguishing features of metabolically healthy but obese individuals [48]. Generally, these studies found that people with metabolically normal obesity, also called uncomplicated obesity [49] or metabolically benign obesity [50], had a similar percentage of body fat but less visceral and liver fat than metabolically abnormal obese people. Moreover, they also had normal values for insulin sensitivity, blood pressure, lipid profile, and inflammatory profile, such as plasma C-reactive protein [45,48-52]. Metabolically normal obese adults who were monitored for up to 11 years did not show a greater risk of developing diabetes or cardiovascular disease than normal-weight, metabolically normal subjects [53]. In contrast, metabolically abnormal, lean or obese subjects had a 4- to 11-fold increased relative risk of diabetes compared with normal-weight, metabolically normal subjects [54].

1.1.2 Epidemiology

The prevalence of obesity worldwide has increased dramatically over the last several decades. The National Health and Nutrition Examination Survey (NHANES) is a cross-sectional, nationally representative serires of surveys conducted by the National Center for Health Statistics of the U.S. Centers for Disease Control and Prevention. All surveys include a standardized physical examination conducted in a mobile examination center with measurement of weight and height, using standardized protocols [55,56]. According to NHANES data, in the United States, about one third of adults between the age of 20 and 74 are considered obese [57]. National population surveys that have been conducted since 1960 have demonstrated that the prevalence of obesity (BMI $\geq$ 30 kg/m^2) has more than doubled, from 13% to 32% [57,58]. More recent data from NHANES show no significant changes in the prevalence of obesity for either men or women between 2003-2004 and 2005-2006 (Figures 1.3, 1.4) [59,60]. This possible stabilization in the obesity trends may be an early sign of a plateau in the obesity epidem. The obesity prevalence was relatively low and stable between 1960 and 1980, but more than doubled from 15% in 1980 to 34% in 2006 [59,60].

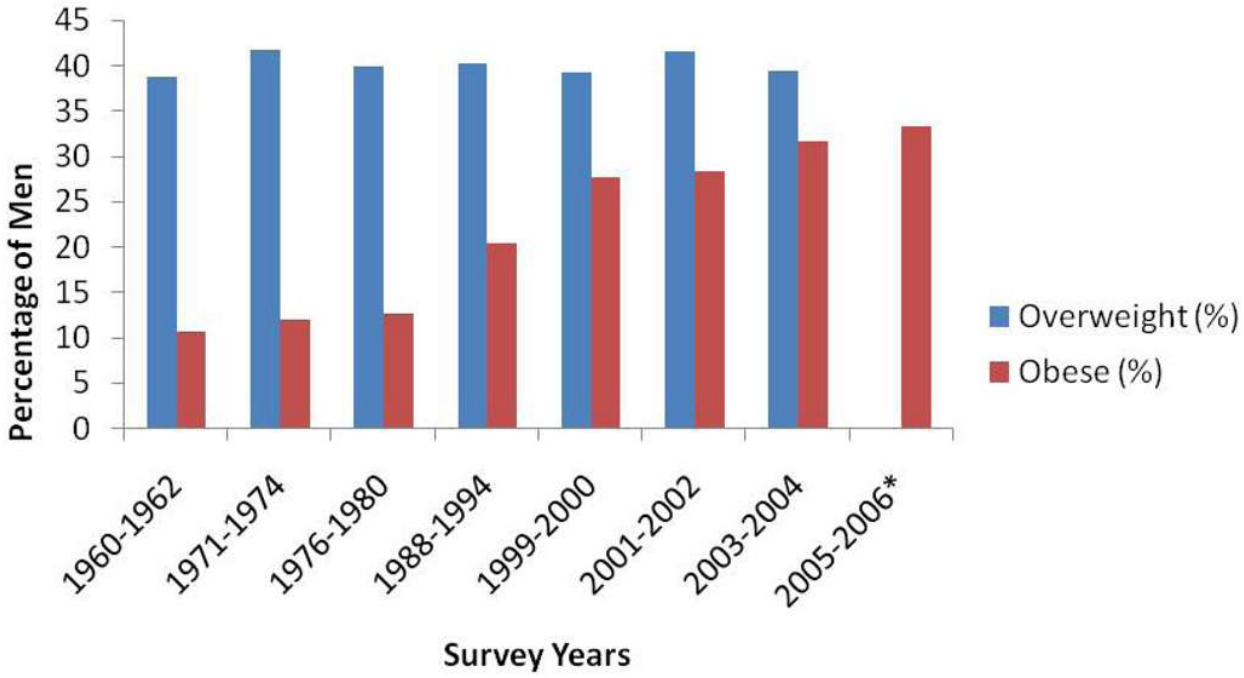

FIGURE 1.3. Prevalence and Trends of Overweight and Obesity Among Men Ages 20–74 Years in the United States: 1960–2006

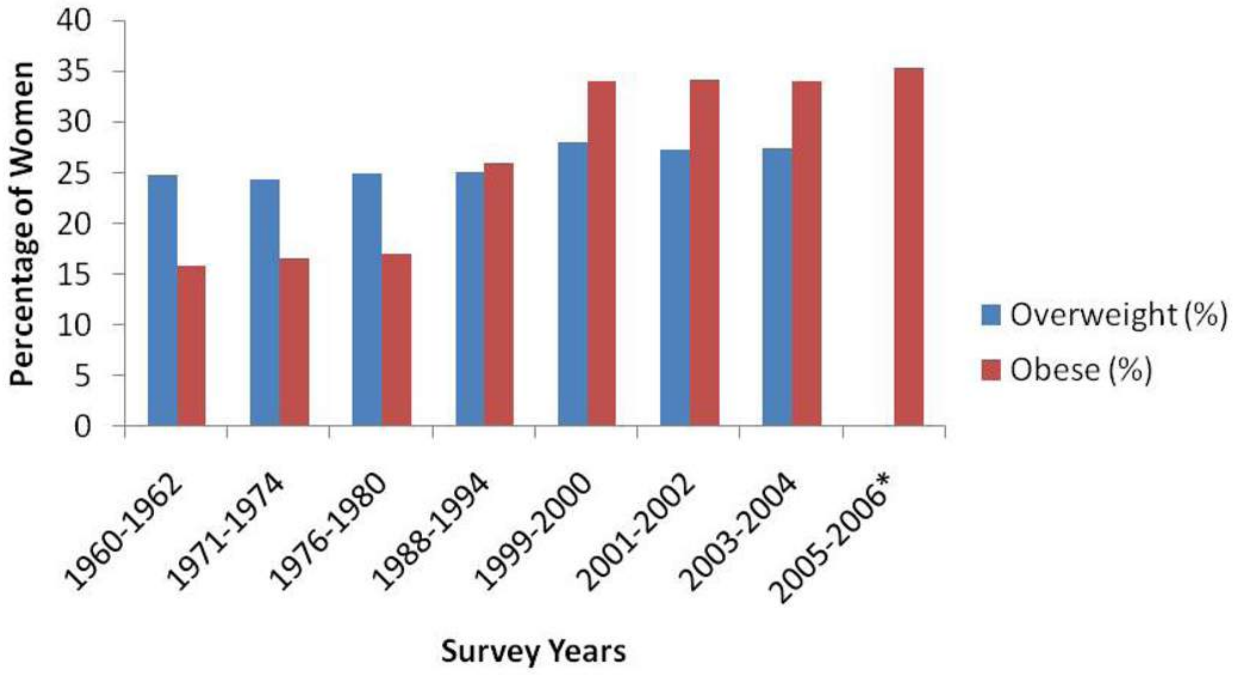

FIGURE 1.4. Prevalence and Trends of Overweight and Obesity Among Women Ages 20–74 Years in the United States: 1960–2006

Gemma Fraterrigo

Data from NHANES show large ethnic differences in the prevalence of obesity, but do not include an adequqte number of minorities other than African American and Mexican American. According to the data from NHANES 2003-2004, African Americans had the highest obesity prevalence of 45% for men and women between 20 and 74 years of age whereas the obesity prevalence was 30.6% for Caucasians and 36.8% for Mexican Americans [60]. The data from NHANES 2005-2006 show large differences in obesity prevalence by ethnic group among women 40-59 years of age, but not significant difference by ethnic group among men. About 53% of African American women and 51% of Mexican American women were obese, compared to 39% Caucasian women [59].

The even more worrisome fact is that the prevalence of obesity has also increased in children and adolescents. The definition of being overweight, in children and adolescents, is having a BMI greater than the 95th percentile for age and gender based on the revised National Center for Health Statistics growth charts. And according to this definition, the prevalence of overweight children and adolescents between the ages 6 and 17 in the United States is 17% [57,61], which is double the numbers reported in earlier surveys. The overweight prevalence changed dramatically between 1980 and 2006 for children 2-19 years of age, increasing from 5.5% to 16.3%. The rapidity with which the prevalence is increasing in children and adolescents is shocking. However, the most recent data from NHANES show no significant changes in the prevalence of obesity for children 2-19 years of age between 2003-2004 and 2005-2006 [62]. It is a well known fact that obesity is commonly associated with certain diseases in adults like T2DM, hypertension, hyperlipidemia, gallbladder disease, nonalcoholic steatohepatitis, sleep apnea, and orthopedic complications and such diseases are being increasingly observed in children [63].

The current epidemic of obesity has been reported in several but not all regions globally. The highest rate of obesity has been reported in the Pacific Islands and the lowest rates have been seen in Asia. The rates in Europe and North American are generally high, while the rates in Africa and Middle Eastern countries are variable [64]. The prevalence of obesity around the world is monitored by the WHO through the Global Database on BMI. The survey data included in the database are identified from the literature or from a wide network of collaborators. However, high quality data from systematic nationally representative samples is sparse. As of November 2004, the database has compiled data covering approximately 86% of the adult population worldwide. The WHO estimates that in 2005 approximately 1.6 billion people

worldwide were overweight and that at least 400 million adults were obese [65]. They further project that, by 2015, approximately 2.3 billion adults will be overweight and that at least 700 million will be obese [65]. According to the data from the Global Database on Body Mass Index, there are wide variations in the prevalence of obesity throughout the world, ranging from India, where 1% or less of the population is obese, to the Pacific Islands, where the prevalence of obesity can reach up to 80% in some regions.

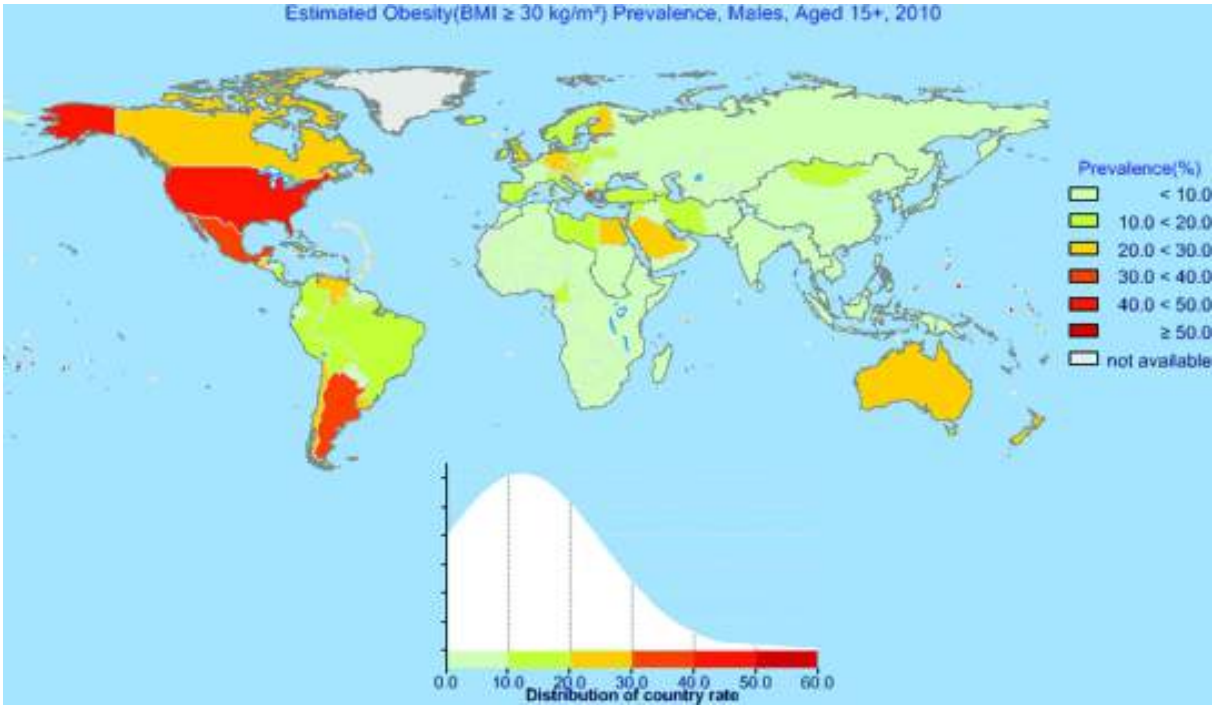

FIGURE 1.5. 2010 Estimated Obesity Prevalence in Males, Ages 15–100 (used by permission, WHO Global Infobase 2010).

The change over time in adult obesity prevalence was calculated for 28 countries that have two or more nationally representative surveys recorded in the Global Database on BMI. Overall, most countries have rising trends of obesity. Only two of the 28 countries showed a falling trend in the prevalence of obesity in men (Denmark and Saudi Arabia), and five of the 28 countries showed a falling trend in the prevalence of obesity in women (Denmark, Ireland, Saudi Arabia, Finland, and Spain) [65].

Because of the increased risk of death and the increased risk of costly chronic diseases associated with obesity, the obesity epidemic places a large financial burden on the economy. The U.S. Department of Health and Human Services has estimated the total economic cost of

Gemma Fraterrigo

overweight and obesity in the United States to be close to $117 billion using data from 1995, updated to 2001 dollars [65]. However, because the prevalence of overweight and obesity has increased since 1995, the costs today are likely to be considerably higher than previous estimates. Trogdon *et al.* estimated that the total indirect cost was $65.67 billion in the United States for 1999, based on data from a systematic review [66]. A recent study by Finkelstein *et al.* projected the annual medical spending due to overweight and obesity approached $92.6 billion in 200228, or about 9% of US health expenditures [67].

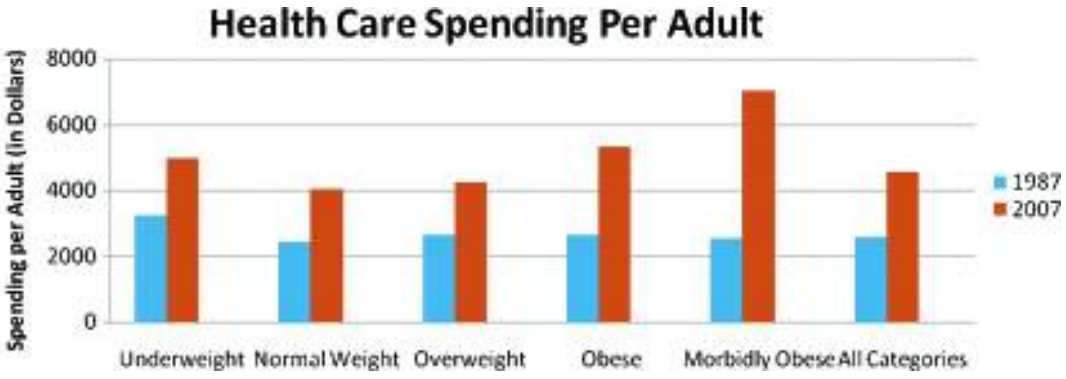

FIGURE 1.6. Health Care Spending per Adult in 1987 and 2007 (CBO data).

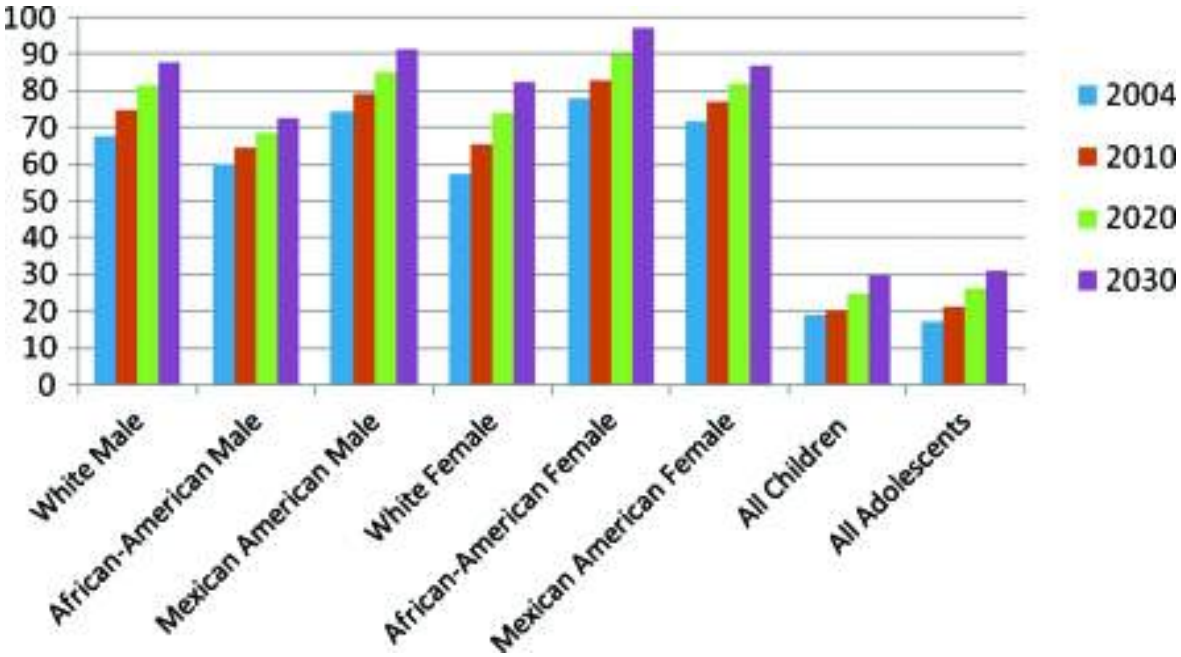

FIGURE 1.7. Estimated Percent Overweight or Obese in the United States.

1.1.3 Pathophysiology

1.1.3.1 Energy Balance

Obesity is the result of an excessive intake of calories compared to the energy expenditure over a long period of time. The gastrointestinal tract can absorb large amounts of nutrients. Even relatively small but chronic differences between energy intake and energy expenditure can lead to large increases in body fat, consequently leading to all the associated increased risk factors. For example, an intake of only 5% more calories than those expended can, in one year, cause an increase of approximately 5 kg in adipose tissue. Even more surprising is that ingesting only 8 kcal more than expended a day, over 30 years will increase body weight by 10 kg. 10 kg is the average amount of weight gained by Americans during the 30-year period between 25 and 55 years of age [68].

1.1.3.2 Genes and Environment

Body weight depends on a complex interaction between genetic background and environmental factors [9]. In humans, genetic background accounts for only about 40% of the variance in body mass [69]. Therefore the significant increase in the prevalence of obesity starting from the 1980s must be largely caused by the changes in environmental factors which led to increased energy intake and reduced physical activity. The lifestyle has changed dramatically involving more dining out, greater availability of processed foods, snack foods, and fast foods, larger serving sizes, and a more sedentary lifestyle and work activities.

1.1.3.2.1 Environmental Effects in High-Risk Populations

Dramatic results of the influence of environment on body weight have been reported globally. Some people of certain genetic background are especially prone to gain weight and develop obesity-related diseases when they are exposed to a "modern" lifestyle

Starting from the 1950s, important changes in the lifestyle of Pima Indians living in Arizona have led to an epidemic of obesity and diabetes in this population [53]. The diet of these urbanized Pimas is much higher in fat (50% of their caloric intake) compared to their traditional diet (15% of their caloric intake). At the same time, these urbanized Pimas are much more sedentary than those you remained in the Sierra Madre Mountains of Northern Mexico and are isolated from Western influences. These "traditional" rural Pimas eat a traditional diet and are

physically active as farmers and sawmill workers; they have much more lower incidences of obesity and diabetes than their Arizona kindred [9].

Another example of a high-risk population whose weight and health have been compromised by exposure to a modern environment is the Aborigine of northern Australia. Urbanized Aborigines have increased body weight compared to their hunter-gatherer kindred, who are usually very lean and they have high prevalences of T2DM and hypertriglyceridemia [70]. The traditional hunter-gatherer lifestyle of the Aborigines consists of a low-fat, low-calorie diet of wild game, fish, and plants and a great level of physical activity. A short-term (7 weeks) of re-exposure to the traditional lifestyle led to weight loss and significant improvements of normalization of glucose tolerance and fasting blood glucose, insulin, and triglyceride levels in urbanized Aborigine with T2DM and hypertriglyceridemia [71].

1.1.3.2.2 Influences of Childhood and Parental Obesity

What determines whether or not a person will become an obese adult? This is determined of course, as we mentioned earlier, both by the genetic and environmental factors but also both by whether he/she was obese as a child and by having at least one obese parent. The risk of adult obesity increases with increasing age and with the severity of obesity in childhood. For example, for people who were obese at 1 to 2 years of age and had nonobese parents have a 8% risk of being obese at 21 to 29 years of age whereas this risk is 79% for those who were obese at 10 to 14 years of age and had at least one obese parent [72]. Even though people who were obese at ages 1 to 2 and had lean parents did not have an increased risk of obesity in adulthood, those who became obese after age 6 had a great than 50% chance of becoming obese as adults.

1.1.3.2.3 Monogenic Causes of Obesity

Although rare, monogenic causes of obesity have been identified and described in recent years.

1.1.3.2.3.1 Leptin Gene Mutation

Leptin gene mutation was discovered when two extremely obese cousins with hyperphagia who belonged to a consanguineous family of Pakistani origin were found to be homozygous for a single nucleotide deletion at position 398 of the leptin gene [73]. This mutation causes a frameshift of the leptin-coding region and consequently premature termination of leptin synthesis. The parents of the cousins were heterozygous for this mutation. Another mutation

Gemma Fraterrigo

involving the leptin gene is a homozygous single-nucleotide transversion in the leptin gene that results in a substitution of Trp for Arg in the mature peptide and consequently causing low serum leptin levels. This was discovered in one adult man and one adult woman, both were hyperinsulinemic [74]. The symptoms in the man were hypothalamic hypogonadism and dysfunction of the sympathetic nervous system while the woman had primary amenorrhea. Treatment with leptin was successful in reversing the obesity in these patients. Treatment with recombinant human leptin resulted in a weight loss of 1 to 2 kg/month over a 12-month period and 95% of the weight lost was fat mass [75]. So does this mean that leptin levels are low in obesity? A study has been done in a large group of subjects but it showed that serum leptin levels increase exponentially with fat mass, suggesting that most obese people are resistant or insensitive to body weight regulation by endogenous leptin [75].

1.1.3.2.3.2 Leptin Receptor Mutation

Leptin receptor mutation was found in three extremely obese sisters from a consanguineous family with extremely high serum leptin levels; more specifically they were found to be homozygous for a single-nucleotide substitution at the splice site of exon 16 of the leptin receptor gene [76]. This resulted in a truncated protein that lacked both the transmembrane and the intracellular domains of the receptor. Symptoms displayed by these sisters were hypogonadotropic hypogonadism, failure of pubertal development, growth delay, and secondary hypothyroidism. This confirms the fact that leptin and its receptor play a role in the central regulation of energy balance and hypothalamic endocrine functions in humans.

1.1.3.2.3.3 Prohormone Convertase I Gene Mutation

A mutation in the gene encoding prohormone convertase 1 (PC1), now called PCSK1, was found in an obese woman with a history of severe childhood obesity [77]. She had impaired glucose tolerance, postprandial hypoglycemia, low plasma cortisol levels, and hypogonadotropic hypogonadism. She also had increased plama proinsulin and POMC levels but very low plasma insulin concentrations. She was a compound heterozygote for two mutations in the PCSK1 gene, which caused loss of the autocatalytic cleavage ability of PC1. Melanocortins, including α-melanocortin-stimulating hormone (MSH), are formed through the processing of POMC by PC1 and this is the reason why a reduced production of melanocortin might have been responsible for obesity in these patients.

1.1.3.2.3.4 Pro-Opiomelanocortin Gene Mutation

Gemma Fraterrigo

A mutation in the Pro-Opiomelanocortin (POMC) gene was found in two obese children with hyperphagia [78]. They also had red hair pigmentation and were deficient in adrenocorticotropic hormone (ACTH). The mutation resulted in complete loss of the ability to synthesize α-MSH and ACTH and the red hair and obesity are believed to be caused by deficiency of α-MSH.

1.1.3.2.3.5 Melanocortin 4 Receptor Mutation

Although rare, mutations in the melanocortin 4 receptor (MC4R) are the most common monogenic cause of obesity [79]. They can be inherited in both a dominant and a recessive mode, in contrast to other monogenic forms of obesity, which have recessive modes of inheritance. In children with MC4R mutations, the degree of obesity and hyperphagia correlates with the extent of impairment of MC4R signaling. However, adult carriers of the mutations cannot be phenotypically distinguished from other obese people [80].

1.1.3.2.3.6 Mutation of the Neurotrophin Receptor TrkB

Neurotrophic factors, secreted by the target tissues, are necessary in the survival and differentiation of neurons in the peripheral nervous system. Neurotrophin signaling occurs through the specific activation of receptor tyrosine kinases of the Trk family. A boy with a complex developmental syndrome and extreme obesity was found to be heterozygous for a de novo missense mutation resulting in a Tyr722Cys substitution in the neuotrophin receptor TrkB. This mutation significantly impaired receptor autophosphorylation and signaling to mitogen-activated protein kinase. Mutation of NTRK2, the gene that encodes for TrkB, seems to lead to a unique human syndrome of hyperphagic obesity [81].

1.1.3.2.3.7 Obesity in Pleiotropic Syndromes

Approximately 30 mendelian disorders with obesity as a clinical feature have been described and it is often associated with mental retardation, dysmorphic features, and organ-specific developmental abnormalities, therefore pleiotropic syndromes. Techniques in positional genetics have allowed the identification of different mutations that underlie these syndromes. But in most cases these genes encode for proteins whose function is yet unresolved [82].

1.1.3.2.4 Obesity Syndromes due to Chromosomal Rearrangements

1.1.3.2.4.1 Prader-Willi Syndrome

The Prader-Willi syndrome is characterized by obesity, mental retardation, short stature, and secondary hypogonadism and is the most common syndromic cause of obesity, and occurs in 1 of every 25,000 births [83]. In this syndrome the paternal segment of 15q11.2-q12 is absent. This absence can be from deletion of the paternal segment in 75% of the cases, or from loss of the entire paternal chromosome 15, with the presence of two maternal homologs instead (uniparental maternal disomy). The role of the genes encoded by the paternal segment and the mechanisms by which they cause obesity have not yet been resolved [83].

1.1.3.2.4.2 SIM1 Gene Mutation

A de novo balanced translocation between chromosomes 1 and 6 was found in an extremely obese girl [84]. This mutation caused a disruption in *SIM1*, the human homolog of the *Drosophila* single-minded *(sim)* gene that regulates neurogenesis. SIM1 encodes a transcription factor that is involved in the formation of the paraventricular and supraoptic nuclei. It is probable that this abnormality altered energy balance in this patient by stimulating food intake because the measured resting energy expenditure of this patient was normal.

1.1.3.2.3 Polygenic Causes of Obesity

Obesity is likely to result from the interaction of many different gene-gene and gene-environment interactions. In contrast to the small number of single-gene mutations that clearly cause obesity in rare patients, a large number of human genes have been identified that show variations in DNA sequences that might contribute to obesity [85]. Many genes with robust associations have been identified but with only modest contributions to overall genetic susceptibility to common obesity or high BMI. It is challenging to figure out how these results fit into current models of the genetic architecture and physiology of obesity since no existing hypothesis explains all the data. Undoubtedly some of the associations will prove to be more important than others. The first major breakthrough provided by the genome-wide association studies was the discovery of the fat mass and obesity-associated gene *(FTO)* as a potential obesity gene.

Data from several studies have shown a strong association between fat mass (or BMI) and a single-nucleotide polymorphism (SNP) in *FTO* in both childhood and adult obesity [86-88]. *FTO* encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Fto messenger RNA (mRNA)

seems to be most abundant in the brain, especially in the hypothalamic nuclei that govern energy balance, and the levels of Fto mRNA in the arcuate nucleus are regulated by feeding and fasting [89]. This suggests that *FTO* participates in the central regulation of energy homeostasis.

1.1.4 Energy Metabolism

Total daily energy expenditure (TEE) consists of resting energy expenditure (REE), which is responsible for about 70% of TEE; energy expenditure in physical activity, about 20% of TEE; and the thermic effect of food (TEF), accountable for about 10% of TEE. REE is the energy expended for normal cellular and organ function under postabsorptive resting conditions. Energy expenditure in physical activity includes the energy costs of both volitional activity, such as exercise, and nonvolitional activity, like spontaneous muscle contractions, maintaining posture, and fidgeting. The TEF is the energy expended in digestion, absorption, and sympathetic nervous system activation after ingestion of a meal.

Cross-sectional studies have investigated whether alterations in energy metabolism are involved in obesity. Obese people usually have greater rates of REE than lean people of the same height because obese people have greater lean and fat mass [90].

No defects in REE or TEE have been detected in those obese people that claim to be "diet-resistant", those who do not lose weight despite the claim of strict adherence to a low-calorie diet [91,92]. The problem seems to be that these patients underestimate their food intake and actually consume twice as many calories as they record in food-intake diaries. At the moment, it is not clear whether obese people expend less total energy in daily physical activity due to the fact that they are less active than lean people. During non-weight-bearing activity such as cycling, obese people expend the same amount of energy as lean people to perform the same amount of work [93]. However, during weight-bearing activies, obese people expend more energy than lean ones, since more work is required to carry their greater body weight.

Data from studies in obese and lean people matched for either fat mass or lean body mass suggests that obese people have a small (about 75 kcal/day) but potentially important reduction in TEF. This might be caused by insulin resistance and blunted sympathetic nervous system activity that occur in obesity [94].

Even though extensive research has so far failed to reveal significant defects in the energy metabolism of people who are already obese, the possibility remains that inherent abnormalities in energy metabolism might play a role in the development of obesity. However, research technology available to us currently only has a limited ability to detect small but chronic defects in energy metabolism that might be clinically significant. Also, it is difficult to establish a cause-and-effect relationship between energy expenditure and the development of obesity since energy metabolism measurements capture only a brief point in time and therefore might not reveal abnormalities that emerge during specific life stages.

Most studies do not support the involvement of a defect in metabolic rate in the development of obesity. One longitudinal study showed that daily TEE at 3 months of age was 21% lower in infants who later became overweight compared to those who maintained a normal weight [95]. However, larger subsequent studies did not confirm this finding [96]. A longitudinal study of 126 Pima Indians showed that those in the lowest tertile of REE at baseline had the highest cumulative incidence of a 10kg weight gain after 1 to 4 years [97]. In contrast, the Baltimore Longitudinal Study on Aging, which followed at 775 men for an average of 10 years, did not detect a relationship between initial REE and weight change [98].

As had been mentioned previously, when energy intake exceeds energy expenditure, weight gain usually occurs. However, genetic factors may influence the amount of weight gained with overfeeding. A study that observed variable weight gain in 12 monozygotic twin pairs who were chronically overfed 1000 kcal/day showed that members of each twin pair gained similar amounts of weight [99]. In another study, an increased body fat after 8 weeks of overfeeding was inversely related to changes in nonvolitional energy expenditure such as fidgeting [100]. This leads to the idea that in some people, nonvolitional energy expenditure during periods of overingestion could be a mechanism that limits weight gain through the dissipation of excess ingested energy.

REE decreased with diet-induced weight loss, promoting weight regain. This observation underlies the set-point theory, which states that body weight is predetermined such that weight loss (or gain) promotes a decrease (or increase) in metabolic rate that acts to restore body weight to a preset level. In both lean and obese people, hypocaloric feeding reduces REE by 15-30%. This reduction cannot be completely accounted for by the accompanying decrease in body size or

lean body mass and is considered a normal part of the physiologic adaptation to energy restriction [101].

The reduction in REE that occurs during negative energy balance is transient and does not persist during maintenance of a lower body weight. As several studies have shown, long-term maintenance of weight loss is not accompanied by an abnormal decrease in REE or TEE when adjusted for changes in body composition [102]. In a meta-analysis of 15 studies, the REE of subjects who were formerly obese was found to be similar to that of subjects who were never obese [103]. Even though the decrease in energy metabolism with weight loss is largely appropriate for the concomitant changes in body composition, this decrease might nonetheless promote weight regain.

1.1.5 Adipose Tissue and Triglyceride Metabolism

The body's major energy reserve is the stored triglycerides within adipose tissue. Triglycerides are a much more compact fuel than glycogen due to fat's energy density and hydrophobic nature. Triglycerides yield 9.3 kcal/g on oxidation and are compactly stored as oil inside the fat cell, accounting for 85% of adipocyte weight. Glycogen, on the other hand, yields only 4.1 kcal/g on oxidation and is stored intracellularly as a gel containing about 2 g of water for every 1 g of glycogen.

Adipose tissue is an effective storage mechanism for transportable fuel that allows mobility and survival when food is scarce. During starvation, the duration of survival is determined by the size of the adipose tissue mass. Lean people die after only about 60 days of starvation, when more than 35% of body weight is lost [104]. On the other hand, obese people have tolerated much longer therapeutic fasts, up to 1 year or longer, without adverse effects. The longest reported fast was a 207 kg man who ingested only acaloric fluids, vitamins, and minerals for 382 days and lost 126 kg, 61% of his initial weight [105].

1.1.5.1 Triglyceride Storage

The primary function of adipocytes is to store triglycerides for future use as energy substrate. Lipogenesis from glucose makes only a limited contribution to triglyceride storage in the adipocyte [106]. Most of the triglyceride in adipocytes is derived from chylomicrons and very-low-

density lipoprotein (VLDL) triglycerides that originate, respectively, from dietary and hepatic sources. These plasma triglycerides are hydrolyzed by lipoprotein lipase (LPL), which is a key regulator of fat cell triglyceride uptake from circulating triglycerides. LPL is synthesized by adipocytes and transported to the endoluminal surface of endothelial cells. The interaction of LPL with chylomicrons and VLDL releases fatty acids from plasma triglycerides, which are then taken up by local adipocytes. Plasma free fatty acids themselves can also be taken up by adipose tissue, independently of LPL.

The principal hormones that are involved in the regulation of LPL activity and expression are insulin and cortisol [107]. The activity of LPL within individual tissues is a key factor in partitioning triglycerides among different body tissues. Insulin influences this partitioning through its stimulation of LPL activity in adipose tissue [108]. Insulin also promotes triglyceride storage in adipocytes through other mechanisms, such as inhibition of lipolysis, stimulation of adipocyte differentiation, and escalation of glucose uptake. The importance of cortisol in fat distribution is supported by the clinical apperance of patients with Cushing's syndrome. The obesity-promoting effect of cortisol can involve a synergistic effect of cortisol and insulin on the induction of LPL in adipose tissue, as has been demonstrated in vitro. Testosterone, growth hormone, catecholamines, tumor necrosis factor (TNF), and other related cytokines inhibit LPL activity [107].

1.1.5.2 Lipolysis

The balance between triglyceride stoage and lipolysis is regulated by complex hormonal and neuronal mechanisms. In order for it to be available as energy substrate, triglycerides stored within adipocytes need to be hydrolyzed by hormone-sensitive lipase (HSL) into fatty acids. These fatty acids can be released from adipocytes into the circulation. The half-life of plasma fatty acids in circulation is only 3 to 4 minutes. During resting conditions, fatty acid release by adipose tissue exceeds the rate of fatty acid oxidation [109]. The excess availability of fatty acids in plasma provides a ready supply of oxidizable substrate to resond to sudden changes in energy requirements, for example during exercise. The plasma fatty acids that escape immediate oxidation are usually re-esterified to triglyceride in adipose tissue, muscle, or liver. These fatty acids are the major precursors of hepatic VLDL triglyceride synthesis [110]. In turn, VLDL triglycerides are secreted by the liver and redistributed throughtout the body, depending on

tissue-specific factors, such as the activity of LPL. Such observations imply that there is continuous redistribution of triglycerides between adipose tissue and the rest of the body.

There is considerable variation within and between subjects in the rate of lipolysis and, therefore, in the level of fatty acids in plasma. The major circulating hormones that influence lipolysis in adipocytes are insulin and catecholamines. Insulin inhibits lipolysis through its effect on HSL, whereas catecholamines stimulate lipolysis. Even small changes in the plasma levels of insulin and catecholamines have major effects of lipolytic rate. At postabsorptive insulin levels, half-maximal suppression of lipolysis occurs, and at insulin levels within the range observed after a regular meal, maximal suppression of lipolysis occurs [111]. Even small increases in resting catecholamine levels stimulate lipolysis. For exaple, growth hormone and cortisol stimulate lipolysis. However, generally, the effects of these other factors are less powerful than the effects of insulin and catecholamines.

Unlike the tight feedback regulation of insulin secretion by glucose levels, insulin and catecholamine concentrations are not regulated by lipolysis or fatty acid levels. Although free acid levels affect glucose-stimulated insulin release, there is no feedback between insulin release and rate of lipolysis. The wide physiologic variations in plasma free fatty acid levels between people can be explained, in part, by the finely tuned dose-response effects of insulin and catecholamines on lipolysis, along with the absence of tight feedback regulation of insulin and catecholamine levels by free fatty acids.

In obese people, basal plasma fatty acid levels are often increased. This higher levels of circulating fatty acids are due to and increased rate of lipolysis from upper-body subcutaneous fat, therefore an increased rate of free fatty acid release into plasma [112,113]. The presence of excess free fatty acid in plasma might lead to increased hepatic free fatty acid uptake, VLDL triglyceride synthesis, intramuscular triglyceride formation, and insulin resistance

1.1.6 Adipose Tissue as an Endocrine Organ

Traidtionally, adipocytes have been viewed as energy depots that store triglycerides during feeding and release fatty acids during fasting in order to provide fuel for other tissues. However, in the recent years, it has been shown that adipose tissue secretes numerous proteins that have important physiologic function (Table 1.2). These factors participate in autocrine and paracrine

regulation within adipose tissue, and, as circulating hormones, they can affect the functions of distant organs such as muscle, pancreas, liver, and the central nervous system, therefore making it an endocrine organ.

TABLE 1.2. Adipocyte-Secreted Proteins

Category	**Protein**
Hormones	Leptin, resistin, angiotensinogen, adiponectin, estrogens, visfatin, angiopoietin 4
Cytokines	Interleukins 1, 6, 8, 10, MCP-1, interferon-y, tumor necrosis factor-α
Extracellular matrix proteins	Various subtypes of collagen-α1, various metalloproteinases, fibronectin, osteonectin, laminin, entactin, thrombospondin 1 and 2
Complement factors	Adipsin, complement C3, factor B
Enzymes	Cholesterol ester transfer protein, lipoprotein lipase
Acute phase response proteins	A-1 Acid glycoprotein, haptoglobin
Other	Fatty acids, plasminogen activator inhibitor 1, prostacyclin

The role of adipose tissue as an endocrine organ has important implications for understanding the pathophysiologic relationship between excess body fat and pathologic states such as insulin resistance and T2DM [114,115]. Not everything released by adipose tissue are produced by adipocytes. There are other cells contained within the adipose tissue, such as endothelial cells, macrophages, and adipocyte precurso cells, can also participate in endocrine functions. Some of the proteins produced by adipose tissue will be reviewed below.

1.1.6.1 Leptin

Leptin is produced by adipocytes and is secreted into the bloodstream. It has pleotropic effects of food intake, hypothalamic neuroendocrine regulation, reproductive function, and energy

Gemma Fraterrigo

expenditure [116,117]. There is a direct relationship between plasma leptin concentrations and BMI or body fat percentage [75]. However, there can be a wide variability in leptin levels among people with the same BMI, which suggests that leptin production is also regulated by factors other than adipose tissue mass. Leptin decreases rapidly within 12 hours after the beginning of starvation. Conversely, it increases in response to overfeeding [118]. Therefore, plasma leptin levels reflect adipose tissue mass and are influenced by energy balance. Leptin is a bidirectional signal that switches physiologic regulation between fed and starved states. Plasma leptin levels increase with increasing fat mass and decrease rapidly during early fasting. The importance of the central versus peripheral effects of leptin in body weight regulation in most obese people is still not clear [119].

1.1.6.2 Resistin

Resistin is another signaling protein secreted by adipocytes [120]. Resistin is increased in mice with diet-induced and genetic forms of obesity and insulin resistance. Administration of recombinant resistin to normal mice led to impaired glucose tolerance and insulin action. Neutralization of resistin levels led to reduced hyperglycemia in obese, insulin-resistant mice, party by improving insulin sensitivity. Based on such findings, resistin has been proposed as a hormone that links obesity to diabetes by inducing insulin resistance.

1.1.6.3 Adiponectin

Adiponectin is the most abundant secretory protein produced by adipocytes. Different from the other secretory products of adipocytes, plasma adiponectin levels are decreased in obesity and insulin resistance. There is a close association between hypoadiponectinemia, insulin resistance, and hyperinsulinemia [121]. Conversely, adiponectin increases with improved insulin sensitivity and weight loss [122]. Interventions that improve insulin sensitivity, such as weight loss or treatment with thiazolidinediones, are associated with increased adipose tissue adiponectin gene expression and plasma concentrations [123]. Also, administration of recombinant adiponectin has glucose-lowering effects and improves insulin resistance in mice with obesity or diabetes [124]. This suggests that decreased plasma levels of adiponectin contribute to some of the metabolic complications associated with obesity.

1.1.6.4 Visfatin

Visfatin is a protein that was previously known and pre-B cell colony-enhancing factor. The initial study showing the insulin-like effects of visfatin [125] was later withdrawn. Subsequent studies reported conflicting results regarding the relation of visfatin to adiposity, subcutaneous or visceral fat, and insulin resistance. The role of this protein in ovesity and insulin resistance is unclear and additional studies are required to elucidate the potential physiologic and pathophysiologic role of visfatin.

1.1.6.5 Estrogens

Adipose tissue has P450 aromatase activity, an enzyme that plays a crucial role in transforming androstenedione into estrone. Estrone is the second major circulating estrogen in premenopausal women and the most important estrogen in postmenopausal women [115]. The conversion rate of androstenedione into estrone increases with age and obesity, and is higher in women with lower-body obesity than in those with upper-body obesity. Besides its role in endocrine regulation, the effects of P450 aromatase on estrogen metabolism might also have a role in autocrine and paracrine action since estrogen receptors are present in adipose tissue.

1.1.6.6 Selected Cytokines

1.1.6.6.1 Tumor Necrosis Factor-α

TNF-α is secreted by adipocytes and its expression is increased in the enlarged adipocytes of obese people [126]. However, plasma TNF-α levels are generally at or below the detection limit of available assays, which suggests that the TNF-α produced in adipose tissue has paracrine, rather than endocrine, functions. The multiple effects of TNF-α on adipocytes include impairment of insulin signaling. For this reason, it has been proposed that TNF-α might partially contribute to insulin resistance in obesity [114].

1.1.6.6.2 Interleukin 6

Interleukin-6 (IL-6) secreted by adipose tissue may account for 30% of circulating IL-6 [127,128]. Obesity is associated with increased plasma IL-6 levels, which may contribute to systemic inflammation and insulin resistance. Insulin sensitivity is inversely related to plasma IL-6 levels [129], and IL-6 directly impairs insulin signaling [130]. Administering IL-6 to humans induced dose-

depend increases in fasting blood glucose, probably by stimulating release of glucagon and other counter-regulatory hormones or by inducing peripheral resistance to insulin action, or both [131].

1.1.7 Adipocyte Biology

1.1.7.1 White Adipose Tissue

Obesity is associated with an increased number of adipocytes. A lean adult has about 35 billion adipocytes, each containing about 0.4 to 0.6 μg of triglyceride whereas an extremely obese adult can have four times as many adipocytes (125 billion), each containing twice as much lipid (0.8 to 1.2 μg of triglyceride) [132].

Understanding of adipocyte differentiation is largely derived from studies conducted in preadipocytes in culture. The current concept is that adipocytes are derived from fibroblast precursor cells after the concerted actions of extraceullular signals and intrinsic transctiption factors and coactivators.

There are many extranuclear factors and intracellular transduction pathways that influence the adipogenic potential of cells in vitro and in vivo (Figure 1.8) [133]. Although in the future it may be possible to regulate adipogenesis in vivo, decreasing adipogenesis without altering energy balance can result in the deposition of triglycerides in other tissues. Excessive amounts of triglycerides in nonadipose tissues can have deleterious effects, as was suggested by the liver steatosis, dyslipidemia, and diabetes observed when adipogenesis was prevented in mice [134].

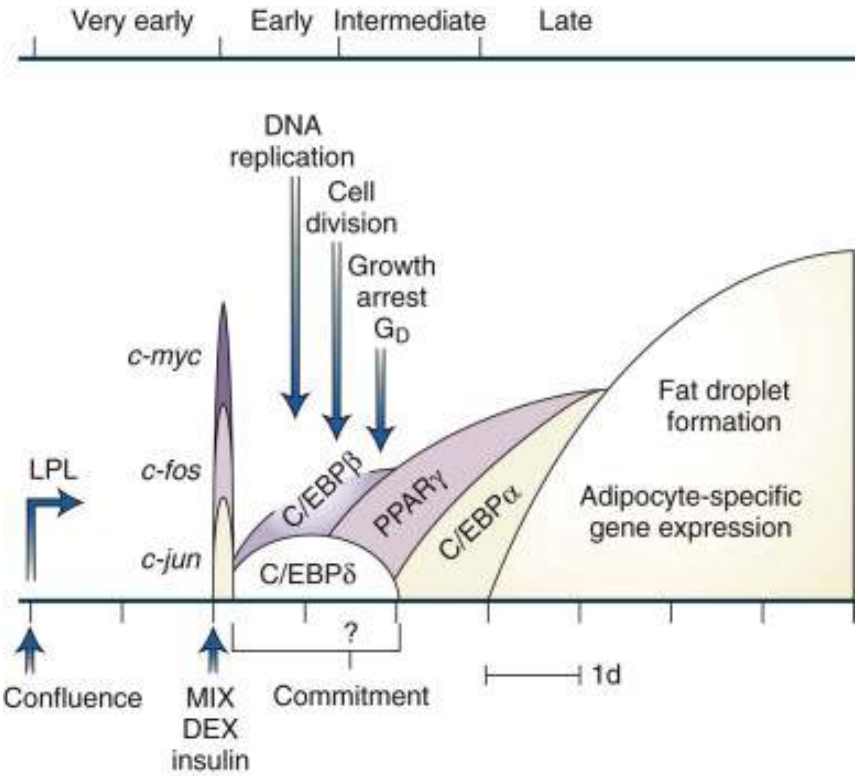

FIGURE 1.8. Progression of 3T3-L1 Preadipocyte Differentiation and Subsequent Changes in Cellular Characteristics. The distinct stages of differentiation (very early, early, intermediate, and late) are shown. C/EBP, CCAAT/enhancer binding protein; DEX, dexamethasone; LPL, lipoprotein lipase; MIX, methylisobutylxanthine; PPAR, peroxisome proliferator–activated receptor.
(From Ntambi JM, Kim Y-C. Adipocyte differentiation and gene expression. J Nutr. 2000;130:3122S-3126S.)

The cornerstone of obesity therapy is to increase the use of endogenous fat depots as fuel by reducing energy intake below energy expenditure. With dieting, weight loss is composed of about 75-85% fat and 15-25% fat-free mass (FFM) [135]. An energy deficit of about 3500 kcal is necessary to oxidize 1 lb of adipose tissue. However, because of the oxidation of lean tissue and associated water losses, a 3500-kcal energy deficit will reduce body weight by more than 1 lb.

Distribution of fat loss is characterized by regional heterogeneity [136,137]. Especially in men and women with initially increased intra-abdominal fat, there are greater relative losses of intra-abdominal fat compared to total body fat mass. A decrease in the size (triglyceride content) of the existing adipocytes is responsible to most, if not all, of the fat loss [138]. In humans, there is also evidence that the number of adipocytes is reduced with large, long-term fat loss [139].

Gemma Fraterrigo

However, it is possible that this perception of decreased fat cell number is false due to inability of standard cell counting techniques to detect adipocytes that have undergone marked shrinkage.

There are two possible mechanisms through which weight loss could eliminate fat cells: 1) dedifferentiation, the morphologic and biochemical reversion of mature adipocytes to preadipocytes, and 2) apoptosis. Adipocyte dedifferentiation has been observed in vitro, but there is no evidence that it occurs in vivo [140]. Adipocyte apoptosis has been induced in vitro [141], and it has been demonstrated to occur in vivo in some patients with cancer [141]. To date, it is not known whether diet-mediated weight loss induces adipocyte apoptosis.

1.1.7.2 Brown Adipose Tissue

Brown adipose tissue (BAT) is structurally different from white adipose tissue; it contains multilocular fat vacuoles and large mitochondria and is intensively innervated by sympathetic nerves. In rodents, BAT is very important for nonshivering thermogenesis. The uncoupling of phosphorylation in BAT results from the activity of uncoupling protein 1 (UCP1) within the inner mitochondrial membrane, which exhausts the electrochemical gradient necessary for oxidative phosphorylation by creating a proton leak. BAT consequently affects energy expenditure by producing heat from uncoupled phosphorylation [142]. Three recent studies in humans provide conclusive evidence that UCP1 activity identified by positron emission tomography or computed tomography provided histoloic confirmation of the presence of supraclavicular BAT [143,144]. UCP1 activity was stimulated by cold exposure [145]. Another stuyd in humans showed a very strong seasonal variation in the presence of BAT [146]. It is possible that altered regulation of BAT activity be involved in the pathogenesis of obesity.

1.1.8 Clinical Features and Complications of Obesity

Obesity causes numerous serious medical complications that impair quality of life and lead to increased morbidity and premature death (Figure 1.9) [13].

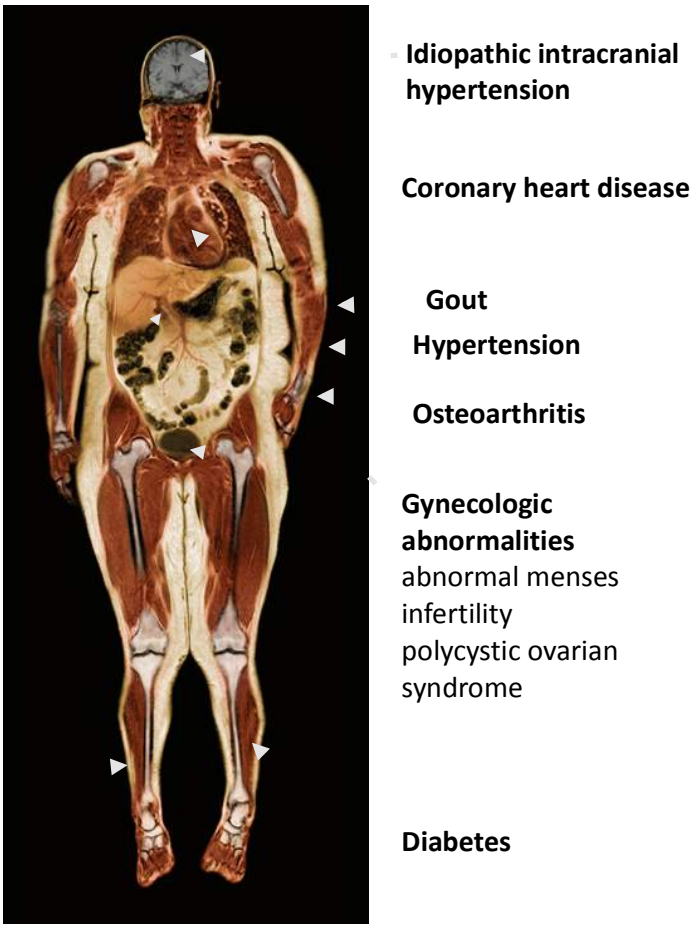

FIGURE 1.9. Medical Complications of Obesity

1.1.8.1 Endocrine and Metabolic Diseases

1.1.8.1.1 The Dysmetabolic Syndrome

The dysmetabolic syndrome is also called the metabolic syndrome, insulin-resistance syndrome, or syndrome X and is characterized by a specific phenotype of central (or abdominal) obesity associated with a cluster of metabolic risk factors for CHD. Features of this syndrome include insulin resistance in conjunction with hyperinsulinemia, impaired glucose tolerance, impaired insulin-mediated glucose disposal, and T2DM; dyslipidemia, characterized by hypertriglyceridemia and low serum HDL-C levels; and hypertension. Other metabolic risk factors such as increased serum levels of apolipoprotein B; low-density lipoprotein (LDL) particles; and plasminogen activator inhibitor 1 (PAI1) with impaired fibrinolysis have also been

Gemma Fraterrigo

associated with abdominal obesity [147,148]. The dysmetabolic syndrome is usually present in obese people, but it has also been reported in normal-weight people who presumably have a high amount of abdominal fat [149].

The dysmetabolic syndrome was originally identified and defined based on epidemiologic associations. The mechanisms behind its pathogenesis and the interrelationships among the individual feature have not been clearly understood. Insulin resistance is thought to be the common denominator of the underlying pathogenic mechanism [150]. However, according to a factor analysis of data obtained from non-diabetic subjects in the Framingham Offspring Study, insulin resistance may not be the only precedent condition, and more than one independent physiologic process may be involved [151].

Abdominal obesity is strongly associated with insulin resistance but it is unclear whether visceral (omental and mesenteric) or subcutaneous abdominal fat is more closely related to insulin resistance because data from different studies show different results. Also to take into consideration is the fact that visceral fat mass often correlates with subcutaneous fat mass, therefore making it difficult to tease out the contribution of each fat depot to insulin resistance. It is also debatable whether visceral fat actually plays a role in the pathogenesis of the dysmetabolic syndrome or merely serves as a marker of increased risk for the metabolic complications of obesity [152].

When there an abundant amount of triglycerides available, they tend to accumulate not only in adipose tissues but also in nonadipose tissues. Ectopic distribution of triglycerides in nonadipose tissue is also closely correlated with the metabolic complications of obesity. Data from different studies showed that insulin resistance to glucose metabolism in skeletal muscle is correlated with the intramyocellular concentration of triglyceride [153]. Excessive intrahepatic triglyceride level is also associated with serious cardiometabolic abnormalities, including T2DM, dyslipidemia (high plasma triglyceride levels, low plasma HDL-C levels, or both), hypertension, the dysmetabolic syndrome, and CHD [154,155]. It is unclear whether triglycerides themselves interfere with insulin action or whether they serve as surrogate markers for some other fatty acid-derived entity that impairs insulin signaling [156].

1.1.8.1.2 Type 2 Diabetes Mellitus

The significant increase in the prevalence of obesity has played an important role in the 25% increase in the prevalence of diabetes that has occurred in the United States over the last 25 years [157]. According to data from NHANES III, two thirds of the men and women in the United States with diagnosed T2DM have a BMI of 27 kg/m^2 or greater [158]. It is a well known fact that the risk of diabetes increases linearly with BMI; for example, the prevalence of diabetes in NHANES III went from 2% in those with a BMI of 25 to 29.9 kg/m^2 to 8% in those with a BMI of 30-34.9 kg/m^2, which is quadruple the risk, and to 13% in those with a BMI greater than 35 kg/m^2 [157]. The risk of diabetes began to increase with BMI exceeded the normal value of 22 kg/m^2 in the Nurses' Health Study [37,159]. Also, the risk of diabetes increased with abdominal fat mass, waist circumference, or waist-to-hip circumference ratio at any given BMI value [160-162]. The risk of diabetes also increases with weight gain as an adult: in men and women aged 35 to 60 years, the risk of diabetes was three times greater in those who had gained 5 to 10 kg since the age of 18 to 20 years, compared to those who had maintained their weight stable (within a 2 kg range) [37,38].

1.1.8.1.3 Dyslipidemia

Obesity is associated with several serum lipid abnormalities such as hypertriglyceridemia, reduced HDL-C levels, and an increased fraction of small, dense LDL particles (VLDL) [163,164] and this association is particularly strong in those with abdominal obesity. Most studies show that serum concentrations of total cholesterol and LDL-cholesterol (LDL-C) are increased in obesity. These abnormalities in serum lipid concentrations associated with obesity are known to be important risk factors for CHD [165-167].

1.1.8.2 Cardiovascular Disease

1.1.8.2.1 Hypertension

A linear relationship between hypertension and BMI has been shown [167,168]. NHANES III showed that the age-adjusting prevalence rates of hypertension, defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or use of antihypertensive medication) in obese men and women were 42% and 38%, respectively. These rates are more than twice than the rate of hypertension, approximately 15%, in lean men and women [169]. The risk of hypertension also increased with weight gain, as in the Framingham Study where there was a 6.5 mmHg increase in blood pressure with every 10% increase in body weight [170].

1.1.8.2.2 Coronary Heart Disease

The risk of CHD is greater in obese persons, and especially more so in those with a greater abdominal fat mass and those who gained weight during young adulthood. Also important is that CHD risk starts increasing at the "normal" BMI levels of 23 kg/m^2 in men and 22 kg/m^2 in women [171]. The Nurses' Health Study has shown that the risk of fatal and nonfatal myocardial infarctions was greater in women who had the lowest BMI but the highest waist-to-hip circumference ratio than it was in women with the highest BMI but the lowest waist-to-hip circumference ratio [172]. Therefore, at any BMI level, the risk of CHD seems to increase with the presence of increased abdominal fat. The risk of CHD also increased with weight gain in adulthood [173]. CHD risk factors related to obesity, such as hypertension, dyslipidemia, impaired glucose tolerance, and diabetes, are largely responsible for the increase in CHD. However, even after adjusting for other known risk factors, several long-term epidemiologic studies still found that overweight and obesity increased the risk of CHD [173]. Based on such findings, the American Heart Association recently classified obesity as a major preventable risk factor for CHD [174,175].

1.1.8.2.3 Cerebrovascular and Thromboembolic Disease

The risk of fatal and nonfatal ischemic stroke is about twice as great in obese compared to lean people and increases progressively with BMI [176,177]. The risks of venous stasis, deep vein thrombosis, and pulmonary embolism are also greater in obesity, especially in those with abdominal obesity [178]. Lower-extremity venous disease may be caused by increased intra-abdominal pressure, impaired fibrinolysis, and the increase in inflammatory mediators [179,180].

1.1.8.3 Pulmonary Disease

1.1.8.3.1 Restrictive Lung Disease

Obesity causes an increased amount of pressure on the chest wall and thoracic cage, which limits pulmonary function by decreasing respiratory compliance, increasing the amount of work required for breathing, restricting ventilation (measured as decreased total lung capacity, decreased forced vital capacity, and decreased maximal ventilator ventilation), and limiting ventilation of the lung bases [181].

1.1.8.3.2 Obesity-Hypoventilation Syndrome

In obesity-hypoventilation syndrome, the partial pressure of carbon dioxide (PCO_2) is less than 50 mmHg because of decreased ventilator responsiveness to hypercapnea or hypoxia (or both) and an inability of the respiratory muscles to meet the increased ventilator demand imposed by the mechanical effects of obesity. Alveolar ventilation is reduced because of shallow and inefficient ventilation related to decreased tidal volume, inadequate inspiratory strength, and elevation of the diaphragm. The resulting increase in intrathoracic pressure further compromises lung function and respiratory capacity. A severe form of the obesity-hypoventilation syndrome is the pickwickian syndrome. This syndrome involves extreme obesity, irregular breathing, somnolence, cyanosis, secondary polycythemia, and right ventricular dysfunction.

1.1.8.3.3 Obstructive Sleep Apnea

Obstructive sleep apnea is characterized by excessive episodes of apnea and hypopnea during sleep, which are caused by partial or complete obstruction of the upper airway despite persistent respiratory efforts. Daytime sleepiness and cardiopulmonary dysfunction result from the interruption in nighttime sleep and arterial hypoxemia. Generally, patients with sleep apnea have a BMI greater than 30 kg/m^2, excess abdominal fat, and a large neck girth (>17 inches in men, >16 inches in women [182-184].

1.1.8.4 Musculoskeletal Disease

1.1.8.4.1 Gout

Hyperuricemia and gout are associated with obesity [185,186].

1.1.8.4.2 Osteoarthritis

In overweight and obese people, the risk of osteoarthritis of weight-bearing joints is greater. Knees are most often involved because much more body weight is exerted across the knees than the hips during weight-bearing activity [187]. The relationship between body size and osteoarthritis is stronger in women than in men. Even small increases in body weight in women can promote arthritis. In a study of twins, symptomatic or asymptomatic lower-extremity osteoarthritis was found in people who were only 3 to 5 kg heavier than their twin sibling [188].

1.1.8.5 Cancer

The risk of cancer is increased in overweight and obese people. According to data from a prospective study in more than 900,000 U.S. adults [189], it was estimated that overweight and obesity could account for 14% of all deaths from cancer in men and 20% in women. In both men and women, BMI was also significantly associated with higher death rates caused by cancers of the esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney, as well as non-Hodgkin's lymphoma and multiple myeloma. There were significant trends of increasing risk in those with higher BMI values for death from cancers of the stomach and prostate in men and for death from cancers of the breast, uterus, cervix, and ovary in women [189]. Most [189,190], although not all [191], epidemiologic studies found a direct relationship between BMI and colon cancer in both men and women. The risks of breast and endometrial cancer mortality increase with obesity and weight gain after age 18 [192]. Interesting is that the risk of breast cancer seems to increase with increasing BMI only in postmenopausal women; in premenopausal women, increased BMI may actually protect against breast cancer [193].

Obesity is often associated with ingestion of a high-fat, high-calorie diet, which is another risk factor for cancer, making it difficult to distinguish how much of the relation between obesity and cancer is attributable to obesity and how much to dietary factors.

1.1.8.6 Genitourinary Disease in Women

Irregular menses, amenorrhea, and infertility are often present in obese women [194]. Pregnant obese women are at greater risk for gestational diabetes and hypertension [195] and delivery complications [196], and their babies are at greater risk for congenital malformations [197]. Incontinence is another problem in extremely obese women, and it has been shown that it typically resolves after considerable weight loss, usually after bariatric surgery [198].

1.1.8.7 Neurologic Disease

The incidence of ischemic stroke increases in obesity. Also idiopathic intracranial hypertension (IIH), also known as *pseudotumor cerebri*, is associated with obesity. This syndrome presents itself with headache, vision abnormalities, tinnitus, and sixth cranial nerve paresis. Although the prevalence of IIH increases with increasing BMI, the risk is increased even in people who are only 10% above ideal body weight [199,200].

1.1.8.8 Cataracts

The prevalence of cataracts is increased in overweight and obesity [201]. Those with abdominal obesity are at greater risk, suggesting that insulin resistance may be involved in the pathogenesis of cataract formation.

1.1.8.9 Gastrointestinal Disease

1.1.8.9.1 Gastroesophageal Reflux Disease

The relationship between gastroesophageal reflux disease and obesity is unclear due to conflicting data from different studies. Most large epidemiologic studies have found a greater incidence of reflux symptoms in obese people [202,203] but not all [204]. Even studies that evaluated gastroesophageal acid reflux by 24-hour pH monitoring have shown conflicting data: reporting the presence of a significant relationship [205] or the lack of relationship [206] between BMI and pathologic reflux (defined as the occurrence of esophageal pH <4 more than 5% of the time).

1.1.8.9.2 Gallstones

There is a linear relationship between the risk of symptomatic gallstones and BMI [40,207]. According to the Nurses' Health Study, the annual incidence of symptomatic gallstones was 1% in women with a BMI greater than 30 kg/m^2 compared to 2% in women with a BMI greater than 45 kg/m^2 [7,207]. The risk of gallstones increases during weight loss, especially during rapid weight loss, and is related to increased bile cholesterol supersaturation, cholesterol crystal nucleation, and decreased gallbladder contractility [208]. When the rate of weight loss is greater than 1.5 kg (or about 1.5% of body weight) per week, the risk of gallstone formation increases exponentially [209]. The incidence of new gallstones in obese people who underwent rapid weight loss with a very-low-calorie (600 kcal/day) and low-fat (1 to 3 g/day) diet [210,211] or with gastric surgery [212] was approximately 25% and 35%, respectively. Gallstone formation is also promoted by the low-fat content of very-low-calorie diets (VLCD), since more than 4 to 10 g of fat in a meal is necessary to stimulate maximal gallbladder contractility [213]. It has been shown that increasing the fat content of a VLCD can prevent the development of new gallstones [214] as well as administration of ursodeoxycholic acid (600 mg/day) during weight loss [215].

1.1.8.9.3 Pancreatitis

It would seem logical that obese people be at increased risk for gallstone pancreatitis because of their increased prevalence of gallstones. However not many studies have studied this issue.

Several studies showed that overweight and obese patients with pancreatitis had a greater risk of local complications, severe pancreatitis, and death than lean patients [216]. The hypothesis is that the increased amount of fat in the peripancreatic and retroperitoneal spaces might predispose obese patients to develop peripancreatic fat necrosis and subsequent local and systemic complications.

1.1.8.9.4 Liver Disease

Obesity is associated with liver abnormalities named nonalcoholic fatty liver (NAFLD), which is characterized by an increase in intrahepatic triglyceride content (i.e., steatosis) with or without inflammation and fibrosis (i.e., steatohepatitis). NAFLD has become an important public health problem due to its high prevalence, potential progression to severe liver disease, and association with serious cardiometabolic abnormalities, including T2DM, the dysmetabolic syndrome, and CHD [154]. The prevalence of NAFLD increases with BMI [217]. In nonobese people, the prevalence rates of steatosis and steatohepatitis are about 15% and 3%, respectively; in people with class I or II obesity (BMI 30 to 39.9 kg/m^2) 65% and 20%; and in extremely obese people (BMI > 40 kg/m^2) 85% and 40% [218-221]. Racial or ethnic background and genetic variation in specific genes play a role in the relationship between BMI and NAFLD [222-224].

NAFLD is an important marker of metabolic dysfunction in obese people, independent of BMI, percent body fat, or visceral fat mass [4,225-228]. It is associated with insulin resistance in liver, skeletal muscle, and adipose tissue [225,229,230]; with increased hepatic de novo lipogenesis [231,232]; and with increased VLDL-triglyceride secretion rate [226]. But it is yet unclear whether the relationship between NAFLD and metabolic abnormalities are causal or simply an association.

Steatosis is not always associated with insulin resistance. An overexpression of hepatic diacylglycerol acyltransferase (DGAT) [233], blockade of hepatic VLDL secretion [234], and pharmacologic blockade of beta oxidation [235] in mice causes hepatic steatosis but not hepatic or skeletal muscle insulin resistance. Steatosis in patients with familial hypobetalipoproteinemia, is due to a genetic deficiency of apolipoprotein B synthesis and decreased VLDL secretion rate, and is not accompanied by hepatic or peripheral insulin resistance [236]. This dissociation between steatosis and insulin resistance seems to suggest that other factors associated with steatosis (e.g.,

Gemma Fraterrigo

inflammation, circulating adipokines, endoplasmic reticulum stress) or other, unidentified metabolites affect insulin sensitivity [9].

An effective therapy for obese people with NAFLD is calorie restriction and subsequent weight loss. A significant decrease in intrahepatic triglyceride content and improvement in hepatic insulin sensitivity occurs very rapidly, within 48 hours after calorie restriction (approximately 1100 kcal/day) begins [237]. A moderate amount of weight loss, of 5% to 10% improves steatosis and inflammation of the liver, decreases the hepatic VLDL-triglyceride secretion rate, and increases muscle insulin sensitivity [237-240]. However, data from most surgical studies showed that weight loss caused by bariatric surgery decreases the cellular factors involved in the pathogenesis of hepatic inflammation and fibrogenesis [241] and improves the histology of the liver: steatosis, inflammation, and fibrosis [242,243].

1.2 METABOLICALLY-NORMAL AND METABOLICALLY-ABNORMAL OBESITY

1.2.1 Introduction and Definition

Obesity is associated with a large number of serious medical complications. The most common complications involve alterations in metabolic function that are risk factors for cardiovascular disease (CVD), namely insulin resistance, diabetes, dyslipidemia (increased serum TG and decreased serum HDL-cholesterol), and increased blood pressure [2]. These metabolic complications have become a major public health problem in the United States and worldwide because the high prevalence of obesity has led to an increase in the prevalence of obesity-related metabolic disease, which has considerable health, quality-of-life and economic implications. However, not all obese persons develop metabolic complications, and ~25% of obese adults are "metabolically normal" based on insulin sensitivity measured by using the hyperinsulinemic euglycemic clamp technique [45,46]. In addition, data from the 1994-2004 National Health and Nutrition and Examination Survey (NHANES) found that 32% of obese adults were metabolically normal, defined as having ≤1 cardiometabolic abnormality (based on blood pressure, homeostasis model assessment of insulin resistance [HOMA-IR] value, and plasma glucose, triglyceride, HDL-cholesterol, and CRP concentrations) [47].

The recognition that a subset of obese persons are resistant to the typical metabolic complications of obesity has led to several studies that have tried to characterize the distinguishing features between metabolically-abnormal obesity (MAO) and metabolically-normal obesity (MNO), also known as metabolically healthy but obese[48], uncomplicated obesity[49], and metabolically benign obesity[50]. In general, the data from these studies found MNO persons had similar percent body fat but less visceral and liver fat compared with MAO persons, and had normal insulin sensitivity, blood pressure, lipid profile, and inflammatory profile (plasma CRP concentration) [45,48-52]. Moreover, when followed for up to 11 years, MNO adults did not show a greater risk of developing diabetes or CVD than normal-weight metabolically-normal subjects [244]. In contrast, metabolically-abnormal lean or obese subjects had a 4- to 11-fold increased relative risk of diabetes than normal-weight metabolically-normal subjects [244].

1.2.2 Potential Mechanisms Responsible for Obesity-related Metabolic Abnormalities

The central hypothesis is that dysfunctional adipose tissue (increased inflammation, remodeling and adipose tissue lipolytic activity with FFA release into plasma) in conjunction with alterations in the regulation of fatty acid transport into other tissues in MAO subjects are involved in the pathogenesis of ectopic fat accumulation, systemic inflammation and insulin resistance.

1.2.2.1 Fatty Acid Metabolism

Alterations in FFA metabolism are likely a major factor in the pathogenesis of insulin resistant glucose metabolism, dyslipidemia and possibly non-infectious inflammation associated with obesity [245,246]. Excessive release of FFA from adipose tissue into plasma and increased plasma FFA concentration can impair the ability of insulin to stimulate muscle glucose uptake [247] and suppress hepatic glucose production [248]. It has been proposed that the cellular mechanism responsible for FFA-induced insulin resistance in skeletal muscle likely involves an increase in intramyocellular fatty acid metabolites, including diacylglycerol (DAG), and ceramide, which interfere with insulin action by activating protein kinase C and mTOR [249], inhibiting Akt, and ultimately preventing the translocation of GLUT-4 from the cytoplasm to the cell membrane for glucose transport [245,250-254]. In addition, activation of the nuclear factor kappa B (IκB) pathway, a major pro-inflammatory pathway [255], has also been implicated in mediating the FFA- induced insulin resistance [251]. This effect is mediated by PKC [256-259], via direct phosphorylation of IκB [260] or by increased production of reactive oxygen species (as a result of excessive intracellular fatty acid availability) which activate IκB-kinase (IKK-β) [251]. IκB activation has also been shown to impair insulin-induced glucose uptake via mTOR mediated serine phosphorylation of IRS1 [261]. Phosphorylation by IKK-β is considered the main pathway by which IκB-α is released from NFκB and subsequent movement of NFκB from the cytosol to the nucleus. Increased FFA delivery to the liver can also affect lipoprotein metabolism by increasing hepatic VLDL-TG production and plasma TG concentration [262]. An increase in plasma triglyceride (TG) concentration increases the transfer of TG from VLDL to HDL, which leads to increased high density lipoprotein clearance and decreased plasma HDL concentration [263].

Gemma Fraterrigo

1.2.2.2 Tissue Fatty Acid Transport and Ectopic Fat Accumulation, Insulin Resistance, and Inflammation

The importance of the fatty acid transporter CD36 in ectopic TG distribution has been demonstrated by studies that found that FFA uptake by the heart and skeletal muscle are decreased in CD36-deficient mice [264,265], myocardial FFA uptake is markedly impaired in men and women who have CD36 deficiency [266,267], and increased skeletal muscle plasmalemma CD36 content is associated with increased muscle FFA uptake and intramyocellular TG accumulation [268-270]. Recent data from studies conducted in rodents found CD36 is required for the development of diet-induced steatosis [264,265]. In $CD36^{-/-}$ mice and null mice rescued for liver CD36, hepatic lipid accumulation in diet-induced obesity was CD36-dependent, and increased CD36 levels enhanced hepatic FFA uptake and promoted steatosis [271]. Three transcription factors, PPARγ, RXR and LXR, which upregulate TG deposition in the liver, converge on activating the CD36 gene and steatosis induced by these agonists is abolished in $CD36^{-/-}$ mice [272]. In addition, CD36 could be directly involved in the pathogenesis of inflammation because CD36 signaling is involved in modulating lipid-induced inflammation through JNK, p-38 and IKK-β [273] and activates NFκB, which induces synthesis of several inflammatory mediators [274]. The findings observed in these animal models are likely relevant to people. There is evidence that adipose tissue CD36 expression and protein content were lower, while skeletal muscle CD36 expression was higher in subjects who had high IHTG content than in those who had normal IHTG content [275], and non-alcoholic fatty liver disease (NAFLD) in human subjects is associated with increased hepatic expression of CD36 [276]. However it is not yet known whether these associations represent a cause-and-effect relationship in people.

Lipin 1 also plays important roles in regulating TG distribution and adipocyte function. Lipin 1 is a bi-functional protein that catalyzes a key step in TG synthesis (phosphatidic acid phosphohydrolase) [277] and acts in the nucleus of the cell to directly regulate gene expression by interacting with DNA-bound transcription factors [278]. Lipin 1 is also an important regulator of systemic metabolism and insulin sensitivity. Mice lacking lipin 1 are lipodystrophic and insulin resistant [279,280] whereas overexpression of lipin 1 in liver of obese insulin resistant mice improves hepatic insulin sensitivity and suppresses VLDL secretion [281]. Lipin 1 expression in liver and adipose tissue has been shown to be inversely related with insulin sensitivity in obese people and increases after marked weight loss [282]. These findings are consistent with the metabolic functions

of lipin 1 in liver, which is to increase fatty acid oxidation and suppress VLDL secretion via transcriptional mechanisms [278,281] and in adipose tissue where it suppresses inflammatory cytokine production expression [283] and facilitates TG esterification [284]. Therefore, increased lipin 1 expression in liver and adipose tissue is associated with improved adipose tissue "function" and diminished intrahepatic TG accumulation.

1.2.2.3 Adipose Tissue-Mediated Inflammation

Adipose tissue produces more than a dozen pro-inflammatory cytokines (adipokines) that can induce insulin resistance, dyslipidemia and steatosis [285]. These adipokines are produced primarily by immune cells located within adipose tissue. Adipose tissue monocyte/macrophage content is increased in obesity, and these cells are active participants in obesity-induced inflammation which can induce metabolic disease [286,287]. In contrast, adiponectin is the most abundant protein secreted by adipocytes, and is inversely associated with increased insulin sensitivity and resistance to metabolic disease [288].

Recent studies conducted in mouse models have demonstrated that obesity is associated with dysfunction of adipose tissue CD4 T helper cells that affect responsiveness to insulin [289,290]. In obese mice, CD4 T helper cells in adipose tissue are skewed towards T helper 1 (T_H1). These cells secrete IFN-γ, which induces adipose tissue macrophages to produce inflammatory cytokines (TNF-α and IL-6), which induce insulin resistance. In contrast, in lean mice, CD4 T cells in adipose tissue of lean mice are skewed towards IL-4-secreting T_H2 cells and regulatory T cells (Treg), which counteract inflammation and protect against insulin resistance. It is not yet known whether these results observed in mice apply to humans.

There has been evidence, in pilot studies, that human adipose tissue contains both CD4 T_H1 and CD4 Tregs, and that many of the CD4 Tregs cells express CD39. CD39 is an ectonucleotidase that degrades ATP generating AMP, which can be a substrate for other nucleotidases that generate adenosine. AMP and adenosine act on P1 and P2 purinoreceptors that are expressed macrophages, inhibiting their capacity to produce inflammatory cytokines [291]. These purinoreceptors are also expressed on adipocytes and regulate sensitivity to insulin, suggesting that CD4 T regs could have a direct effect on adipocyte insulin sensitivity. It has also been found that a CD4 T cell subset in human adipose tissue from obese subjects produces IL-26, an IL-10 family cytokine that is exclusively expressed in humans. IL-26 activates STAT3

and induces the production of IL-10 in target cells. IL-26 acts on stromal cells, not on hematopoietic cells, and the receptor for IL-26 is also expressed on hepatocytes and adipocytes. Therefore, these data suggest that CD4 T cells secreting IL-26 might directly affect insulin sensitivity in stromal cells and other organs. In summary, it seems as though there is a role for CD4 T cells in regulating insulin responsiveness of human adipose tissue and indicate that CD4 T cells may act via novel mechanisms that have not yet been explored.

1.2.2.4 Adipose Tissue Remodeling

Adipose tissue remodeling is likely involved in the pathogenesis of some of the metabolic abnormalities associated with obesity [292]. The accumulation of large adipocytes is associated with an increase in the rate of adipocyte death, tissue inflammation, and the onset and progression of insulin resistance. High-fat diet induced overfeeding and weight gain in mice causes a marked increase in adipocyte size and adipocyte death [293]. Adipose tissue macrophages localize to dead adipocytes, aggregating to form crown-like structures that envelope and ingest the dying adipocyte and its lipid droplet [294]. Clearance of dead adipocytes by adipose tissue macrophages is an initial remodeling event that promotes pro-inflammatory activation of macrophages and is required for the differentiation of new adipocytes generating a bimodal distribution of small and large adipocytes. Adipose tissue MCP-1secretion recruits circulating monocytes (macrophages) that express C-C chemokine receptor (CCR)2 to infiltrate adipose tissue [286,295,296]. In humans, CCR2+ macrophages express differentiation markers EMR1, ITGAM, ITGAX and CD68, and are different from non-inflammatory resident adipose tissue macrophages. During basal conditions, mouse adipose tissue macrophages are characterized by low expression of differentiation markers whereas overfeeding causes a "phenotypic switch" in the macrophage population, as monocytes are recruited from the circulation [297,298].

1.2.3 Ectopic Fat Accumulation and Metabolically-Abnormal Obesity

Excessive ectopic fat accumulation, defined as increased intracellular TG present in "non-adipose tissues" such as liver and muscle, is associated with insulin resistance in those tissues [153,299]. It has been shown that excessive IHTG content is a robust marker of metabolic dysfunction (insulin resistance in liver, muscle and adipose tissue and increased VLDL-

triglyceride [VLDL-TG] secretion rate), independent of body mass index (BMI), percent body fat, and visceral fat mass in obese persons [226-228,300]. It has been hypothesized that ectopic fat accumulation is caused by an inadequate capacity of adipose tissue to store TG [301,302]. This notion is supported by data from studies conducted in lipodystrophic and lipoatrophic animal models and humans, which found that decreased adipose tissue mass is associated with ectopic fat accumulation in liver and muscle and with metabolic disease, particularly insulin resistance, diabetes, and dyslipidemia [282,303-306]. Moreover, expanding adipose tissue mass in animal models, by either fat transplantation or genetic manipulation, reduced ectopic fat distribution and normalized metabolic function [302,307]. However, it seems unlikely that the small amount of TG that accumulates in "ectopic" organs cannot be accommodated by the large adipose tissue mass in obese humans. For example, the amount of IHTG (average of 25% of liver volume) in the obese subjects with NAFLD represent <1% of the total TG present in adipose tissue (<0.4 kg of TG in the liver and ~40 kg of TG in adipose tissue). There is data that suggests ectopic fat accumulation involves redirecting FFA uptake and TG synthesis away from adipose tissue and toward liver and muscle [275].

1.2.4 Effect of Overfeeding on Metabolic Function in Human Subjects

There are >100 publications that evaluated the effects of overfeeding and weight gain on metabolic function in adult human subjects. More than 30 publications focused almost exclusively on energy metabolism, whereas 71 studies evaluated different aspects of substrate metabolism.[99,118,308-336,337-371] Most studies (69%) evaluated the effect of short-term overfeeding without weight stabilization (2-28 d), and many (23%) evaluated the effect of increasing only 1 (or primarily 1) macronutrient, such as carbohydrate.[315-317,325,331,339,345,347,351,362,368,370, 334,348,372] Eight studies evaluated insulin sensitivity by using the hyperinsulinemic-euglycemic technique [333,351,355,356,362,368,370,373,374] Of these 8 studies, 7 were conducted in lean subjects and 1 was conducted in "reduced-obese" subjects (10% of weight loss) [355]. Two studies evaluated the effect of overfeeding with mixed macronutrients [367] or fructose alone [368] on ectopic fat accumulation, but both studies were conducted in lean subjects, and the duration of overfeeding was 6[368] to 28[367] days. Only 6 studies included obese subjects[339,355,357,358,366,369]. Data obtained from lean subjects reported conflicting results in glucose homeostasis; overfeeding improved [312,375], did not change[333], or worsened [366,373] oral glucose tolerance or insulin sensitivity. Data from two studies

support the hypothesis that certain individuals are prone to develop adverse effects of weight gain while others are more protected [376,377]. In one study, a 5 kg weight gain, induced by overfeeding in lean men, resulted in a deterioration in arterial compliance; the decline in compliance correlated directly with the increase in visceral fat, demonstrating differences in individual responses to overfeeding.[376] In the other study, the increase in blood pressure after weight gain was smaller in subjects who had high cardiorespiratory fitness than those who had low cardiorespiratory fitness, demonstrating different responses based on baseline function.[377] In summation, these data suggest that overfeeding worsens insulin sensitivity and metabolic function in susceptible persons, and underscore the need for more studies to provide a detailed evaluation of the metabolic effects of overfeeding in high and low risk populations.

1.3 HYPOTHESIS

Despite the strong evidence that obesity is associated with multiple metabolic risk factors for cardiovascular disease, about one-third of obese adults do not have obvious metabolic abnormalities. The reason behind why weight gain and body fat accumulation causes metabolic abnormalities in some persons but not in others still remains unclear. Different studies have identified excessive intrahepatic triglyceride (IHTG) content as a robust marker of obese persons who have metabolic dysfunction (insulin resistance in liver, muscle and adipose tissue and increased VLDL-triglyceride [VLDL-TG] secretion rate), independent of body mass index (BMI), percent body fat, and visceral fat mass. On the other hand, obese persons who have normal IHTG content appear to be resistant to developing obesity-related metabolic complications. The mechanisms responsible for the accumulation of ectopic fat and the development of metabolic disease in some, but not all, obese persons are not known.

The hypothesis of this study is that the difference in susceptibility to metabolic abnormalities between the MNO and MAO will be due to differences in the response to overfeeding. We hypothesize that overfeeding will induce abnormalities in lipid metabolism (increased adipose tissue lipolytic activity, increased hepatic and muscle tissue FFA uptake, impaired hepatic fatty acid oxidation and increased hepatic lipogenesis) which will simultaneously cause ectopic fat accumulation, adipose tissue remodeling, inflammation, and multi-organ insulin resistance but that these adverse effects will be significantly greater in the susceptible MAO group. Furthermore, the hypothesis of this study is that increased adipose tissue lipolytic activity and

inflammation ("sick" adipose tissue), in conjunction with alterations in the regulation of fatty acid transport into other tissues, will result in increased release of free fatty acids (FFA) into plasma, increased FFA uptake "by non-adipose" tissues, ectopic fat accumulation, and systemic inflammation (increased circulating proinflammatory proteins, metabolites and cells), which in turn cause insulin resistance and increase cardiometabolic disease risk. More specifically, the hypothesis is that alterations in fatty acid metabolism/trafficking and adipose tissue remodeling/inflammation will be associated with ectopic fat accumulation and insulin resistance, and human adipose tissue CD4 T cells will exhibit skewed functional polarization toward a proinflammatory phenotype in MAO group, which in turn will impair insulin signaling and cause insulin resistance.

1.4 AIMS AND OBJECTIVES

About 25%-30% of obese persons do not have typical obesity-associated metabolic abnormalities and are at low risk of developing diabetes and CVD. However, the adaptive responses to overfeeding that might protect some obese persons from metabolic disease are not known. A better understanding of the differences between these unique cohorts and how they respond to an overfeeding-weight gain challenge will likely provide important insights into the mechanism responsible for obesity related metabolic disease. More studies that provide a detailed evaluation of the metabolic effects of overfeeing in high and low risk populations are necessary. The aim of this thesis is to lay the groundwork for understanding why some obese persons are resistant, while others are prone, to developing obesity-related metabolic disease, which could have considerable future research and clinical implications. Furthermore, a targeted characterization of the factors and metabolic pathways that distinguish MNO and MAO will make it possible to identify novel targets for drug therapy and new biomarkers to assess and monitor metabolic health

Therefore, the objectives of this thesis are: 1) to provide a better understanding of the metabolic mechanisms responsible for the accumulation of excessive ectopic fat (intrahepatic triglyceride [IHTG]) and metabolic dysfunction in obese persons, 2) to determine the specific metabolic adaptations that prevent the adverse metabolic effects of weight gain in obese persons who have normal IHTG content compared with those who have nonalcoholic fatty liver disease (NAFLD), 3) to determine the specific cellular and organ system metabolic and immunologic alterations

that are associated with insulin resistance and inflammation in order to identify putative mechanisms and novel biomarkers involved in the pathogenesis and progression of inflammatory and cardiometabolic diseases and to determine potential mechanisms responsible for the relationship between fatty acid metabolism and inflammation and between the immune system and metabolic dysfunction.

Specifically, to determine the effect of overfeeding an additional 1000 kcal/d for ~8-12 wks, until a moderate ~5-7% weight gain is achieved, on body triglyceride distribution and adipose tissue remodeling, multi-organ insulin sensitivity, and the mechanisms responsible for these changes in obese subjects who have either normal IHTG (metabolically-normal obesity; MNO) or high IHTG (metabolically-abnormal obesity; MAO) content, matched on age, sex, ethnicity, BMI and percent body fat was studied. Furthermore, the inter-relationships among insulin sensitivity, fatty acid metabolism, and immune system/inflammatory activity in healthy, lean subjects with normal IGHT content, MNO, BMI-matched MAO, and extremely obese subjects undergoing bariatric surgery who have a range of IHTG and from whom liver tissue can be obtained during surgery was evaluated.

CHAPTER 2 Methods

The following methods were performed to assess the metabolic effects of overfeeding in obese subjects with normal IGTG or high IHTG content. These studies were approved by the Human Research Protection Office of Washington University School of Medicine (Protocol number 10-0708). All study participants had provided written informed consent before participating in this study, which was approved by the Human Research Protection Office of the Washington University School of Medicine. All parts of this study were performed at the Clinical Research Unit, Washington University School of Medicine in St Louis.

2.1 SUBJECT SELECTION

2.1.1 Overfeeding Study Subject Selection

Subjects were recruited through the Volunteers for Health Database at Washington University School of Medicine and by local postings. After a telephone interview, prospective volunteers were required to complete 2 screening visits. Subjects were screened with a history and physical examination, routine blood tests, lipid panel, liver biochemistries,12-lead electrocardiogram, and an oral glucose tolerance test [378]. Women who were able to bear children had a pregnancy test. Subjects completed the Michigan Alcohol Screening Test [379], a Barriers Interview and the Questionnaire on Eating and Weight Patterns-Revised. Subjects were both male and female, of 18-65 yrs of age, and included all races and ethnic groups. Subjects who had active or previous history of other liver diseases, history of alcohol abuse, at the moment were consuming ≥20 g alcohol/day, or scored ≥4 points on Michigan Alcohol Screening Test, had diabetes, had severe hypertriglyceridemia (>300 mg/dL), smoked tobacco, determined to be at high risk for an eating disorder per the scoring guidelines, or were taking medication that might confound the study results were excluded. Women who were pregnant or lactating were also excluded. Subjects had to be sedentary (regular exercise <2hr/week or <2 times/week for the last 3 months) to eliminate the confounding effect of physical activity on insulin sensitivity. To minimize potential weight gain-induced health risks to the study subjects, subjects with extreme obesity (BMI ≥40 kg/m^2), intrahepatic triglyceride content (IHTG) >25%, diabetes, poorly controlled obesity co-morbidities (e.g. blood pressure >150/100 mmHg) or other serious diseases were excluded.

Between August 2010 and February 2014, a total of 71 potential subjects were screened for this

study, of which 40 were considered eligible. Subjects, men and women, were considered eligible for the study if they had a body mass index [BMI] between 30 and 40 kg/m^2 (clase I and II obesity). Then these obese subjects were divded in two groups based on their IHTG content determined by MRS into: **Group 1** (**High IHTG)** will consist of 20 subjects, both men and women, with class I and II obesity (BMI 30.0-44.9 kg/m^2) and who have > 10% IHTG content determined by MRS. **Group 2 (Normal IHTG)** will consist of 20 subjects, both men and women, with class I and II obesity (BMI 30.0-39.9 kg/m^2) who have normal IHTG content (<5%).

Thirteen subjects discontinued the study because of relocation (n=1), withdrawal of consent (n=11), and non-study related surgery (n=1); Therefore, data were collected and final analyses were performed on a total of 27 subjects.

2.1.2 Characterization of MNO and MAO Subject Selection

The study population consisted of 4 groups of subjects (of all races and ethnic groups with an equal number of men and women in each group), who were 18-65 years old: 1)9 lean subjects (IHTG content ≤5%; BMI 18.5-24.9 kg/m^2); 2) 12 obese subjects with normal IHTG content (MNO; IHTG content ≤5%; BMI 30.0-44.9 kg/m^2); 3) 13 BMI- and sex- matched obese subjects with increased IHTG content (MAO; IHTG content ≥10%; BMI 30.0-44.9 kg/m^2), and 4) 19 extremely obese subjects (BMI 35.0-55.0 kg/m^2) undergoing bariatric surgery, who had a range of IHTG content and from whom liver biopsies was obtained during surgery (based on our previous experience, we anticipated that IHTG will range from normal to up to 50% of liver volume, and believed that 24 subjects in this group would allow us to study subjects with a robust range in IHTG, including subjects with normal IHTG content.

Participants were recruited by reviewing our database of research subjects containing thousands of lean and obese research study volunteers, by local postings, and by attending the bariatric surgery clinic. After a personal or telephone interview, prospective volunteers were screened with a history and physical examination, routine blood tests, lipid panel, liver biochemistries, and an oral glucose tolerance test [378]. Women who are able to bear children got a pregnancy test. Subjects completed the Michigan Alcohol Screening Test [379]. Subjects who had active or previous history of other liver diseases, history of alcohol abuse, currently consuming ≥20 g

alcohol/day, diabetes, severe hypertriglyceridemia (>300 mg/dL), smoke tobacco, or take medication that might confound the study results were excluded. Women who were pregnant or lactating were also excluded. Subjects had to be considered somewhat sedentary (regular exercise <2 h/week for the last 2 months) to eliminate the confounding factor of activity on insulin sensitivity. Data collected on subjects as part of a separate protocol, which shares with this protocol the same inclusion/exclusion criteria, may be combined with data from this study for final analyses.

2.2 OVERFEEDING STUDY PROTOCOL

The specific aims will be evaluated by obtaining outcome measures before and after 8-12 wks of overfeeding, after subjects have gained ~5-7% body weight and have been weight stable (<2% change) for 3 weeks. An overview of the study protocol is shown in Table 2.1 and Figure 2.1.

All baseline body composition analyses, metabolic studies and other studies (leasted below) were repeated at weeks ~17 -19, after ~8-12 wks of high-calorie diet feeding, after the subjects had been weight stable (<2% change) for 3 weeks.

TABLE 2.1. Overview of the Study Protocol

Week	Research Procedures
Weeks 0-2:	Medical screening and meeting with dietitian for food diary instructions
	Body composition analyses and return food diary
Week 3:	Admit to inpatient Clinical Research Unit (CRU)
	Euglycemic-hyperinsulinemic clamp procedure with adipose and muscle tissue biopsies
	Individual session with research dietitian and behavioral psychologist regarding diet plan
Week 4:	Admit to inpatient Clinical Research Unit
	Metabolic studies: VLDL kinetics, and *de novo* lipogenesis.

Resting Energy Expenditure

Begin high-calorie diet (1000 kcal/d overfeeding above estimated total daily energy expenditure) at discharge

Weeks 5-16: Weekly blood tests, medical evaluation, body weight and individual diet session with research dietitian to ensure compliance with 1000 kcal/d overfeeding.

Maintain weight stability at 5-7% weight gain for 3 weeks: Repeat body composition analyses once weight stability achieved

Week 17 Admit to inpatient Clinical Research Unit

Repeat euglycemic-hyperinsulinemic clamp procedure with adipose and muscle tissue biopsies

Week 18 Admit to inpatient Clinical Research Unit

Repeat metabolic studies: VLDL kinetics, and *de novo* lipogenesis.

Repeat Resting Energy Expenditure

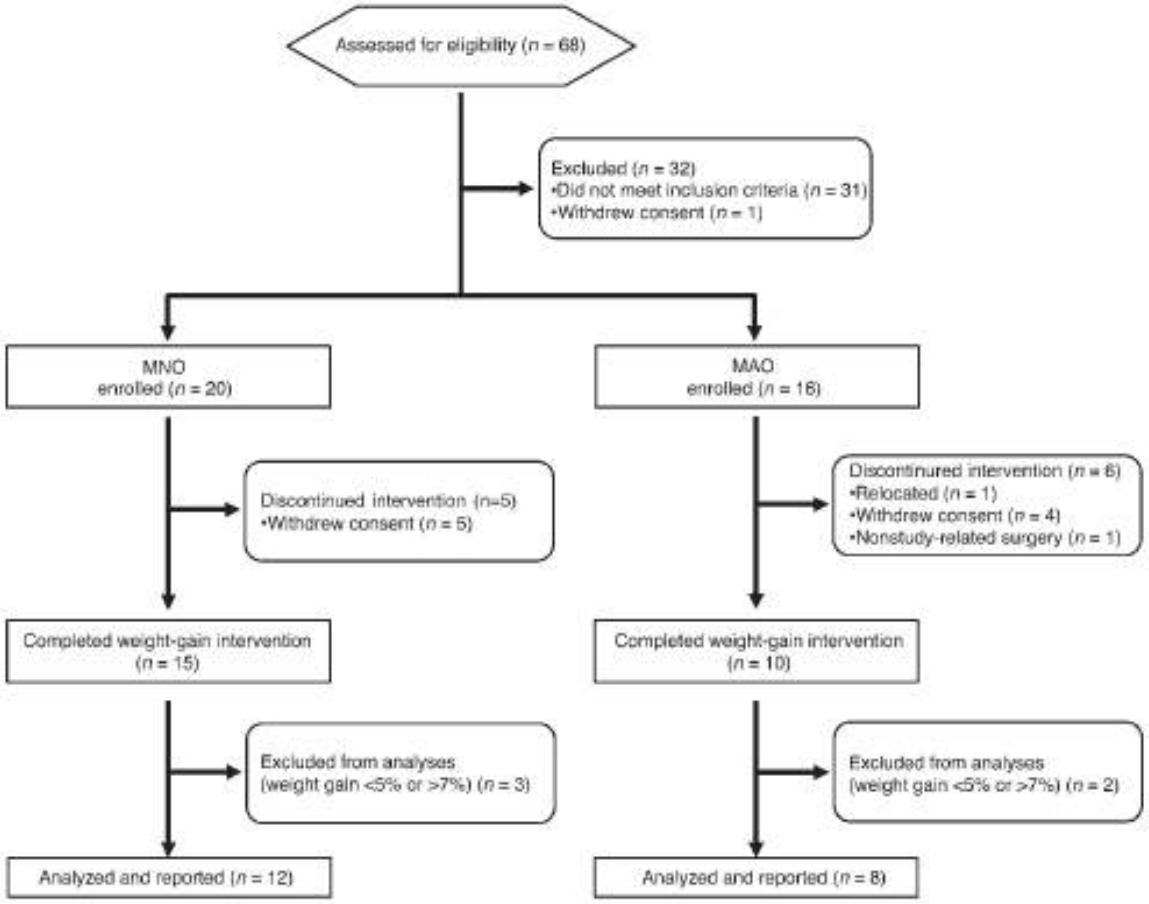

FIGURE 2.1 Study Flowchart

2.3 BODY COMPOSITION ANALYSES

2.3.1 Determination of Total Body Fat and Fat-Free Mass

Total body fat (FM) and fat-free mass (FFM) were determined by using dual energy x-ray absorptiometry (DXA) (Lunar iDXA, GE Healthcare), which permits reliable evaluations in subjects who weigh up to 425 lbs (Figure 2.2).

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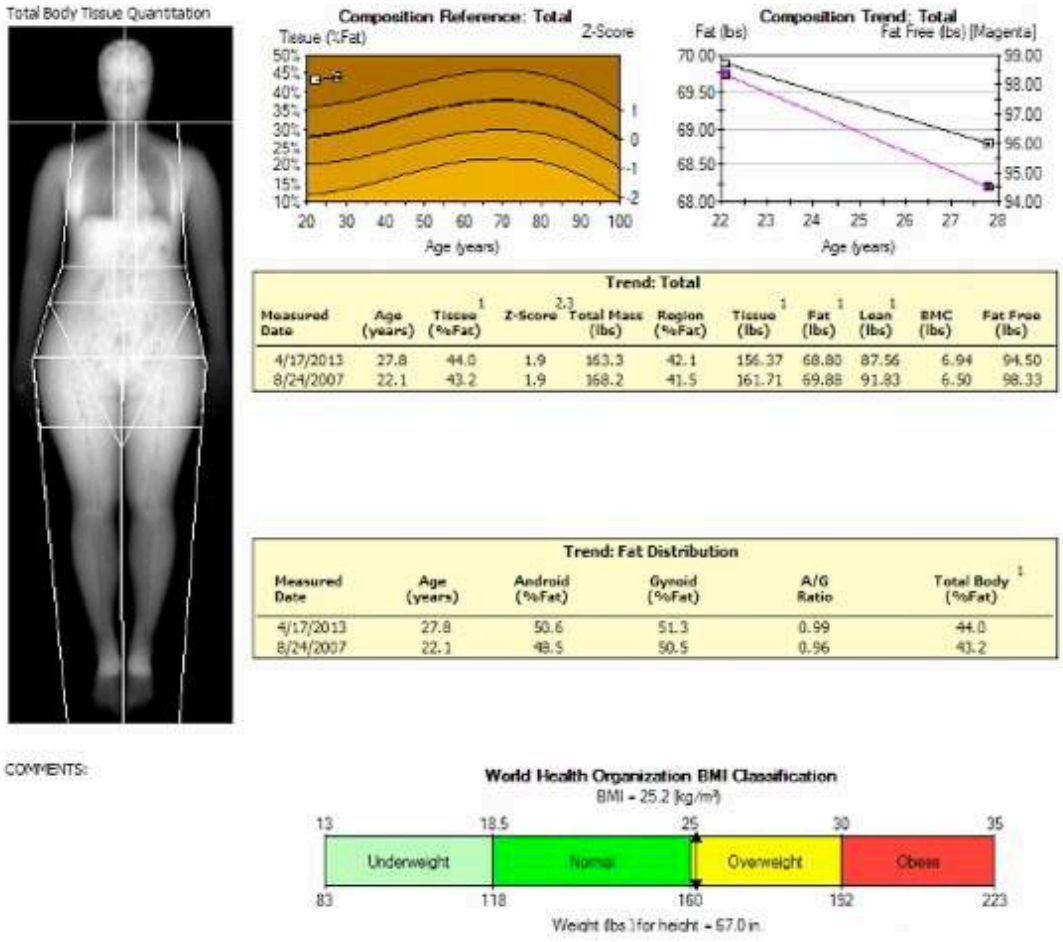

FIGURE 2.2 DXA Scan for Body Composition

2.3.2 Determination of Visceral and Subcutaneous Abdominal Adipose Tissue Volumes

Visceral adipose tissue (VAT) and subcutaneous abdominal adipose tissue (SAT) volumes were quantified by using magnetic resonance imaging, (Siemens, Iselin, NJ; ANALYZE 7.0 software, Mayo Foundation, Rochester, MN); 8, 10-mm-thick slice images, obtained at the L4-L5 interspace and proximally, were analyzed for visceral abdominal and subcutaneous abdominal fat content [226,300] (Figure 2.3).

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FIGURE 2.3 MRI Machine

2.3.3. Determination of Intrahepatic Triglyceride Content

Intrahepatic TG content was determined by using proton magnetic resonance spectroscopy (MRS) (3T whole-body system; Magneton Vision Scanner; Siemens, Erlanger, Germany) [380] . Three 2 x 2 x 2 cm^3 voxels were examined in each subject, and the values were averaged to determine IHTG content. The coefficient of variation of replicate values of the triplicate determinations for 3 voxels was 1.5%. Hepatic fibrosis was determined by using magnetic resonance elastography (MRE).

2.3.3.1 Basic Principles of the Determination of IHTG

Both proton MRS and MRI were performed with the same instrument and involved the use of a strong magnet and nonionizing radio frequency waves to acquire data. The information obtained from these 2 techniques are different, however, MRI provides tissue structural information, whereas MRS provides quantitative information on the biochemical profile (metabolites) within tissues. MRS is based on the influence of the cellular chemical environment on the local magnetic

Gemma Fraterrigo

field experienced by nuclear protons. Protons in different chemical environments oscillate at different frequencies, known as the resonance frequency. Therefore, chemically different atoms, for example the ^{1}H in water and fat, and morphologically different atoms, such as the ^{1}H in intramyocellular and extramyocellular fat, can be distinguished by their resonance frequencies. The change in resonance frequency is called the chemical shift and is used to distinguish between different metabolites. For imaging, Fourier transformation was used to deconvolute the frequency data into spatial intensities. For spectroscopy, Fourier transformation was used to deconvolute multiple frequencies (chemical shifts) into their individual resonances and generate spectra that provide information on the chemical nature and intensity (amount) of individual components. The nuclear magnetic resonance spectrum is made of peaks, generated by the tissue and appropriate standards, plotted against frequency. The frequency axis is usually reperesented in parts per million (ppm), which allows the same ppm values to be obtained for the same metabolite resonance, independent of the magnetic field strength used to generate the data. The area under the peak represents the total number of protons, but it is also influenced by other factors, such as relaxation time of metabolites and acquisition parameters. The effect of these other factors can be determined, however, so quantitative concentrations of tissue metabolites can be obtained.

The low concentration of intracellular metabolites results in a low signal to noise ratio and affects the precision of MRS measurements. This is the reason why multiple repetitive scans are usually obtained, and the data from each scan are averaged together. A relaxation delay is necessary between each scan so that the nuclei can return to their equilibrium state. The return to equilibrium, or longitudinal relaxation, is characterized by a first order rate constant, T_1. The value for T_1 is approximately 1 second for in vivo ^{1}H within water and fat when using a 1.5T magnet and increases with increasing field strength. Since nuclei in different environments have longitudinal relaxation rates, the relaxation delay must be long enough to achieve adequate relaxation for all species. Inadequate relaxation introduces a bias that decreases experimental accuracy. Transverse relaxation reflects the decoherence or decay of magnetization and is characterized by a first order rate constant, T_2. During the localization process, which is needed to ensure that signals are originating only from the region of choice, transverse relaxation can occur and must be accounted for by using a correction, because different species have different rates. Typically, T_2 if 40 milliseconds (ms) and 80 ms for in vivo water and fat, respectively.

2.3.3.2 Technical Application of Determination of IHTG

Localized hepatic MRS to determine IHTG content requires initial scout images to determine the appropriate voxel iste (ie, region of interest) and ensure the biliary tree, adipose tissue, and blood vessels are avoided because these tissues would lead to unaccurate results. In our studies, 3 voxels were positioned in the right lobe of the liver (Figure 2.4). Although IHTG content is often homogeneous throughout the right lobe of the live, we averaged the values from 3 voxels to help correct for any potential regional differences in IHTG content.

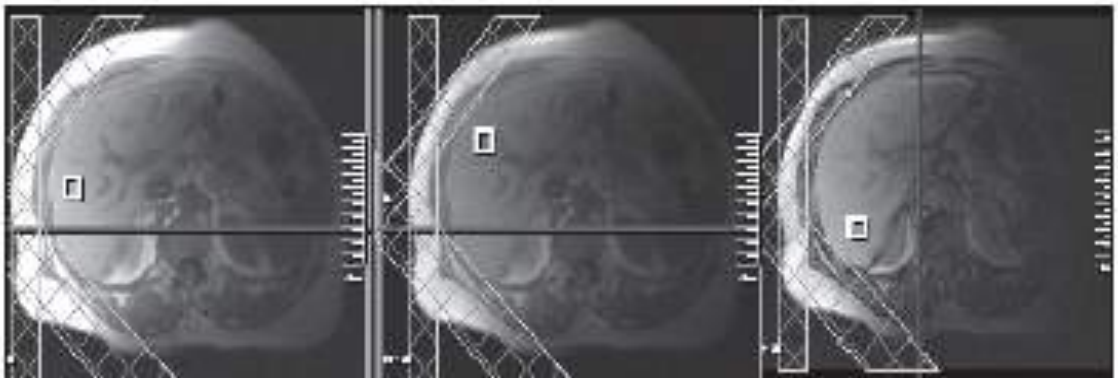

FIGURE 2.4 Scout images were obtained by magnetic resonance imaging, indicating the 3 voxel positions used to measure intrahepatic triglyceride content.

The NMR spectra obtained from a study subject of shown in Figure 2.5. A voxel located at each position identified in Figure 2.4 was evaluated by using a Siemens 3T whole-body imaging system (MAGNETOM Trio; Siemens, Erlangen, Germany). The strength of the external magnetic field influences resonance separation. Therefore, increasing the magnetic field can sometimes provide a more reliable assessment of individual NMR peaks. For eample, the H_2O and fat resonances shown in Figure 2.5 are chemically shifted by approximately 350 Hz or 3.5 ppm from each other. The separation of these 2 resonances would be reduced to 175 Hz if a 1.5T system had been used but would still have been adequate to quantify the areas of both peaks.

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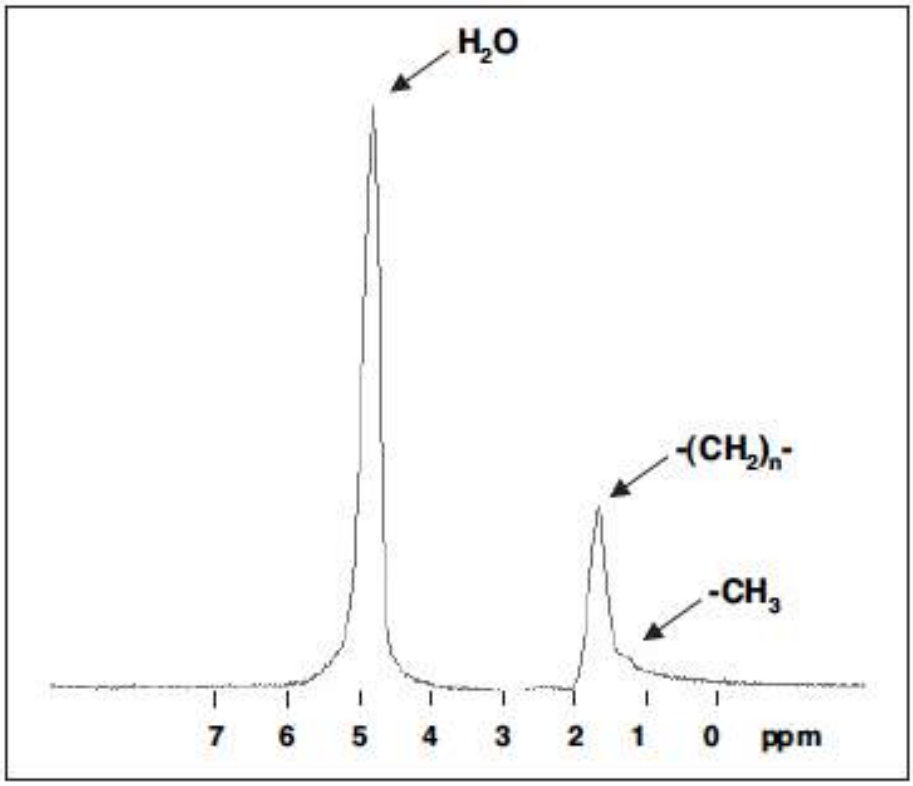

FIGURE 2.5 ^{1}H-magnetic resonance spectra in an obese man. Percent intrahepatic triglyceride content is calculated as the ratio of the area under the resonance peak for methylene groups in fatty acid chains of intrahepatic triglycerides (CH_2n) and the combined area under the resonance peaks for both CH_2n and H_2O. Intrahepatic triglyceride content in this subject was 43%. ppm = parts per million.

Assessment of IHTG content by using MRS usually took <30 minutes. Obtaining reliable data required that subjects were able to remain supine for the durating of the procedure, that subjects were able to respond to simple verbal commands, and that the subjects were able to hold their breath for 20 seconds during different times of the scanning procedure.

2.4 METABOLIC STUDIES

All metabolic studies were performed in the Clinical Research Unit (CRU). Subjects were asked to avoid caffeine and alcohol for at least 24 h before admission. Subjects were also instructed to abstain from any exercise for at least 3 days before each admission and were required to keep a complete 3-day food log to judge compliance with their weight stability diet. If their diet was judged to be inappropriate (e.g. inadequate in carbohydrate or energy intake) the study was

Gemma Fraterrigo

rescheduled and further diet instructions were given. At 1700 h on the day of admission to the CRU, subjects consumed a dinner meal that contained one-third of the calories based on 12 calories per kg of fat free mass determined by DXA. This meal was consumed by 1900 h. After this meal, subjects fasted, except for water, until completing the metabolic study the next day.

2.4.1 Two-Stage Euglycemic-Hyperinsulinemic Clamp Procedure to Assess Insulin Action and Fatty Acid Oxidation

At 0500 h, after subjects had fasted overnight, a catheter was inserted into a radial artery to obtain arterial blood samples and into an antecubital vein to infuse isotope tracers, dextrose and insulin. If an arterial line could not be placed, an intravenous line was placed in the hand. A warming box was then used for hand vein blood draws. At 0600 h a two-stage hyperinsulinemic euglycemic clamp procedure with stable-isotopically labeled tracer was initiated to determine hepatic (suppression of glucose production), skeletal muscle (stimulation of glucose disposal), and adipose tissue (suppression of lipolysis) insulin sensitivity, and hepatic fatty acid oxidation. After baseline blood samples were obtained, a primed-constant infusion of [6,6-$^{2}H_2$]glucose (22 μmol/kg prime and 0.22 μmol·kg^{-1}·min^{-1} constant infusion) was started at t=0 min and continued throughout the clamp procedure. At t=0 a constant infusion of [2,4-$^{13}C_2$]-hydroxybutyrate (0.03 μmol/kg/min) was also started and continued for 3.5 h. After 90 min (0730 h), a constant infusion of [U-^{13}C]palmitate (6 nmol·kg FFM^{-1}·min^{-1}) was started to determine the rate of appearance (Ra) of plasma palmitate . At 930 h, after the basal period was completed, a two-stage euglycemic, hyperinsulinemic pancreatic clamp was initiated and continued for 6 h. Euglycemia was achieved by a variable rate infusion of 20% dextrose enriched to approximately 2.5% with [6,6-$^{2}H_2$]glucose to minimize changes in glucose isotopic enrichment (adding tracer to the dextrose infusion provides more accurate measures of glucose Ra by minimizing changes in plasma glucose enrichment [381]). During stage 1 of the clamp (3.5 h to 6.5 h of isotope infusion), insulin was infused at a rate of 7 mU·m^2·min^{-1} for 3 h (initiated with a two-step priming dose of 28 mU·m^2·min^{-1} for 5 min followed by 14 mU·m^2·min^{-1} for 5 min). During stage 2 of the clamp (6.5 h to 9.5 h of the isotope infusion), insulin was infused at a rate of 50 mU·m^2·min^{-1} for 3 h (initiated with a two-step priming dose of 200 mU·m^2·min^{-1} for 5 min followed by 100

Gemma Fraterrigo

$mU \cdot m^2 \cdot min^{-1}$ for 5 min). The plasma insulin concentrations achieved with these insulin infusion rates provided an optimal range for evaluating insulin's effect on adipose tissue lipolysis (low-dose insulin infusion), hepatic glucose production (low-dose insulin infusion) and glucose uptake by skeletal muscle (high-dose insulin infusion). The infusion of [$^2H_2$]glucose and [U-^{13}C]palmitate was decreased by 50% of basal during stage 1 and by 75% during stage 2 because of the expected decreases in hepatic glucose production and lipolytic rate. Plasma samples were taken before beginning the isotope infusion to obtain baseline measurements of substrate enrichment. Plasma samples were taken every 10 min during the last 30 min of the basal period and each stage of the insulin clamp to determine glucose and FFA concentrations and kinetics, and plasma insulin concentrations. Plasma samples were obtained every 10 min at the end of the basal period and throughout the entire clamp period to monitor plasma glucose concentration.

2.4.2 Stable Isotope Tracer Studies to Assess VLDL Kinetics and Hepatic DNL

This study was performed on a separate day, at least a week from the two-stage euglycemic-hyperinsulinemic clamp procedure following the same preparation necessary for a metabolic study as already explained above.

At 0530 h, a catheter was inserted into a hand vein to obtain blood samples and into an antecubital vein of the contralateral arm to infuse isotope tracers. If a hand vein could not be accessed, an arterial line was placed. At 0600 h: 1) an intravenous bolus of [$^2H_5$]glycerol (75 µmol/kg) was given to assess VLDL-TG kinetics, 2) a 12-h constant infusion of [U-^{13}C]palmitate (6 nmol·kg FFM^{-1}·min^{-1}) was started to determine the proportion of fatty acids within VLDL-TG derived from systemic plasma FFA (derived primarily from lipolysis of subcutaneous adipose tissue TG) and non-systemic fatty acids (derived from lipolysis of intrahepatic and intraperitoneal TG, hepatic lipolysis of circulating TG, and *de novo* hepatic fatty acid synthesis) by accounting for isotopic dilution between plasma and VLDL-TG palmitate using a multi-compartmental model, 3) a 12-h primed (4.2 µmol/kg) constant (0.06 µmol·kg^{-1}·min^{-1}) infusion of [$^2H_3$]leucine was started to determine VLDL-apolipoprotein B kinetics, 4) a 12-h constant infusion of [1,2-$^{13}C_2$]acetate (2 µmol/kg/min) was started to quantify DNL in VLDL-TG. Blood samples were taken before the isotope infusion to determine baseline

substrate concentrations and background isotopic enrichments and at 5, 15, 30 min and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10 and 12 h during isotope infusion to determine leucine enrichment in VLDL-apoB, glycerol and palmitate enrichment in VLDL-TG, glycerol enrichment in plasma, and plasma FFA concentrations. Although this is a long infusion protocol (12 h infusion), it was well-tolerated and was necessary to achieve sufficient curvature and approach toward plateau enrichment of VLDL-apoB and VLDL-TG palmitate to assure reliable modeling of kinetic parameters in obese subjects.

2.4.3 Fatty Acid Oxidation Study

Subjects were admitted to the Clinical Research Unit at Washington University School of Medicine on the evening before the fatty acid oxidation study. At 1900 h subjects were served a standard meal and then fasted until study completion the next day. At 0600 h the following morning, a constant infusion (0.03 μmol/kg /min) of [2,4-$^{13}C_2$]β-hydroxybutyrate (Cambridge Isotope Laboratories, Andover, MA) was started and continued for 3.5 hours. Blood samples were collected before beginning the tracer infusion to determine glucose, insulin, plasma cholesterol and triglyceride, and hydroxybutyrate concentrations, and background plasma hydroxybutyrate tracer-to-tracee-ratio (TTR), and every 10 min during the final 30 min of the infusion protocol, to determine hydroxybutyrate kinetics.

2.5 RESTING ENERGY EXPENDITURE

Resting energy expenditure was measured during the VLDL metabolic study. Oxygen consumption and carbon dioxide production were measured by indirect calorimetry (metabolic cart and a ventilated hood system; DeltaTrac, SensorMedics, Yorba Linda, CA) (Figure 2.6) for 30 min, but only the last 20 min will be used for calculations.

Gemma Fraterrigo

FIGURE 2.6 Metabolic Cart and Ventilated Hood System

2.6 ADIPOSE TISSUE AND MUSCLE TISSUE BIOPSIES

Tissue samples were obtained from subcutaneous abdominal and femoral adipose tissue and the *vastus lateralis* muscle during the euglycemic-hyperinsulinemic clamp procedure. They were obtained 60 min after starting the glucose tracer infusion (basal stage of the clamp procedure). Muscle and fat biopsies were also obtained 60 min after starting stage 2 of the clamp procedure to determine the effect of insulin infusion on insulin signaling and adipocyte markers. The biopsy sites were cleaned and draped, and tissues were obtained under sterile conditions. After anesthetizing the skin and underlying tissues with lidocaine, ~1 g of adipose tissue was obtained from the periumbilical area by aspiration through a liposuction cannula, ~200 mg of muscle and ~1 g of femoral adipose tissue was obtained by using a 6 mm Bergstrom needle inserted through a small (0.5 cm) skin incision. All tissue samples were immediately and gently rinsed with ice-

Gemma Fraterrigo

cold saline. Adipose tissue samples were divided into 3 aliquots: 1) immediately frozen in liquid nitrogen for subsequent determination of cell factors and quantitative PCR; 2) placed in osmium tetraoxide to assess adipocyte size and number; and 3) placed in formalin for subsequent microscopy and immunohistochemistry analyses to determine macrophage content and characteristics and adipose tissue remodeling (antibodies for myeloid content and characteristics: CD68, CD11c, CD11b, CD14, CD16, CD1a, CD123, BDCA-2, MHC class II, Scavenger Receptors type I and type II, CD163, DC-SIGN, Mannose receptor, Langerin and DC-Lamp; antibodies for adipocyte differentiation stage and for differentiate dead from viable adipocytes: perilipin 1 and perilipin2).[294,382-389] Muscle samples were divided into three aliquots. Each aliquot was immediately frozen in liquid nitrogen and stored at –80°C. These tissues were used to evaluate: 1) cellular factors involved in insulin action (muscle: GLUT 4, phosphorylation of IRS-1 and Akt, and JNK, diacylglycerol and ceramide content; adipose tissue: GLUT 4, phosphorylation of IRS-1 and Akt, markers of ER stress [Grp78 and spliced XBP-1 expression, and phosphorylated eIF2α], and adiponectin expression); 2) adipocyte differentiation/proliferation (adipocyte size and number, and bimodal distribution of cell size); 3) markers of inflammation (adipose tissue macrophage characteristics and gene expression of IL-6 and TNFα); and 4) factors that regulate fatty acid trafficking and ectopic fat accumulation (muscle and adipose tissue CD36 and FAS expression and content, and LPL activity).

2.7 LIVER BIOPSIES AND VISCERAL FAT IN BARIATRIC SURGERY SUBJECTS

Liver tissue, visceral fat and subcutaneous fat were obtained from subjects undergoing roux-en-Y gastric bypass, laparoscopic adjustable gastric banding, or sleeve gastrectomy surgery for obesity. Liver samples were obtained by needle biopsy and visceral fat and subcutaneous fat were obtained by surgical excision at the beginning of the surgical procedure, before gastric stapling and intestinal resection or banding was performed. Two pieces of liver tissue were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Another piece of tissue was placed in formalin for subsequent histological and immuno-histochemical analyses. Adipose tissue samples were divided into 3 aliquots: 1) immediately frozen in liquid nitrogen for subsequent determination of cell factors and quantitative PCR; 2) placed in osmium tetraoxide to assess adipocyte size and number; and 3) placed in PBS for immunological analyses.

2.8 DIETARY INTERVENTION

After baseline metabolic studies were completed, subjects began an 8-12wk high-calorie diet intervention. The recommended dietary energy intake was 1000 kcal a day more than the estimated baseline total energy requirements (calculated as 1.25 times the measured REE), but maintain the same relative macronutrient composition as their baseline diet, until they gained ~6% (acceptable range of 5% to 7%) of their initial body weight. This additional food intake was achieved by having subjects eat specific menu choices from among five fast-food restaurant chains (Burger King, Kentucky Fried Chicken, McDonald's, Pizza Hut, and Taco Bell). An individualized diet plan was developed for each subject by the study dietitian based on estimated energy requirements, and the subject's food preferences and dietary habits. The cost of the additional calories from the fast food restaurants was covered by the study via checks made out to the participants. The macronutrients of the additional fast food calories provided approximately 45% calories as carbohydrate, 15% as protein and 40% as fat. The use of commercially-available liquid and shakes and meals from fast food restaurants helped provide a structured meal plan and made it easier for subjects to maintain accurate dietary records. Participants were required to keep daily records of food intake for the entire study to reinforce continued self-monitoring. Dietary compliance was judged based on food records and an expected 1% increase in bodyweight every 1-2 weeks. Energy intake was modified as needed to achieve the targeted weight gain. Four consecutive days of food records were collected, validated and analyzed at baseline and at ~4 weeks after starting the high-calorie diet to assess total daily energy intake and dietary macronutrient composition.

During the intervention, subjects were seen weekly by the study research coordinator and dietitian to: 1) provide a medical evaluation (history and physical examination), 2) obtain body weight by using a calibrated scale, 3) review any problems, 4) review compliance, and 5) check plasma liver biochemistries, fasting glucose and electrolytes. The investigators and the research nurse reviewed laboratory tests every week. If values for plasma alanine aminotransferase (ALT) or aspartate aminotransferase (AST) increased by more than 100% above baseline or were more than twice the upper limit of normal, the subject was seen by a physician. If necessary, the subject's medical issues were discussed with the other members of the DSMB and a decision was made regarding stopping the high-calorie diet. The overfeeding protocol was *stopped* in

subjects who developed an increase in ALT or AST >2.5 times the upper limit of normal, or any other changes in weekly laboratory tests or clinical evaluation that the DSMB or physician considered dangerous.

After subjects achieved the targeted ~6% gain in body weight, they were kept weight-stable for >2 weeks before repeating the same procedures conducted at baseline. To help maintain weight stability, subjects were instructed to reduce their energy intake by ~200 kcal/day. At each weekly visit, the subject was weighed and dietary recommendations were adjusted as needed to keep weight stable.

2.9 WEIGHT LOSS AFTER STUDY COMPLETION

After subjects completed the 8-12 wk high-calorie diet intervention and repeated the metabolic studies, we provided them with a 6-month weight loss program to lose all weight gained by the high-calorie diet and an additional 3%-5% of body weight loss to promote health, supervised by the study dietitian and behavioral therapist. There was no cost to the subjects for this program, with considerable experience in achieving successful diet-induced weight loss in study subjects[237,240,390-403]. This weight loss program included dietary and behavioral education topics, provided in individual weekly sessions for the first month, and monthly sessions for the remaining 5 months. Each session was led by our study dietitian and lasted ~60 min. The behavioral program used cognitive-behavioral techniques, emphasized strategies of self-monitoring and goal-setting, and included problem-solving, overcoming high-risk situations for unhealthy eating, relapse prevention, and strategies for long-term weight maintenance. Handouts were provided for study subjects to allow them to record the setting and reaching of dietary goals, as well as summarized the key points of the educational content. The dietary intervention provided information on energy content of foods, eating at restaurants and parties, holiday eating, portion sizes, making healthy choices while food shopping or dining out, reading food labels, healthy meal patterning, and modifying recipes. A structured meal plan was emphasized. The nutrition intervention promoted a diet consistent with an expert panel, convened by the National Heart, Lung, and Blood Institute.[10] Participants were encouraged to keep daily records of food intake during the entire treatment program to reinforce continued self-monitoring. Food records were reviewed by the program dietitian, who provided written feedback. If the participants were not able to lose the weight gained by the high calorie diet within the first 6 months after the post

metabolic studies, they were offered a choice of 1) another 6 months in the previously mentioned weight loss program or 2) enrolling in the Washington University Weight Management Program for 6 months at a reduced cost.

All participants had a CMP and Lipid panel drawn once during the weight loss phase for safety monitoring. Selected participants had an MRS performed during the weight loss phase.

2.10 ANALYSIS OF SAMPLES AND CALCULATIONS

2.10.1 VLDL-TG and VLDL-apoB Kinetics

Lipoprotein fractions (VLDL, IDL, and LDL) were isolated by sequential ultracentrifugation within 12 h of blood collection. VLDL was separated from plasma by density gradient ultracentrifugation [240] . Briefly, 2 ml of plasma were transferred into Opti Seal tubes (Beckman Instruments, Palo Alto, CA), overlaid with a NaCl-EDTA solution (1.006 kg/l), and centrifuged in a 50.4 Ti rotor (Beckman Instruments) at 100,000 *g* for 16 h at 8°C. The top layer, containing VLDL, was removed by tube slicing (Beckman Instruments). The density of the remaining sample was adjusted to 1.019 kg/l by use of a NaCl-KBr solution (1.3115 kg/l), and the sample was recentrifuged for the isolation of IDL. To separate LDL, the density of the remaining sample was adjusted to 1.063 kg/l and recentri- fuged, and the top layer that contained LDL was collected. The exact amounts of the fractions containing VLDL, IDL, and LDL recovered by tube slicing (~ 1.3 ml) were recorded to calculate TG and apoB-100 concentrations. The isolated lipoprotein fractions of each sample were stored at -70°C until final analyses were performed.

Glycerol, palmitate and leucine TTRs in plasma and in VLDL-TG or VLDL-apoB100 were determined by using gas chromatography-mass spectrometry [4,226]. The m+0 through m+4 isotopomers of VLDL-TG palmitate were measured with corrections for concentration-dependency of methyl palmitate GC/MS [404] and spectral overlap [405] to assess de novo lipogenesis, and the m+16 isotopomer was measured to assess the contribution of plasma palmitate to VLDL-TG production.

Total plasma apoB100 concentration was measured by using an immunoturbidimetric kit (Wako Chemicals). VLDL was isolated, processed, and analyzed, and VLDL-TG and VLDL-apoB kinetics were determined by using compartmental modeling [226,240,406].

Gemma Fraterrigo

The fractional turnover rate (FTR) of VLDL-TG and VLDL-apoB100 (in pools/h) was determined by fitting the TTR of glycerol and leucine in plasma and in VLDL-TG and -apoB100 to a multicompartmental model, as previously described [240]. The rate of VLDL-TG (in μmol/min) and -apoB100 (in nmol/min) secretion into plasma was calculated by multiplying the FTR of VLDL-TG and -apoB100 (in pools/min) by the steady-state concentration of VLDL-TG (mmol/L) and -apoB100 (in nmol/L), respectively. The proportion of fatty acids within VLDL-TG derived from systemic plasma FFA (generated by lipolysis of subcutaneous adipose tissue triglyceride) and non-systemic fatty acids (generated by lipolysis of intrahepatic and intraperitoneal triglyceride, hepatic lipolysis of circulating triglyceride, and/or de novo hepatic fatty acid synthesis) was calculated by accounting for isotopic dilution between plasma and VLDL-TG palmitate by using a multi-compartmental model [226]. The proportion of fatty acids within VLDL-TG derived from de novo synthesis of fatty acids was calculated by mass isotopomer distribution analyses [407] based on the incorporation of one or two labeled $^{13}C_2$-acetates into VLDL-TG palmitate (m+2 and m+4 isotopomers, respectively). Plasma VLDL-TG and -apoB100 clearance (in mL/min) was calculated by multiplying the FTR of VLDL-TG and -apoB100 (in pools/h) by plasma volume (in L), divided by 60.

2.10.1.1 Calculations

The fractional catabolic rate (FCR) of VLDL-TG (in pools/h), which represents the fraction of the VLDL-TG pool that leaves the pool per unit of time, was calculated by fitting the glycerol TTR in plasma and in VLDL-TG to a multicompartmental model [406] as described below. During steady-state conditions, the VLDL-TG FCR is equal to the VLDL-TG fractional secretion rate. The absolute rate of VLDL-TG secretion (equal to the absolute rate of VLDL-TG catabolism) was calculated as 1) total secretion rate, which represents the total amount of VLDL-TG produced by the liver, normalized to FFM; and 2) secretion per unit of plasma, which represents the rate of release of VLDL-TG from the liver into the bloodstream, as follows

VLDL-TG secretion rate (in μmol • kgFFM-1 • min^{-1})

= [(VLDL-TG FCR/60) x $C_{VLDL-TG}$ x PV]/FFM

VLDL-TG secretion into plasma (in μmol • 1 $plasma^{-1}$ • min^{-1})

Gemma Fraterrigo

= (VLDL-TG FCR/60) x $C_{VLDL\text{-}TG}$

where $C_{VLDL\text{-}TG}$ is the concentration of VLDL-TG in plasma, and PV is plasma volume, which was estimated on the basis of each subject's FFM (PV = 0.055 liter x kgFFM) [408,409]. It was assumed that PV was equal to the VLDL-TG volume of distribution, because VLDL is restricted to the plasma compartment and does not enter the interstitial space of the lymphatic system [410].

The relative contribution of systemic plasma FFA to VLDL-TG-bound fatty acids was calculated by fitting the palmitate TTR in plasma and VLDL-TG to a multicompartmental model [406] to determine the fraction of VLDL-TG-bound palmitate that is derived from systemic plasma palmitate. The contributions of systemic plasma fatty acids ($VLDL\text{-}TG_{PFA}$) and nonsystemic plasma fatty acids ($VLDL\text{-}TG_{NPFA}$) to VLDL-TG secretion were calculated as follows

$VLDL\text{-}TG_{PFA}$ = VLDL-TG secretion x fraction of VLDL-TG derived from systemic plasma palmitate

$VLDL\text{-}TG_{NPFA}$ = VLDL-TG secretion – $VLDL\text{-}TG_{PFA}$

The systemic plasma fatty acid pool includes fatty acids from the systemic circulation that are taken up by the liver and directly incorporated into VLDL-TG or temporarily incorporated into rapidly turning over intrahepatic and intraperitoneal TG stores before incorporation into VLDL-TG. The nonsystemic fatty acid pool includes fatty acids derived from 1) preexisting lipid stores in the liver and intraperitoneal fat depots, 2) hepatic uptake and lipolysis of plasma lipoproteins, and 3) hepatic de novo lipogenesis.

VLDL-TG clearance from plasma (ml/min) was calculated by dividing the rate of VLDL-TG disappearance from plasma (VLDL-TG catabolic rate in μmol/min) by the plasma VLDL-TG concentration (in μmol/ml).

2.10.1.2 Muticompartmental Model

To provide a more comprehensive analysis of VLDL-TG tracer kinetics, a multicompartmental model based on the model developed by Zech et al. (8) was used (Figure 2.7) for VLDL-TG following a bolus of [2-^{3}H]glycerol. The time course of plasma glycerol or palmitate TTR was described as a "forcing function", defined as a linear interpolation between observed time points.

This time course accounted for systemic recycling of glycerol and palmitate tracers between plasma and peripheral tissues. As proposed by Zech et al. (8), there are two biosynthetic pathways between plasma glycerol or palmitate and VLDL-TG. The "fast" pathway comprises a time delay (typically 0.5 h) to account for TG synthesis, VLDL packaging, and secretion. A "slow" pathway represents a pool of tracer (presumably labeled hepatic glycerolipids) that turn over at a rate of approximately 0.3 pools/h (adjustable parameter), which provides a source of non-systemic tracer recycling. Inclusion of the "slow" pathway was necessary to fit the terminal tail of VLDL-TG enrichment when bolus tracers were used. A single compartment was adequate to describe VLDL-TG tracer kinetics, and VLDL-TG kinetic heterogeneity was not evident in any of our subjects. The fractional catabolic rate (FCR) or VLDL-TG was the fraction of the plasma VLDL-TG pool lost per h. Compartmental modeling was performed using the SAAM II program (SAAM Institute, University of Washington, Seattle).

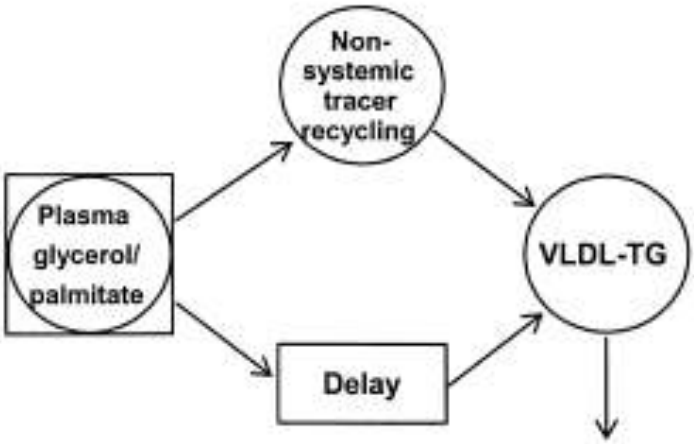

FIGURE 2.7 Compartmental Model for Turnover Kinetics of VLDL-TG by Using Stable Isotopically Labeled Glycerol and Palmitate. Glycerol or palmitate tracer is injected into plasma. A "fast" synthetic pathway comprises a short time delay for synthesis, assembly, and secretion of VLDL-TG, and a "slow" pathway accounts for non-systemic tracer recycling (tracer that is removed from plasma, placed into pools that turn over, and contribute tracer to VLDL-TG secretion after the bolus has cleared through the system). The primary kinetic parameter measured by this model is the fractional catabolic rate of VLDL-TG (loss of VLDL-TG tracer from the system).

A nonlinear function was used to describe the fraction of plasma glycerol or palmitate that is taken up by the VLDL-TG synthetic pathways because the bolus doses of palmitate and glycerol do not represent massless quantitites of tracer. This function is calculated as:

Gemma Fraterrigo

$$k_t = k_0 / (1 + TTR_t)$$

where k_t is the fractional rate constant for a given time t (different values for the fast and slow pathways), k_0 is the fractional rate constant at the limit of zero enrichment and TTR_t is the plasma tracer enrichment at time t. This nonlinear relationship ensures that the amount rather than fraction of plasma glycerol or palmitate used for VLDL-TG synthesis remains constant over time.

2.10.2 Plasma Glucose, Insulin, Palmitate, and Hydroxybutyrate Kinetics

Plasma glucose concentration was measured by using an automated glucose analyzer (Yellow Spring Instruments Co, Yellow Springs, OH). Plasma insulin concentration was measured by using electrochemiluminescence technology (Elecsys 2010, Roche Diagnostics USA) and by using a chemiluminescent immunoassay method (Immulite 1000, Diagnostid Products Corporation, Los Angeles, CA). Plasma β-hydroxybutyrate concentrations were determined by using gas chromatography.

Plasma glucose tracer-to-tracee ratio was determined by using electoron impact ionization gas chromatography-mass spectrometry [4]. Plasma was processed to recover these substrates and derivatize them for gas chromatography-mass spectrometry (GC/MS) analyses (MSD 5973; Hewlett-Packar)[226,228,237,300,411]. Isotopic steady-state conditions were achieved during the final 30 minutes of the basal period of the clamp procedure and Steele's steady state equation[412] (i.e., dividing the palmitate TTR value from 60 to 180 min) was used to calculate substrate kinetics. Glucose rate of disappearance from plasma was assumed to equal the glucose rate of appearance during basal conditions; during the clamp procedure, glucose rate of disappearance was assumed to equal the sum of endogenous glucose rate of appearance and the rate of infused glucose. Basal palmitate rate of appearance (R_a) in plasma was also calculated by using the Steele equation for steady-state kinetics.

2.10.3 *De Novo* Lipogenesis

Plasma lipid profile was measured by enzymatic colorimetric assay and run on the Cobas c501 analyzer (Roche Diagnostics USA). Plasma FFA concentrations were determined by using gas chromatography.

The quantification of DNL (using [1,2-$^{13}C_2$]acetate infusion) by mass isotopomer distribution analysis was performed [413,414].

2.10.3.1 Mass Spectrometric Analyses

Gas chromatography (GC) / MS. Fatty acid (FA) methyl esters were analyzed by means of GC/MS (model 5970, Hewlett-Packard Co., Palo Alto, CA). Conditions were isothermal (200°C) with a 20-m fused silica column. Molecular anions m/z 299 and 298 were compared (for 18:0) ance 271 and 270 (for 16:0) for determination of percent excess enrichment of the FA methyl esters.

2.10.3.2 Model for Calculation of *De Novo* Lipogenesis

Traditionally, studies of biosynthesis have used radioactive isotopes or, when stable isotopes are used, the macromolecule has been hydrolyzed to its precursor units (i.e., enriched protein to [^{13}C]leucine). Comparison of precursor to product labeling has been straightforward because the existence of isotopomers has not been an issue. This is so for radioisotopes because the average value for all molecular species is measured, i.e., specific activity is measured as the toal disintegrations per minute per total mass present, and the existence of different subpopulations of labeled molecules is not relevant. In contrast, when one analyzes intact lipid molecules (C-16, C-18) using MS, each isotopomer will appear as a separate species. If, for example, one FA molecule ends up with two labeled acetates and another with zero, this is not identical analytically to two molecules having one labeled acetate in stable isotope studies whereas they would be identical with radioisotopes. The effect of isotopomer distribution on biosynthetic calculations therefore had to be addressed.

The relationship between an enriched precursor and the distribution of label in a macromolecular product is shown (Figure 2.8) The frequency of isotopomers in a product (e.g., VLDL-stearate, containing nine acetate units) synthesized from a precursor (e.g., enriched acetyl-CoA) can be predicted using the binomial expansion. This gives the likelihood of choosing *x* special (enriched) unites out of *n* total units:

$$b(x\,;\,n,\,p) = [^{n}{}_{x}](p)^{x}(1 - p)^{(n-x)},$$

where *n* is the number of precursor units in prodct, *x* is the number of enriched precursor units in

Gemma Fraterrigo

product, p is the probability of each precursor unit being enriched, and $[^{n}_{x}] = (n)!/[(n - x)!(x)!]$.

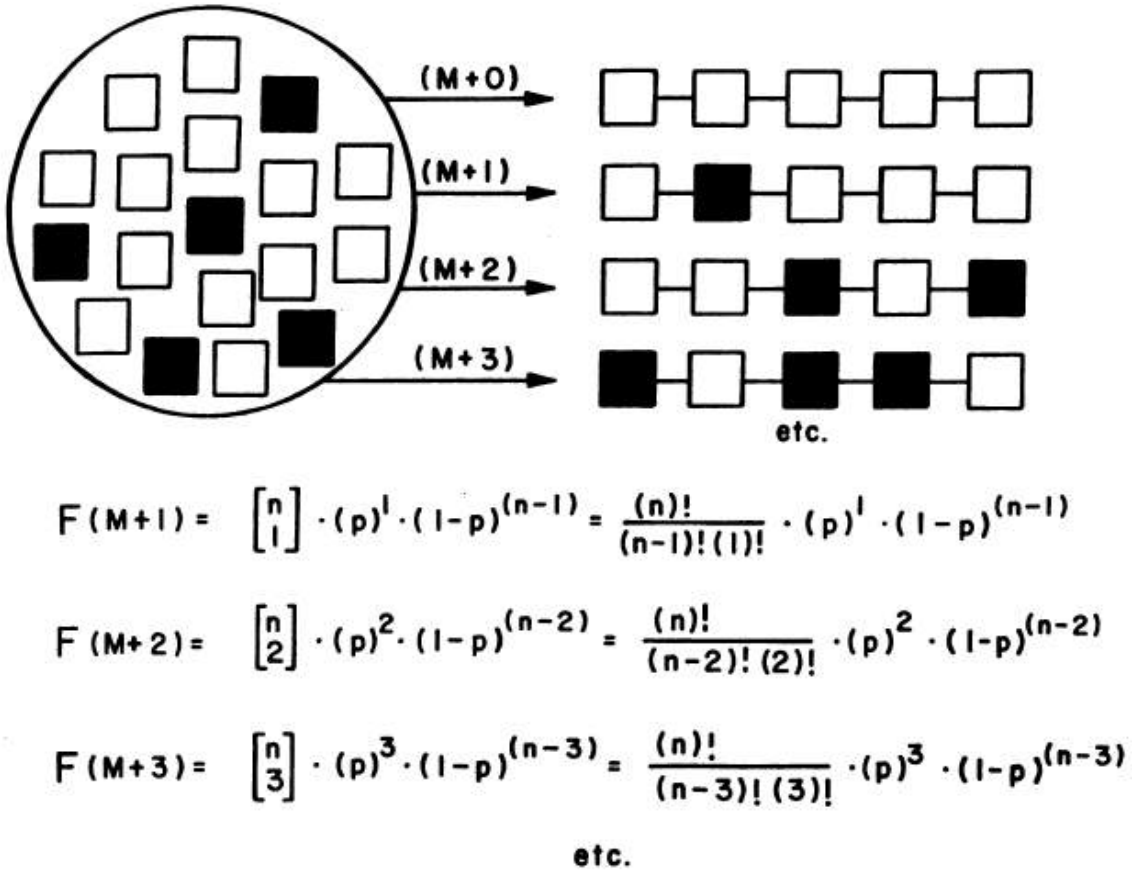

FIGURE 2.8 Schematic Model of the Relationship Between Isotope Abundance in Precursor Units (■) and Abundance of Isotopomers in a Polymeric Product. The frequency (F) of each isotopomer containing x labeled units (■) in a product containing n total units is a function of the probability (p) that each precursor unit is labeled, according to the binomial expansion: $F(M + x) = [^{n}_{x}](p)^{x}(1 - p)^{n-x}$. In this example, the product would contain five precursor units $(n = 5)$, the probability that each precursor unit is labeled would be $^{5}/_{18}$ (p = 0.278), and calculation of the abundance of each isotopomer species [F(M + 1), F(M + 2), and F(M + 3), where $x = 1$, $x = 2$, and $x = 3$, respectively] using the binomial expansion is shown.

In intuitive terms, this is the problem of how often you will get one black ball and eight white balls if you draw nine balls from a box and the probability of each ball being black is p. What makes the application slightly more complex is that there is a natural background p as well as the experimentally induced p. Moreover, our goal is not just to calculate the theoretical frequency in

Gemma Fraterrigo

order to calculate a fractional replacement (synthesis) rate.

An example of the method for calculation de novo lipogenesis for VLDL-stearate follows. If infusion of [^{13}C]acetate resuls in an acetyl-CoA enrichment of 7.0 MPE and natural abundance of acetate is assumed to be 2.22%, the ratio of M + 1/(M + 0) + (M + 1) isotopomers before and after administration of tracer will be:

$$\text{Background} \quad \frac{(M+1)}{(M+0)+(M+1)} = \frac{b(1;9,0.022)}{b(0;9,0.022)+b(1;9,0.022)} = \frac{0.1657}{0.8186+0.1657} = 0.1683$$

$$\text{Enriched} \quad \frac{(M+1)}{(M+0)+(M+1)} = \frac{b(1;9,0.092)}{b(0;9,0.092)+b(1;9,0.092)} = \frac{0.3826}{0.4195+0.3826} = 0.4770$$

$$\text{Excess} \quad \frac{(M+1)}{(M+0)+(M+1)} = 0.4770 - 0.1683 = 0.3087$$

This can be readily translated into kinetic terms. At time zero, the ratio of M + 1 abundance relative to (M + 1) + (M + 0) is 0.1683 in VLDL-stearate. If all VLDL-stearate were then replaced by newly synthesized molecules derived from acetyl-CoA during the experiment, M + 1/(M + 0) + (M + 1) would increase to 0.4770, or the excess, termed EF[M + 1/(M + 0) + (M + 1)] would be 0.3087. If only 50% of VLDL-stearate were derived from acetyl-CoA and the remainder were from reesterification of preformed stearate, M + 1/(M + 0) + (M + 1) would be half-way between 0.1683 and 0.4770 (0.3227), or the excess would be half of EF[M + 1/(M +0) + (M + 1)]; and so on. The value for EF [M + 1/(M + 0) + (M + 1)] thereby represents the asymptote or maximal value toward which the product enrichment may approach, and the relationship between product and precursor specific activity or enrichment used in fractional synthesis calculations.

Gemma Fraterrigo

Accordingly, calculation of fractional VLDL-FA synthesis from acetyl-CoA during an experiment is straightforward. The measured enrichment in the FA [M + 1/(M + 0) + (M + 1)] is divided by the calculated asymptotic ("precursor") value, EF[M + 1/(M + 0) + (M + 1)]. The latter is calculated from the equation relating *p* to EF[M + 1/(M + 0) + (M + 1)] (Figure 2.9.A). SMX-acetate enrichments are used to represent hepatic acetyl-CoA enrichments (*p*).

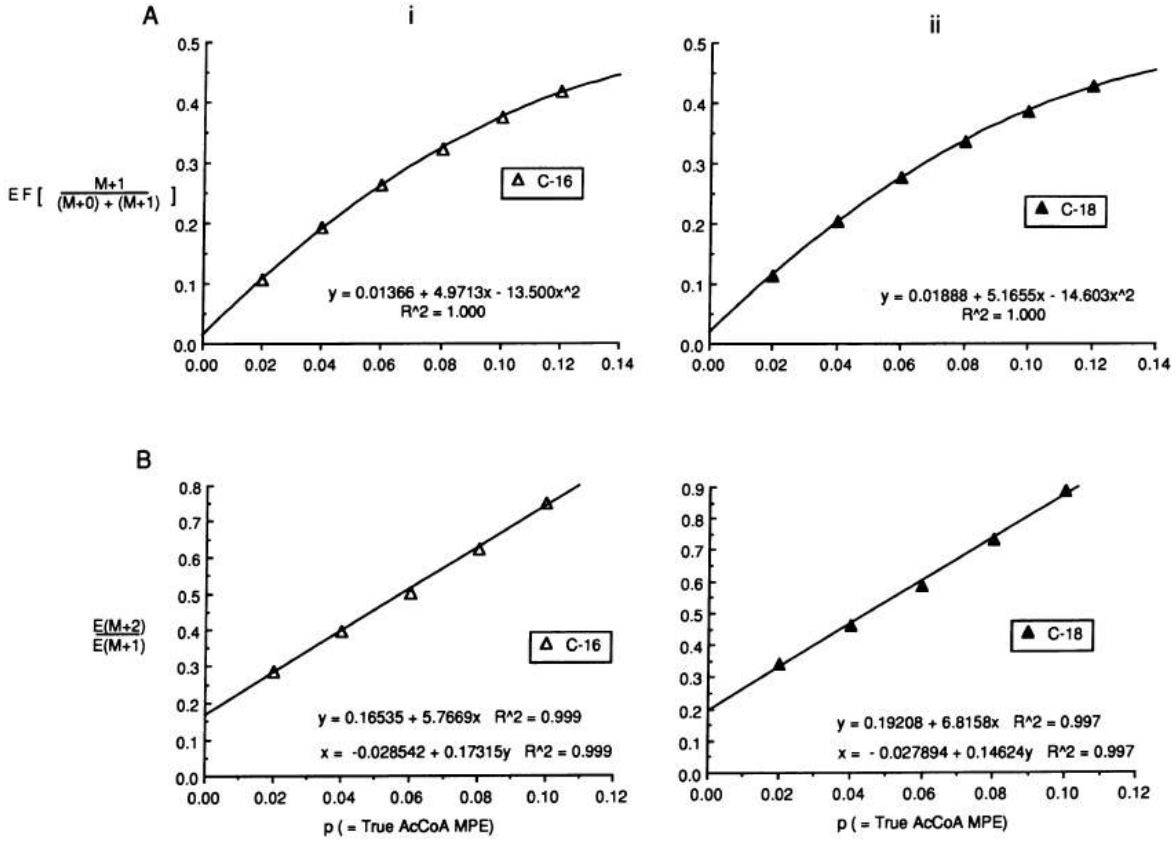

FIGURE 2.9 (*A*) The theoretical relationship between *p* (enrichment of acetyl-CoA) and EF[M +]1)/(M + 0) + (M + 1) isotopomers in VLDL-FA if all VLDL-FA were derived from acetyl-CoA. Background (M + 1)/(M + 0) + (M + 1) is subtracted assuming a natural ^{13}C abundance of 2.22% in acetate, according to to Eq. in the text. (*B*) The theoretical relationship between *p* and the ratio of excess M + 2/excess M + 1 (EM + 2/EM + 1) enrichments in VLDL-FA, calculated as excess M + 2/(M + 0) + (M + 1) + (M + 2) divided by excess M + 1/(M + 0) + (M + 1) + (M + 2). Curves in *A* and *B* are computer simulations. The relationship in *A* is used for calculating the percent of VLDL-FA synthesized by the de novo pathway (observed M + 1/(M + 0) + (M + 1) divided by EF [M + 1/(M + 0) + (M + 1)]). The relationship in *B* permits inference of *p* from EM + 2/EM + 1 ratios in VLDL-FAs, which can be compared to SMX-acetate enrichments for internal validation of the model.

With regard to the theoretical basis of the calculations, three further points should be made. First,

Gemma Fraterrigo

precursor-product models are simpler mathematically if a steady-state enrichment is present in the precursor pool but the product enrichment need not attain a plateau during the experimental period for valid biosynthetic calculations. Secondly, the excess M + 2/excess M +1 (EM + 2/EM +1) isotopomer ratio is uniquely determined by the enrichment of the precursor (Figure 2.9.B). Conversely, the EM + 2/EM + 1 ratio uniquely predicts a true precursor enrichment. This relationship can be exploited experimentally as an internal check of the validity of the model, by comparing true precursor enrichments inferred from isotopomer frequencies to measured SMX-acetate enrichments. Finally, it is worth noting that lipid elongation will not be described by this model. However, this is unlikely to account for a significant amount of label in FAs because only one acetyl-CoA rather than a string of acetyl-CoA units is added, in addition to its probably minor input quantitatively.

2.10.4. Skeletal Muscle DAG (Diacylglycerol) and Ceramide Content

Quantification of DAG and ceramide molecular species was performed by using electrospray ionization tandem mass spectrometry, as described below [415], [416]

2.10.4.1 Quantification of DAG Content

All synthetic DAG standards were obtained as solutions in chloroform at a concentration of 1 mg/ml from Avanti Polar Lipids, Inc. (Alabaster, AL) and Sigma (St Louis, MO). They were used as received. All solvents were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA). Anhydrous pyridine and methylene chloride were from Sigma (St Louis, MO). *N*-chlorobetainyl chloride was from Tyger Scientific Inc. (Ewing, NJ).

Between 1 and 100 nmol of DAG was dissolved in 0.5 ml anhydrous methylene chloride in a dry 5-ml glass tube with cap. Anhydrous pyridine (10 µl) and 10 mg *N*-chlorobetainyl chloride were added to the reaction solution. The tube was flushed with dry nitrogen, capped and incubated at 42°C with stirring. When the reaction was complete (within ~ 4 h as judged by TLC), the solvent was removed by a nitrogen stream, and the residues were extracted by using the Bligh and Dyer technique[417] to remove the salts. The lipid extracts were dried under a stream of nitrogen gas, dissolved in chloroform, filtered through a 0,2 µm Gelman acrodisc CR PTFE syringe filters, re-extracted, and dried again under a nitrogen stream. The final lipid residue was re-suspended in a 1:1 chloroform/methanol mixture prior to ESI-MS analysis.

Lipids of each sample were extracted by a modified Bligh and Dyer technique[417] utilizing 50-mM NaCl in the acqueous layer in the presence of 12:0/12:0 DAG (1 nmol/mg of wet weight) used as an internal standard. This molecular species of DAG represents <1% of the total endogenous DAG mass. The lipid extracts were dried under a nitrogen stream and dissolved in chloroform. DAG derivatives were analyzed by ESI/MS in the positive-ion mode and quantified by comparisons of the individual ion peak intensities with those of the internal standar (i.e., 12:0/12:0 DAG) after correction for ^{13}C isotope effects.

ESI mass spectra were obtained with a Finnigan Classic LCQ ion-trap mass spectrometer (San Jose, CA) with an ESI source. Samples were directly introduced into the instrument at a flow rate of 5 µl/min. The spray voltage was 5 kV, and the capillary temperature was 200°C. All data were acquired in the positive-ion mode at unit mass resolving power by scanning between ions of *m/z*500 and *m/z*800. Normally, 3-min scans were averaged and processed using Finnigan Xcalibur 1.1 software.

2.10.4.2 Quantification of Ceramide Content

All synthetic ceramides were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The purity for all ceramides was examined by ESI/MS prior to utilization.

A stock solution for each ceramide molecular species in 2:1 of chloroform/methanol (v/v) was quantitatively prepared and stored under nitrogen at -20°C. The ceramide solutions were brought to room temperature right before utilization of the solutions. The mixtures of ceramide molecular species were prepared from these stock solutions using gas-tight syringes. The concentration of each ceramide molecular species in the mixtures was ranged from 1 too 1000 nM. Since chlorine ions could complicate the ESI mass spectra of ceramide and interfere with the quantitative analyses of ceramide molecular species, all the mixed solutions were extracted at least twice by a modified Bligh and Dyer technique[417] utilizing 50 mM LiOH in an aqueous layer to minimize the presence of chlorine ions in the solutions. The extracts were dired under a nitrogen stream, dissolved in chloroform, reextracted, and dried under a nitrogen stream. The final residues of ceramide mixtures were resuspended in 0.2 ml of 1:1 chloroform/methanol for ESI/MS analyses.

Lipids were extracted by a modified Bligh and Dyer technique[417] utilizing 50 mM LiOH in an aqueous layer in the presence of N17:0 Cer (5 nmol/mg of protein) (used as an internal standard

for ceramide quantification). The lipid extracts were dried under a nitrogen stream, dissolved in chloroform, desalted with Sep-Pak columns, and filtered with 0.2-m Gelman ac- rodisc CR PTFE syringe filters (Gelman Science, Ann Arbor, MI), reextracted at least once, and then dried under a nitrogen stream. The final lipid residue was resuspended in 0.4 mL of 1:1 chloroform/methanol for ESI/MS analyses.

ESI mass spectral analyses of ceramides were similarly performed utilizing a Finnigan TSQ-7000 spectrometer equipped with an electrospray ion source. Typically, a 5-min period of signal averaging for each spectrum of a ceramide sample or a 10-min period of signal averaging for each tandem mass spectrum of a lipid extract in the profile mode was employed. Lipid extract samples were directly infused into the ESI chamber using a syringe pump at a flow rate of 1 µl/min. prior to the ceramide analyses of lipid extracts from biological samples, LiOH in methanol (50 nmol/mg of protein) was added. Ceramides were directly analyzed in the negative-ion ESI/MS. Tandem mass spectrometry of ceramides after ESI was performed with a collision energy of 32 eV and a collision gass pressure of 2.5 mTorr. Ceramide molecular species were directly quantitated by comparisons of ion peak intensities with that of internmal standar in both ESI/MS and ESI/MS/MS analyses after correction for ^{13}C isotope effects.

2.10.5 Adipocyte Size and Number

In order to isolate adipocytes from fresh adipose tissue, adipose tissue was minced and digested with collagenase (collagenase, Worthington Biochemical, Lakewood, NJ) and fix them with 2% osmium tetraoxide in collidine-HCl buffered solution [418,419]. The tissue was dispersed into small fragments within 1 hour of incubation with collagenase [420]. Adipocytes were liberated from the tissue fragments by gentle stirring with a rod. Liberation of the cells was manifested by an increased turbidity in the medium. Fragments of tissue still remaining after this treatment were removed with forceps. The suspension of cells was centrifuged in polyethylene centrifuge tubes for 1 minute at 400 x *g*. The adipocytes floated to the surface, and the stromal-vascular cells (capillary, endothelial, mast, macrophage, and epithelial cells) were sedimented. The stromal-vascular cells were removed by aspiration, and the adipocytes were washed by suspending them in 10 ml of warm (37°C) albumin buffer containing the desired concentration of glucose and

centrifuging for 1 mintue at 400 x *g*. This procedure was repeated.

Adipocyte cell number was determined as well as cell size distribution (per 10-μm bin), mean cell size, median cell size and percent of cells greater than 100 microns.

2.10.6 Adipose Tissue Macrophage Infiltration

Adipose tissue samples were fixed overnight in zinc-formalin (Anatech Ltd., Battle Creek, MI) and embedded in paraffin. Five-micron sections (cut at 50-μm intervals) were mounted on glass slides, and stained for expression of macrophage CD68, CD11c, CD11b, CD14, CD16, CD1a, CD123, BDCA-2, MHC class II, scavenger receptors type I and type II, CD163, DC-SIGN, Mannose receptor, Langerin and DC-Lamp. High expression of CD14 and round morphology identified monocytes. CD16, CD68, scavenger receptors, CD163, mannose receptor and DC-SIGN were highly expressed by different macrophage populations, while CD1a and Langerin specifically labeled dendritic cells of epidermal origin. Co-expression of BDCA-2, CD68 and CD123 were indicative of plasmacytoid monocytes or plasmacytoid DC. Finally, CD11c, CD83 and DC-Lamp marked mature dendritic cells.

2.10.7 Adipose Tissue Lymphocyte Populations

Because of the limited number of CD4+ T cells recovered from the adipose tissue samples, T cells extracted from adipose tissue were expanded in culture by using phytohemagglutinin and IL-2 to obtain a sufficient number of lymphocytes for further analysis.

We expanded lymphocytes obtained from adipose tissue to evaluate polarization of CD4 T cells. Adipose tissue stromal-vascular fraction was obtained after 1-hour incubation of abdominal subcutaneous adipose tissue biopsies with 2 mg/ml collagenase D at 37°C, followed by centrifugation to remove adipocytes. The stromal-vascular fraction was stimulated with 1 μg/ml phytohemagglutinin and cultured in RPMI media supplemented with 10% fetal bovine serum, glutamax, sodium pyruvate,

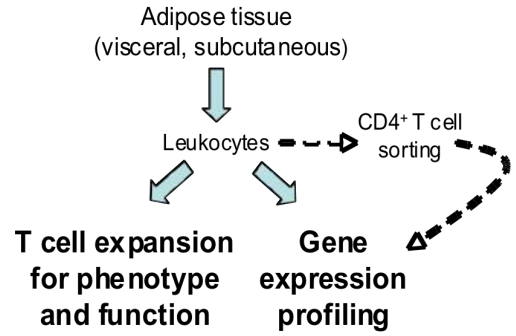

FIGURE 2.10 Adipose Tissue Processing

nonessential amino acids, kanamycin sulfate, and IL-2 to expand T cells. After 2 to 3 weeks, the expanded stromal-vascular fraction of cultues were restimulated with 10^{-7} M phorbol myristate acetate and 1 µg/ml ionomycin to induce activation, and monensin was added to the cultures after 2 hours to prevent secretion of cytokines. After 7 hours of stimulation, the cytokine production from CD4 T cells was evaluated by flow cytometry (FACSCalibur and Cell-Quest softward; BD Biosciences, Mountain View, CA) at a single cell level after staining with anti-CD4 followed by intracellular staining for IFN-gamma, IL-13, IL-17, and IL-22. All antibodies were purchased from Pharmingen except the anti—IL-22 antibody, which was obtained from R&D Systems, Minneapolis, MN.

2.10.8 Tissue Metabolic Factors, Gene Expression, Content of Specific Proteins and Activity

Gene expression was measured by quantitative real-time PCR, which was optimized for the genes of interest. Gene expression of liver and skeletal muscle IL-17 and IL-22 receptors and gene expression of adipose tissue total CD4+ T-lymphocyte polarization was evaluated by using flow cytometry.

2.10.8.1 Immunoprecipitation and Western Blot Analysis

Protein content of insulin signaling pathways, fatty acid trafficking and CD36 localization were measured by western blot [421]. Whole cell extracts were prepared using lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate) containing protease inhibitor mixture (Sigma). The cell lysates were clarified by centrifugation at 10,000 x *g* (10 min, 4°C), and the clarified lysates were incubated with primary antibodies (overnight at 4°C). Immune complexes were captured by adding 10 µl of protein A-Sepharose beads (Santa Cruz) followed by incubation with end-over-end rotation overnight at 4°C followed by washing five times in lysis buffer. Protein was eluted by boiling (10 min) in 30 µl of SDS sample buffer. All of the samples were separated on a 4-20% polyacrylamide-reduced gradient gel and electroblotted onto Immobilon-P polyvinylidene difluoride membranes. The membranes were blocked in TBST (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Tween 20) with 5% nonfat milk and then incubated with primary antibodies in 2% BSA/TBST overnight at 4°C or 2 h at room temperature. Incubation with secondary horseradish

peroxidase-conjugated goat anti-rabbit or anti-mouse IgG followed, and the proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

2.10.8.2 Tissue LPL Activity

Tissue LPL activity was determined as follows[422]. To ensure optimal LPL activity, adipose biopsy samples were flash frozen in homogenization buffer and stored at -80°C until assay. Muscle samples were flash frozen, stored at -80°C and cryopulverized prior to homogenization and assay for LPL activity. Activity was measured by incubating tissue homogenates in the presence of serum (source of apolipoprotein C-II) with a substrate emulsion consisting of Intralipid and ^{3}H-triolein, incorporated by sonication (75 W, 10 min). The emulsion was stored at 4°C. The incubation medium was prepared from 10 µl heat-activated serum as a source of apolipoproetin CII, 60 µl of deionized water and 100 µl of the incubation buffer which contained 12% fatty acid free bovine serum albumin, 0.02% standard heparin, 0.2 M NaCl and 0.3 M Tris-HCl, pH 8.5. The total volume was adjusted to 200 µl with the sample volume diluted 5-fold in water (20 µl, i.e. 4 µl of tissue homogenate). The final substrate concentration was 6 mM. Incubation was carried out for 60 min at 25°C in a shaking water bath. The reaction was stopped by addition of distilled water (-.5 ml) and 2 ml of isopropanol/heptane/H_2SO_4 (48:48:3:1 v/v/v). Total lipids were extracted and fatty acids separated from TG as follows: after centrifugation (2000 x *g* for 3 min, 4°C), a sample of the upper phase (800 µl) containing total lipids was transferred to new tubes into which 1 ml alkaline ethanol (ethanol 95%/water/2 M NaOH, 500:475:25 v/v/v) and 3 ml heptane were added. After a second centrifugation, the upper heptane phase containing unhydrolysed TG was discarded. A new extraction was performed with 3 ml of heptane. Finally, an aliquot (800 µl) of the remaining alkaline ethanol phase containing fatty acids was counted. All incubations were performed in triplicate. Extraction efficiency of fatty acids was determined to be around 42%. Hydrolyzed fatty acids were extracted and counted in a liquid scintillation counter to determine LPL activity (1 milliunit lipase activity equals 1 nmol fatty acids produced per min). LPL activity in mU per g tissue net weight was calculated taking into account dilution sample, extraction efficiency of fatty acids, incubation time, specific activity of the substrate and tissue/volume ratios of homogenates.

2.10.8.3 Skeletal Muscle, Liver, and Adipose Tissue Gene Expression

Complementary DNA (Vilo cDNA synthesis; Invitrogen, Carlsbad, CA) was generated from pooled human liver, skeletal muscle, and adipose tissue RNAs (Trizol; Invitrogen) to identify expression of the receptors IL-17RA, IL-17RC, and IL-22RA in liver and skeletal muscle, and to assess total CD4+ T-cell content and cytokines involved in lymphocyte recruitment (CCL5) and differentiation (IL-7) in adipose tissue. For gene expression validation, complementary DNA was serial diluted and amplified by using SYBR Green chemistry (Applied Biosystems, Carlsbad, CA). Target gene expression was considered valid if assay polymerase chain reaction efficiency was between 90% and 100% and signal could be detected for at least 3 dilutions. Primer pairs used for transcript detection are listed in (Table 2.2). For $2^{-\Delta Ct}$ relative abundance calculations, results were normalized to the housekeeping gene 36B4.

TABLE 2.2 Primer Pairs Used for Transcript Detection

Gene	Forward primer	Reverse primer
IL-17RA	AGGCGGTGGCGTTTTACCTTCA	TGCTCACAGTCAGGCACAAGGA
IL-17RC	GTCACTGTGGACAAGGTTCTC	CTCCAACAGTAGCACATCGTC
IL-22RA	CGCCTGCACCTCCCAACTCC	TGAAGCGCAGCGGCTGGAAA
$CD4^+$	GGAGTCCCTTTTAGGCACTTG	CAGTTCCACTGTATCCCCTTTTT
CCL5	CTGCTTTGCCTACATTGCCC	CACACTTGGCGGTTCTTTCG
IL-7	TCCTGCGGTGATTCGGAAAT	GATCCGCCAGCAGTGTACTT

2.10.9 Plasma Adipokines.

TNF-α and adiponectin were determined by commercially available ELISA kits (Linco Research, St. Charles, MO, and R&D Systems, Minneapolis, MN). Plasma concentrations of interleukin (IL)-6, CCL5, and IL-7 were measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Plasma concentrations of IL-22 and IL-17 were measured using an enzyme-linked immunosorbent assay (eBioscience, San Diego, CA) as per manufacturer's protocol, with the following modification: standard curves were extended to allow for better detection sensitivity (IL-22 > 1,95 pg/ml, IL-17 > 0.39 pg/ml).

2.10.10 Hepatic Insulin Sensitivity Index

Hepatic insulin sensitivity was determined by calculating the reciprocal of the Hepatic Insulin

Gemma Fraterrigo

Resistance Index (the product of basal endogenous glucose production rate and fasting plasma insulin concentration) [423]. Skeletal muscle insulin sensitivity was assessed as the percent increase in glucose rate of disappearance during insulin infusion [225]. The computerized, updated homeostasis model assessment was used to provide an index of whole-body insulin resistance [424].

2.10.11 Cell Culture and Rodent Studies

2.10.11.1 Metabolic Effects of IL-17 and IL-22 on Human Primary Hepatocytes

The effect of IL-17 and IL-22 on (1) insulin signaling was determined by measuring phosphorylated Akt in hepatocytes incubated with or without human IL-17 or IL-22, followed by stimulation with human insulin; (2) on glucose release was determined by measuring glucose content in media after incubating hepatocytes with or without human IL-17 or IL-22 followed by stimulation with glucagon with or without human insulin; and (3) rate of glycolysis was determined by measuring the rate of ^3H_2O formation from [5-^{3}H]glucose by hepatocytes after a pretreatment period with IL-17 or IL-22, followed by incubation with or without insulin and IL-17 or IL-22.

Human primary hepatocytes (Invitrogen; lot no. Hu4242) were plated onto collagen-coated plates at a density of 0.7 x 105 cells/cm^2 and cultured in 10% fetal bovine serum/Dulbecco's modified Eagle medium for 12 to 16 hours before initiation of experiments. To determine the effect of IL-17 and IL-22 on insulin signaling, hepatocytes were incubated with or without 7.5 ng/ml of human IL-17 or IL-22 (Cell SignalingTechnology, Danvers, MA) in serum-free Dulbecco's modified Eagle medium for 6 hours, followed by stimulation with human insulin (0.5 μM) for 5 minutes. The cells were then washed with ice-cold phosphate-buffered saline and immediately lysed with a cell lysis buffer containing 1 x protease inhibitor mixture (Roche, Indianapolis, IN) plus 1 mM NaF, 2 mM Na_3VO_4, and 20 mM $Na_4P_2O_7$. The protein extracts were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting for total and phosphorylated Akt using primary antibodies (Cell Signaling Technology). To examine the effect of IL-17 or IL-22 on glucose release from the hepatocytes, cells were pretreated with IL-17 or IL-22 for 6 hours as described here. At the end of this pretreatment period, the hepatocytes were washed with phosphate-buffered saline and then incubated for 2 hours in a glucose-free Hank's-HEPES buffer containing 10 mM sodium lactate, 5 mM pyruvate, and 0.5 μM glucagon with or without 0.5 μM insulin and IL-17 of IL-22.

Glucose content in the media was determined using an enzymatic glucose assay kit (Sigma-Aldrich Corp., St Louis, MO). Rates of glucose release were expressed as nmol/h x mg protein. Experiments were carried out in duplicates and repeated 3 times. Rates of glycolysis were determined after a 6-hour pretreatment period with IL-17 or IL-22 by measuring rates of formation of $^{3}H_2O$ from [5-^{3}H]glucose (American Radiolabeled Chemicals, St Louis, MO) by the hepatocytes. Briefly, after the pretreatment period, hepatocytes were washed and incubated for 3.5 hours in low glucose Dulbecco's modified Eagle medium containing 0.5 μCi [5-^{3}H]glucose/ml with or without insulin (0.5 μM) and IL-17 or IL-22 (7.5 ng/ml). After this incubation period, radioactivity ($^{3}H_2O$) in the media was determined after removal of [5-^{3}H]glucose by using mini-columns containing Dowex 1X4 anion exchange resin (200—400 mesh) [425]. The rates of utilization of [5-^{3}H]glucose were expressed as nmol of glucose metabolized per hour x mg cell proteins. Experiments were carried out in triplicate and repeated 3 times.

2.10.11.2 Effect of IL-17 and IL-22 on Skeletal Muscle Glucose Uptake in Rat Soleus and Epitrochlearis Muscles

This protocol was approved by the Animals Studies Committee of Washington University. Strips of soleus and epitrochlearis muscles taken from male Wistar rats were incubated with glucose, with or without human IL-17 or rat IL-22, and with or without the addition of a maximally effective insulin concentration. Glucose transport activity was measured by using 2-deoxygluxose.

Male (~ 70 g) Wistar rats were obtained from Charles River and given normal rat chow (Constant Formula Purina Rodent Chow no. 5001; Purina Mills, St Louis, MO). Food was removed at 6:00 PM the date before the experiment. The next morning, rats were anesthetized by using an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight), and the epitrochlearis and soleus muscles were removed [426]. Before incubation, the soleus muscle was split longitudinally into strips with an average weight of 20 to 25 mg. Muscles were incubated for 90 minutes at 35°C in 2 ml of oxygenated Krebs-Henseleit buffer containing 8 mM glucose, 32 mM mannitol, and 0.1% bovine serum albumin with or without 100 ng/ml of human IL-17 (Cell Signaling Technology) or rat IL-22 (US Biological, Swampscott, MA). The muscles were then transferred to medium of the same composition with or without the addition of a maximally

effective insulin concentration (2 mU/ml), and incubated for 30 minutes. Muscles were then washed for 10 minutes at 30°C in Krebs-Henseleit buffer containing 40 mM mannitol and 0.1% bovine serum albumin, with or without insulin, and with or without IL-17 and IL-22, to removed glucose from the extracellular space. The flasks were gassed with 95% O_2-5% CO_2 and shaken continuously in a Dubnoff incubator (Precision Scientific, Chicago, IL) during the incubations.

Glucose transport activity was measured by using 2-deoxygluxose (2-DG), since 2-DG can avoid the problem of efflux and therefore an underestimation of glucose transport activity, which is present when using 3-O-methyl-D-glucose (3-MG)[427]. Phosphorylation serves to trap 2-DG in the cell, in as much as glucose-6-phosphatase activity is very low in skeletal muscle. This makes it possible to measure sugar transport for a sufficiently long period to render relatively unimportant the contribution of the underestimation of the rate of sugar transport during the equilibration period. Thus the accumulation of intracellular 2-deoxyglucoe 6-phosphate (2-DG-6-P) can be used to measure glucose transport activity when transport rates are high.

After the rinse, muscles were incubated for 20 minutes at 30°C in 2 ml Krebs-Henseleit buffer containing 4 mM 2-[1,3-^{3}H]DG (1.5 μCi/ml), 36 mM [^{14}C]mannitol (0.2 μCi/ml, 0.1% bovine serum albumin, and insulin with and without IL-17 or IL-22). Osmolarity and temperature were maintained, and the flasks were gassed continuously as described previously. Muscles were processed by boiling for 10 min in 1 ml of water. Extracts were transferred to an ice bath, vortexed, and then centrifuged at 1,000 *g*. Aliquots of the muscle extracts and of the incubation media were placed in scintillation vials containing 10 ml of ScentiVerse (Fisher) and counted in a Packard liquid scintillation counter with channels preset for simultaneous ^{3}H and ^{14}C counting. The amount of each isotope present in the samples was determined and this information was used to calculate the extracellular space and the intracellular concentration of 2-DG [428]. The intracellular water content of the muscles was calculated by subtracting the measured extracellular space water from total muscle water. Total muscle water was assumed to be 80% of muscle weight, which is the average value for rat epitrochlearis muscles in such experimental conditions [428]. This was how extracellular space and intracellular 2-deoxygluxose concentrations were determined.

2.11 STATISTICAL ANALYSES

Data analyses were based on a pre/post design in which the goal was to determine whether the

impact of overfeeding in obese subjects was different in subjects with high IHTG compared with those who have normal IHTG content. The basic analytic strategy was similar for all outcome measures, because all of them were continuous variables. Initial analyses involved t-tests that compared baseline values of variables across groups. Analysis of covariance was used to evaluate the effect of the intervention, because that approach is generally preferred to t-tests that compare differences when, as was anticipated here, there are substantial baseline between-group differences in outcome measures. Specifically, these analyses of covariance treated the post intervention value as the dependent variable and the baseline value and study group as predictors. In all analyses, we ensured that the conditions necessary for the valid use of a proposed statistical procedure were satisfied. Therefore, we routinely evaluated the equal variance and normality assumptions of the t-test and assessed regression residuals to ensure that analyses of covariance were appropriate. When conditions were violated, we explored the use of data transformations intended to produce data that satisfy normality and equal variance assumptions. If an appropriate transformation could not be identified, we used non-parametric methods as an alternative to the more standard analyses. Therefore, in some cases, it was necessary to use Wilcoxon's test instead of the t-test or a semi-parametric analysis of covariance that is based on the ranks of the data as an alternative to a more standard analysis of covariance.

CHAPTER 3 Analysis of the Adverse Effects of Moderate Weight Gain in Metabolically Normal and Abnormal Obese

3.1 INTRODUCTION

This chapter focuses on the adverse effects of overfeeding and weight gain in metabolically normal (high IHTG content) and metabolically abnormal (normal IHTG contet) obese subjects. This work was supported by NIH grants UL1RR024992 (Clinical Translational Science Award), DK 56341 (Nutrition and Obesity Research Center), DK 37948 and DK 20579 (Diabetes Center Grant), and UL1TR000450 (KL2 Award); a Central Society for Clinical and Translational Research Early Career Development Award, and by grants from the Longer Life Foundation and the Kilo Foundation.

Obesity is associated with several metabolic abnormalities, including insulin resistance, dyslipidemia, and nonalcoholic fatty liver disease (NAFLD), which are important risk factors for type 2 diabetes, the metabolic syndrome, and coronary heart disease [1,2]. Data from a series of studies have demonstrated that increased intrahepatic triglyceride (IHTG) content (i.e., NAFLD) is a robust marker of metabolic dysfunction in obese people [4,226,429,430], and that the amount of IHTG is directly correlated with the degree of insulin resistance in the liver, skeletal muscle, and adipose tissue [225]. However, not all obese persons develop NAFLD, insulin resistance, and cardiometabolic disease. About 35% of obese adults have normal IHTG content [224], and 2%–50% of obese adults are "metabolically normal," depending on the criteria used to define metabolic normality and the sex and age of the study cohort [3,45,47,431-437]. Moreover, the risk of developing diabetes and future cardiovascular events is much lower in metabolically normal obese (MNO) people than in metabolically abnormal obese (MAO) people [5,244]. These observations suggest that some obese people are prone to develop alterations in fat distribution and metabolic disease, whereas others are protected from the adverse metabolic effects of weight gain and increased adiposity. However, it is also possible that MNO people are not protected, but simply require additional weight gain to develop adverse metabolic outcomes.

With overfeeding and weight gain, it is expected that the total fat mass (FM) of both metabolically normal and metabolically abnormal obese subjects will increase whereas there will be a different distribution of the ectopic fat in the two groups. The hypothesis was that

overfeeding in obese subjects with high IHTG content will increase ectopic fat (IHTG content), because of changes in: hepatic lipid metabolism (i.e. increase in DNL without adequate increase in VLDL-TG and apoB-100 secretion, and in fatty acid oxidation), and cellular factors that increase muscle (and presumably liver) fatty acid uptake (increased muscle CD36 expression and content and muscle lipoprotein lipase [LPL] activity). In contrast, overfeeding in obese subjects with normal IHTG content will not cause a significant increase in ectopic fat accumulation, because of metabolic adaptations in: i) hepatic lipid metabolism that increase hepatic VLDL-TG secretion rate and fatty acid oxidation and ii) cellular factors that increase adipose tissue TG accumulation (increased subcutaneous adipose tissue CD36 expression and content and adipose tissue lipoprotein lipase [LPL] activity, and subcutaneous adipocyte proliferation). Another phenomena that might be expected would be a greater increase in VAT rather than SAT in the obese subjects with high IHTG content after overfeeding compared to a greater increase in SAT rather than VAT in the obese subjects with normal IHTG content.

IHTG content was assessed by using magnetic resonance spectroscopy (MRS), *in vivo* hepatic lipid metabolic kinetics was assessed by using stable isotope tracer infusion, and cellular factors were assessed by obtaining adipose tissue and skeletal muscle biopsies.

It is also expected that overfeeding in obese subjects with high IHTG content will cause an impairment in *in vivo* insulin sensitivity in the liver (suppression of glucose production), skeletal muscle (glucose uptake) and adipose tissue (suppression of lipolysis), and in the cellular components of the insulin signaling cascade in muscle and adipose tissue (GLUT 4, phosphorylation of IRS1 and Akt), because of changes in cellular metabolites that influence insulin action: intramyocellular factors that inhibit insulin signaling (phosphorylation of JNK and concentrations of specific lipid metabolites (diacylglycerol and ceramide) and adipose tissue factors that are associated with insulin resistance, including endoplasmic reticulum (ER) stress, adipose tissue remodeling, and inflammation (increased recruitment/differentiation of preadipocytes generating a bimodal distribution of adipocytes, adipocyte necrosis and pro-inflammatory macrophage infiltration, and expression of inflammatory adipokines). In contrast, overfeeding in obese subjects with normal IHTG content should not cause a deterioration of *in vivo* multi-organ insulin sensitivity or muscle insulin signaling, because it should not induce adverse changes in myocellular and adipose tissue factors that inhibit insulin signaling.

Multi-organ insulin sensitivity was assessed *in vivo* by using a two-stage euglycemic-hyperinsulinemic clamp procedure with stable isotope tracer infusion, and cellular markers in muscle and adipose tissue was assessed by obtaining muscle and adipose tissue biopsies during basal conditions and insulin infusion.

3.2 AIMS AND OBJECTIVES

The aim of this study was to test the hypothesis that obese people who have normal IHTG content (MNO) would be resistant, whereas those who have increased IHTG content (MAO) would be prone, to developing adverse metabolic effects after gaining weight, and to evaluate some of the putative molecular mechanisms in adipose tissue responsible for the adverse metabolic effects of weight gain. Accordingly, hepatic, skeletal muscle, and adipose tissue insulin sensitivity was evaluated (by using a 2-stage hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotopically labeled glucose and palmitate tracer infusion); hepatic lipoprotein kinetics was evaluated (by infusing a stable isotopically labeled leucine tracer in conjunction with mathematical modeling); body fat distribution was determined (by using magnetic resonance spectroscopy and imaging along with DXA); and assessment of adipose tissue global transcriptional changes by microarray analyses was made (by obtaining subcutaneous adipose tissue biopsies) in obese subjects with normal (<5,6%) [438] and increased (≥10%) IHTG content, before and after they gained about 6% body weight by consuming an additional 1,000 kcal/day of macronutrient-balanced foods obtained from selected fast-food restaurants.

Such a study could lay the groundwork for understanding why some obese persons are resistant, while others are prone, to developing obesity-related metabolic disease, which could have considerable future research and clinical implications.

3.3 RESULTS

3.3.1 High-calorie Diet Intervention

The increase in daily total energy intake during the high-calorie diet intervention was similar in the MNO and MAO groups (1,171 ± 403 kcal and 1,324 ± 522 kcal, respectively; P = 0.491) (Table 3.1). There was a small decrease in the percentage of calories derived from protein

compared with that observed before the high-calorie diet, but the percentage of calories from carbohydrates and fat did not change significantly in either group (Table 3.1). This goes to show that the differences observed after weight gain were not caused by a difference in the macronutrients in the diet. Weight gain caused a similar increase in resting energy expenditure (REE) in both groups (Table 3.1) (P value for the intervention × group interaction = 0.216; P value for the intervention = 0.011), as would be expected. The targeted weight gain was achieved after about the same duration of time of the high-calorie diet intervention in the MNO (6.8 ± 2.7 weeks) and MAO (8.3 ± 2.4 weeks) groups (P = 0.239).

TABLE 3.1 Energy Expenditure, Calorie Consumption, and Diet Composition Before and During Overfeeding

	MNO		MAO	
	Before	During	Before	During
REE (kcal/d)	1,468 ± 391	1,596 ± 576^	1,499 ± 205	1,838 ± 414^
Energy intake (kcal/d)	2,022 ± 568	3,193 ± 512^	2,206 ± 333	3,531 ± 555^
Protein intake (%)	15 ± 2	13 ± 2^	15 ± 2	14 ± 2^
Carbohydrate intake (%)	49 ± 6	51 ± 6	46 ± 8	46 ± 6
Fat intake (%)	36 ± 5	36 ± 5	39 ± 6	39 ± 5

Values represent the mean ± SD. ^P< 0.05, value was significantly different from the corresponding value before weight gain.

3.3.2 Body Composition

The percentage of body fat and BMI values did not differ between the MNO and MAO groups at baseline. However, IHTG content and visceral adipose tissue (VAT) volume were much higher in MAO subjects than in MNO subjects (Table 3.2). IHTG content was 15.2% at baseline in MAO subjects compared to 2.4% at baseline in MNO subjects. VAT volume was 1714 cm^2 at baseline in MAO subjects compared to 885 cm^2 at baseline in MNO subjects. High-calorie dietary intake caused a similar increase in body weight in MNO (mean = 6.2 ± 0.7%, range = 5.1% - 7.2%) and MAO (mean = 5.8 ± 0.7%, range = 4.6% - 7.0%) subjects (P = 0.307) (Table 3.2). The weight increase consisted of about 72% fat mass (FM) and about 28% fat-free mass (FFM) in both groups. Although the relative increase in IHTG content was similar between groups, the

Gemma Fraterrigo

absolute increase was greater in MAO subjects than in MNO subjects (Table 3.2).

TABLE 3.2 Body Composition Characteristics Before and After Weight Gain

	MNO (n = 12)			MAO (n = 8)			ANOVA		
	Before	After	Change	Before	After	Change	P_x	P_t	P_g
BMI (kg/m²)	34.0 ± 3.0	36.0 ± 3.2	6%	35.7 ± 3.9	37.8 ± 4.2	6%	0.953	<0.001	0.285
BW (kg)	95.8 ± 13.7	101.7 ± 14.4	6%	103.0 ± 11.0	109.0 ± 11.6	6%	0.842	<0.001	0.239
Body FM (%)	44.9 ± 6.7	46.2 ± 6.2	3%	43.2 ± 5.3	44.8 ± 4.8	4%	0.544	<0.001	0.561
FM (kg)	42.8 ± 7.4	46.8 ± 7.6	9%	44.2 ± 5.4	48.6 ± 5.2	10%	0.494	<0.001	0.596
FFM (kg)	52.5 ± 11.4	54.2 ± 12.3	3%	58.3 ± 10.2	59.9 ± 10.1	3%	0.863	<0.001	0.277
IHTG content (%)	2.4 ± 1.1	3.9 ± 2.6[A]	63%	15.2 ± 4.0[B]	22.8 ± 4.3[A,B]	50%	<0.001	-	-
VAT volume (cm³)	885 ± 240	987 ± 295	12%	1,714 ± 585	1,912 ± 645	12%	0.243	0.001	<0.001
SAAT volume (cm³)	3,008 ± 796	3,071 ± 809	2%	3,145 ± 871	3,308 ± 928	5%	0.503	0.141	0.625

Values represent the mean ± SD. [A]$P < 0.05$, value was significantly different from the corresponding value before weight gain; [B]$P \leq 0.05$, value was significantly different from the corresponding value in the MNO group. P_x denotes ANOVA P values for the intervention × group interaction; P_t denotes ANOVA P values for the effect of the intervention; P_g denotes ANOVA P values for the differences between groups. SAAT, subcutaneous abdominal adipose tissue.

3.3.3 Metabolic Characteristics

At baseline, the concentrations of homeostasis model assessment of insulin resistance (HOMA/IR), plasma glucose, insulin, LDL cholesterol, triglycerides, VLDL apolipoprotein B100 (apoB100), alanine aminotransferase (ALT), and branched-chain amino acids (BCAAs) (valine, leucine, and isoleucine) were higher in MAO subjects than in MNO subjects (Table 3.3). Weight gain caused some cardiometabolic outcome measures such as plasma triglycerides, VLDL apoB100, ALT, and adiponectin concentrations and blood pressure to worsen in the MAO group but not in the MNO group. On the other hand, weight gain did not cause a significant change in other metabolic variables such as in plasma insulin, free fatty acids, LDL cholesterol, HDL cholesterol, and BCAA concentrations in either group. Weight gain also increased fasting plasma glucose and leptin concentrations in both groups (Table 3.3).

Gemma Fraterrigo

TABLE 3.3 Metabolic Characteristics Before and After Weight Gain

	MNO (*n* = 12)			MAO (*n* = 8)			ANOVA		
	Before	After	Change	Before	After	Change	P_s	P_i	P_g
Glucose (mg/dl)	93 ± 4	96 ± 5	3%	105 ± 9	110 ± 13	5%	0.564	0.033	0.001
Insulin (mU/l)	8.6 (6.8, 11)	9.4 (6.8, 13)	9%	23.3 (15.2, 35.8)	27.4 (17.5, 42.8)	18%	0.579	0.104	<0.001
HOMA-IR	2.0 (1.5, 2.5)	2.2 (1.6, 3.1)	10%	6.0 (3.8, 9.5)	7.3 (4.4, 12.2)	22%	0.570	0.062	<0.001
Free fatty acid (μmol/ml)	0.52 ± 0.14	0.44 ± 0.11	-15%	0.53 ± 0.03	0.54 ± 0.13	2%	0.198	0.415	0.216
LDL cholesterol (mg/dl)	106 ± 24	107 ± 26	1%	133 ± 20	128 ± 29	-4%	.537	0.480	0.041
HDL cholesterol (mg/dl)	49 ± 10	51 ± 9	4%	43 ± 8	44 ± 8	2%	0.262	0.004	0.116
Triglycerides (mg/dl)	89 ± 43	89 ± 32	0%	134 ± 61[B]	170 ± 52[A,C]	27%	0.017	-	-
VLDL apoB100 (mg/dl)	3.0 ± 2.4	3.2 ± 2.3	7%	4.9 ± 2.4[C]	7.0 ± 2.3[A,C]	43%	0.048	-	-
ALT (IU/l)	14 ± 6	15 ± 8	7%	22 ± 6.3[B]	31 ± 9[A,C]	14%	0.017	-	-
Total BCAAs (μM/l)	388 ± 74	390 ± 69	1%	458 ± 75	455 ± 48	-1%	0.941	0.847	0.035
Adiponectin (μg/ml)	9.4 (5.9, 15.0)	9.5 (6.1, 14.6)	1%	6.6 (5.0, 8.8)	5.9 (4.3, 8.1)	-11%	0.046	-	-
Leptin	54 ± 24	67 ± 24	24%	56 ± 25	67 ± 24	20%	0.798	0.008	0.952
SBP (mmHg)	123 ± 12	118 ± 13	-4%	128 ± 13	139 ± 6[A,C]	9%	0.045	-	-
DBP (mmHg)	67 ± 9	67 ± 14	0%	71 ± 8	81 ± 6[A,C]	14%	0.056	-	-

Values represent the mean ± SD or 95% CIs. [A]$P < 0.05$ and [B]$P = 0.06$, values significantly different from the corresponding values before weight gain; [C]$P \leq 0.05$, value significantly different from the corresponding value in the MNO group. P_s denotes ANOVA *P* values for the intervention × group interaction; P_i denotes ANOVA *P* values for the effect of the intervention; P_g denotes ANOVA *P* values for the differences between groups. DBP, diastolic blood pressure; SBP, systolic blood pressure; TG, triglyceride.

3.3.4 Substrate Kinetics

There was no difference in the endogenous glucose rate of appearance (Ra) (and glucose rate of disappearance [Rd]) during the basal stage (no insulin infusion) before weight gain in the MNO group (15.9 ±1.6 μmol/kg FFM/min) compared with that observed in the MAO group (17.0 ± 1.7 μmol/kg FFM/min), and this did not change with weight gain in either the MNO (16.5 ± 1.4 μmol/ kg FFM/min) or MAO (18.7 ± 1.4 μmol/kg FFM/min) group. The basal palmitate Ra before weight gain in the MNO group (2.8 ± 0.8 μmol/kg FM/min) was also not different from that seen in the MAO group (2.5 ± 0.8 μmol/kg FM/min), and this did not change with weight gain in either the MNO (2.4 ± 0.8 μmol/kg FM/min) or MAO (2.3 ± 0.8 μmol/kg FM/min) group.

Before weight gain, the endogenous glucose Ra during low-dose insulin infusion and the relative

Gemma Fraterrigo

suppression of glucose Ra (index of hepatic insulin sensitivity) were 52% higher and 22% lower ($P < 0.001$), respectively, in MAO subjects compared with that observed in MNO subjects (Figure 3.1.A). The endogenous glucose Ra during low-dose insulin infusion was higher after weight gain than before weight gain in MAO subjects ($P = 0.014$), but did not change in MNO subjects (P value for intervention x group interaction = 0.018) (Figure 3.1.A).

Before weight gain, glucose Rd during high-dose insulin infusion and the relative increase in glucose Rd (index of skeletal muscle insulin sensitivity) were 52% and 75% lower, respectively, in MAO subjects compared with the levels seen in MNO subjects (both $P < 0.001$) (Figure 3.1.B). After weight gain, both glucose Rd ($P = 0.07$; P value for intervention x group interaction + 0.024) and the percentage increase in glucose Rd during high-dose insulin infusion ($P = 0.043$; P value for intervention x group interaction = 0.004) decreased in MAO subjects, but did not change in MNO subjects (Figure 3.1.B).

Before weight gain, palmitate Ra during low-dose insulin infusion and the relative suppression of palmitate Ra (index of adipose tissue insulin sensitivity) were 64% higher and 33% lower, respectively, in MAO subjects compared with that seen in MNO subjects (both $P < 0.001$) (Figure 3.1.C). After weight gain, the relative suppression of palmitate Ra during low-dose insulin infusion decreased in MAO subjects, but did not change in MNO subjects ($P = 0.046$, P value for intervention x group interaction = 0.019) (Figure 3.1.C).

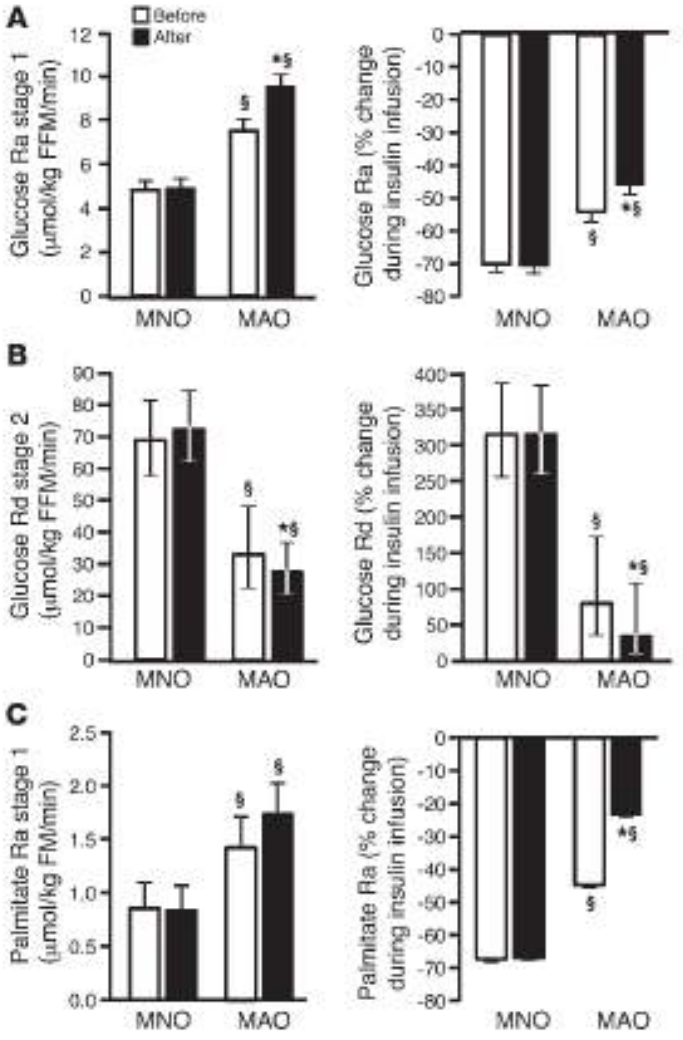

FIGURE 3.1 Hepatic, Skeletal Muscle, and Adipose Tissue Insulin Sensitivity. (**A**) Hepatic, skeletal muscle, and adipose tissue insulin sensitivity in MNO (n = 12) and MAO (n = 8) subjects before (white bars) and after (black bars) weight gain. Endogenous glucose Ra in plasma and percentage of suppression of glucose Ra during low-dose insulin infusion (stage 1) of the clamp procedure (an index of hepatic insulin sensitivity). (**B**) Skeletal muscle glucose Rd from plasma and percentage of stimulation of glucose Rd during high-dose insulin infusion (stage 2) of the clamp procedure (an index of skeletal muscle insulin sensitivity). (**C**) Palmitate Ra in plasma and percentage of suppression of palmitate Ra during low-dose insulin infusion (stage 1) of the clamp procedure (an index of adipose tissue insulin sensitivity). Repeated-measures ANCOVA was used for statistical analysis, with the intervention as the within-subjects factors (MNO vs. MAO), and sex and race as covariates. $^{\xi}P < 0.01$, value different from the corresponding MNO value; $^{*}P < 0.05$, value different from the before-weight-gain value. Data represent the mean ± SEM (**A** and **C**) or the mean and 95% CIs (**B**).

Gemma Fraterrigo

Before weight gain, the rates of hepatic VLDL apoB100 secretion into plasma were higher in MAO subjects than in MNO subjects ($P < 0.01$) (Figure 3.2). Weight gain caused a further increase in VLDL apoB100 secretion rates in MAO ($P = 0.004$), but not in MNO, subjects. The rates of VLDL apoB100 clearance from plasma were similar in the MNO and MAO groups before weight gain (18.7 ± 6.2 and 17.6 ± 6.3 ml/min, respectively).

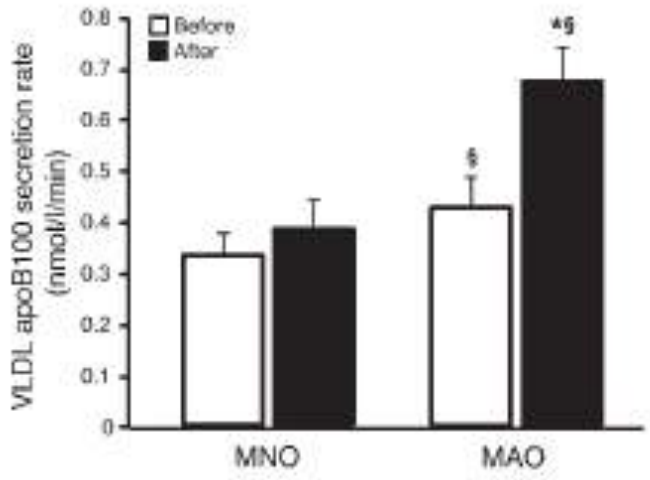

FIGURE 3.2 VLDL ApoB100 Kinetics. VLDL apoB100 secretion rates in MNO (n = 12) and MAO (n = 8) obese subjects before (white bars) and after (black bars) weight gain. Repeated-measures ANCOVA was used for statistical analysis, with the intervention as the within-subjects factor (MNO vs. MAO), and sex and race as covariates. $^{\S}P < 0.05$, value different from the corresponding MNO value; $^{*}P < 0.01$, value different from the before-over-feeding value. Data represent the mean ± SEM.

3.3.5 Adipose Tissue Gene Expression Profile

Parametric analysis of gene set enrichment (PAGE) of microarray data [439,440] was used to evaluate global transcriptional changes in subcutaneous adipose tissue induced by weight gain in the MNO and MAO subjects. Biological pathways related to lipid metabolism and synthesis were markedly increased by weight gain in the MNO, but not in the MAO, group (Figure 3.3.A; e.g., FATTY_ACID_BIOSYNTHETIC_PROCESS [Z score = 7.8 in the MNO group, 0.77 in the MAO group]; LIPID_BIOSYNTHETIC_PROCESS [Z score = 6.1 in the MNO group, 1.5 in the MAO group]; LIPID_METABOLIC_PROCESS [Z score = 5.4 in the MNO group, 0.16 in the MAO group]). Consistent with the upregulation in lipogenic pathways, adipose tissue gene

Gemma Fraterrigo

expression of key lipogenic enzymes (fatty acid desaturase 1 [FADS1], fatty acid desaturase 2 [FADS2], and fatty acid elongase 6 [ELOVL6]) were also significantly increased by weight gain in the MNO group, but did not change in the MAO group (Figure 3.3.B).

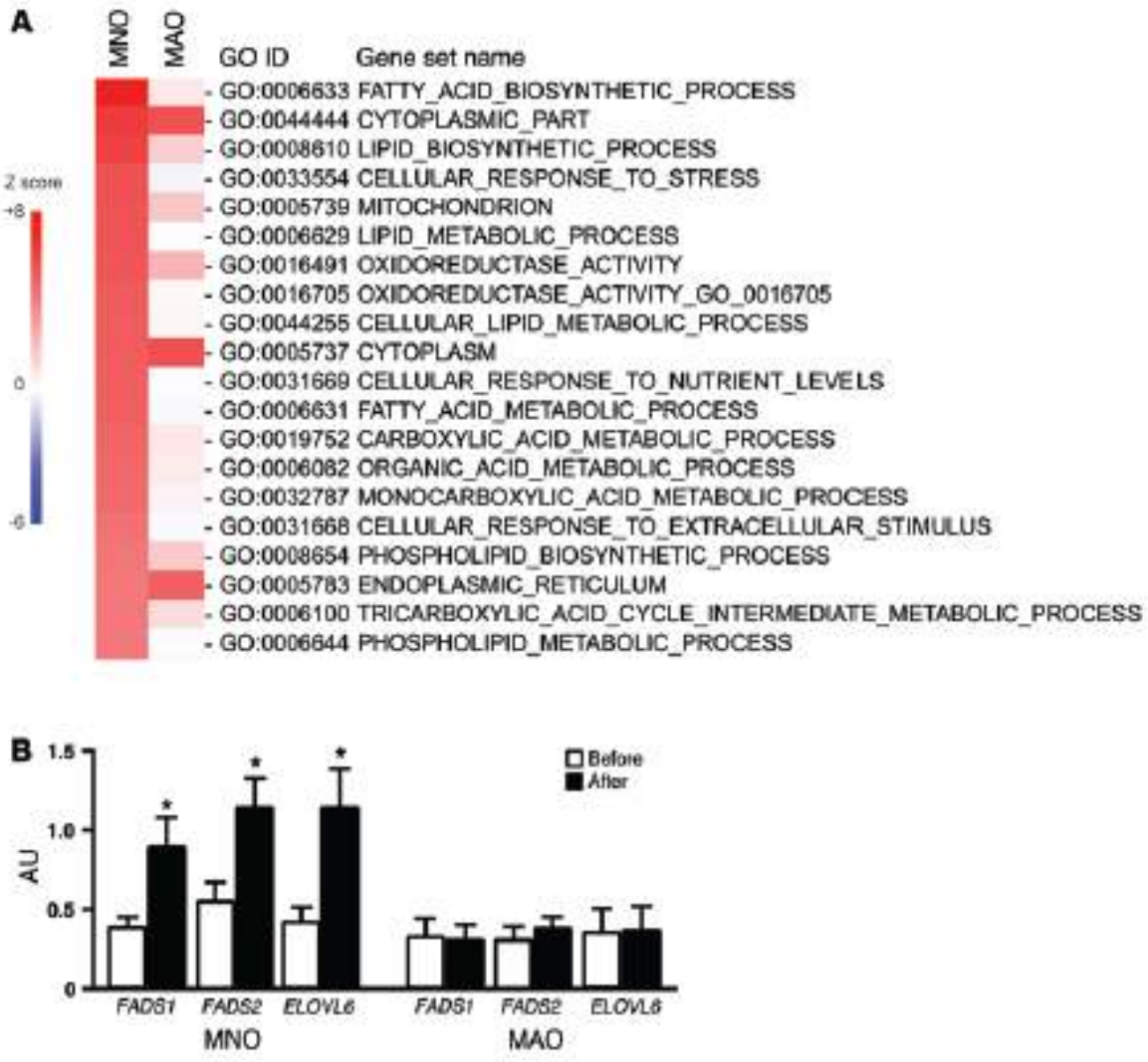

FIGURE 3.3 Adipose Tissue Gene Expression Profile. PAGE was performed on microarray data to identify pathways in subcutaneous adipose tissue that changed with weight gain in MNO (n = 12) and MAO (n = 8) subjects. (A) The top-20 significantly upregulated pathways in MNO subjects are listed on the basis of their Z scores values before and after weight gain. (B) Gene expression of key lipogenic enzymes in subcutaneous adipose tissue was determined by real-time PCR in MNO and MAO subjects before (white bars) and after (black bars) weight gain. ANCOVA was used for statistical analysis, with the intervention as the witihin-subjects factor (before vs. after weight gain), the group as the between-subjects factor (MNO vs. MAO), and sex and race as covariates. *P < 0.02, value different from the before-weight-gain value. Data represent the mean ± SEM.

Gemma Fraterrigo

3.4 DISCUSSION

3.4.1 Moderate Weight Gain Exacerbates Metabolic Risk Factors in MAO

Although obesity is commonly associated with a constellation of risk factors for cardiovascular disease, including insulin resistance, diabetes, dyslipidemia, and increased blood pressure, some obese people do not have metabolic abnormalities. In the present study, we used the accumulation of IHTG to identify obese subjects who are metabolically normal and those who are metabolically abnormal and challenged each group with a high-calorie diet until the subjects achieved moderate (~6%) weight gain. Our data demonstrate distinct differences in the response to weight gain in MNO and MAO subjects. In MAO subjects, but not MNO subjects, moderate weight gain exacerbated several metabolic risk factors for cardiovascular disease, including increased blood pressure, plasma triglyceride levels, VLDL apoB100 concentrations, and VLDL apoB100 secretion rates, and decreased plasma adiponectin concentrations and insulin sensitivity in the liver, skeletal muscle, and adipose tissues. Weight gain also caused a greater absolute, but not relative, increase in IHTG content in MAO subjects compared with that seen in MNO subjects. These data demonstrate that obese people with normal IHTG content represent a distinct obese phenotype that is resistant to the adverse metabolic effects of moderate weight gain, as if they were somewhat protected from the negative effects of moderate weight gain whereas obese people with high IHTG content are more prone to such adverse effects and therefore more at risk. These findings have important clinical implications and suggest that the intensity of obesity therapy should be based on metabolic function rather than BMI values alone. Also, such "at risk" obese subjects should be followed more closely and monitored more intensively.

3.4.2 IHTG Content as a Robust Marker of the Adaptive Response to Weight Gain

The concept that some obese people are predisposed to, while others are protected from, cardiometabolic disease was first proposed nearly 60 years ago [441]. Since then, a series of studies have used the components of the metabolic syndrome or some measure of insulin sensitivity to identify a subset of obese people who are metabolically normal. However, there is no consensus as to how metabolic normality should be defined, so the reported prevalence of MNO ranges

from 2% to 50%, depending on the specific criteria used and the population studied[3,45,47,431-437]. In the present study, we used IHTG content to identify obese people who have already demonstrated either a propensity for or a resistance to accumulating triglycerides in organs other than adipose tissue. This approach also separated our subjects into those who were insulin resistant or insulin sensitive with respect to hepatic, muscle, and adipose tissue insulin action. Our results demonstrate that IHTG content is a robust marker of the adaptive response to weight gain and that obese people who have normal IHTG content tolerate moderate weight gain without serious adverse metabolic effects compared with those who have NAFLD. Moreover, our data suggest that the absolute, not the relative, increase in IHTG content tracks with the adverse metabolic effects that occur with weight gain. Therefore, it would seem as though those with NAFLD should be treated and monitored in a more intense manner. However, the association between IHTG content and metabolic dysfunction does not prove a cause-effect relationship. In fact, a dissociation between hepatic steatosis and insulin resistance has been observed in patients with familial hypobetalipoproteinemia who have increased IHTG content because of a genetic truncation of apoB100 that impairs VLDL export [236,442]. Overall, these findings support the notion that MNO people with normal IHTG content represent a distinct phenotype and are not simply in a transition phase toward MAO. Nonetheless, we cannot exclude the possibility that our MNO subjects would develop metabolic abnormalities with greater weight gain.

3.4.3 Potential Underlying Mechanism that Protects the MNO from the Adverse Effects of Weight Gain

The cellular mechanisms responsible for the differences in metabolic function between MNO and MAO people are not clear. One prominent hypothesis is that a decreased capacity of adipose tissue to transport glucose and convert carbohydrate precursors into triglycerides is associated with adverse effects on metabolic health. It has been found by others and confermed by other studies that, compared with MNO subjects, MAO subjects have decreased adipose tissue expression of genes involved in glucose uptake and lipogenesis [4,443-447]. In the present study, we extended these findings by demonstrating that several biological pathways and genes related to lipid metabolism and synthesis were significantly increased by weight gain in the MNO, but not MAO, subjects. These data are consistent with the results from a study conducted in a rodent

model indicating that an experimental increase in the expression of adipose tissue lipogenic genes prevented weight gain-induced insulin resistance and the metabolic complications associated with obesity [448]. Together, these data suggest that increased adipose tissue capacity for lipogenesis helps protect against the adverse metabolic effects of weight gain. However, an important limitation of our study is that we evaluated adipose tissue samples from only one compartment (subcutaneous abdominal fat) and did not obtain samples from other adipose tissue depots (e.g., visceral or gluteo femoral fat), skeletal muscle, or other tissues. Therefore, we cannot exclude the possibility that we missed other adipose tissue factors [449] or potential mediators of insulin resistance, such as diacylglycerol, ceramide, and acylcarnitines, in other tissues [450-452] that could help explain the different responses to weight gain in our MNO and MAO subjects.

3.5 CONCLUSION

The results from this study demonstrate that moderate weight gain elicits very different physiological responses in MNO and MAO people and that IHTG content can be used to identify obese people who are prone to, or protected from, the development of metabolic disease. These data support the need for more aggressive weight-management therapy in the subset of obese people who have NAFLD and are at high risk for continued deterioration of metabolic function with additional weight gain.

CHAPTER 4 Analysis of the Effects of Moderate Weight Gain on Hepatic Lipid Metabolism in Metabolically Normal and Abnormal Obese

4.1 INTRODUCTION

This chapter focuses on the adverse effects of overfeeding and weight gain in metabolically normal (high IHTG content) and metabolically abnormal (normal IHTG content) obese subjects. This work was supported by NIH grants UL1RR024992 (Clinical Translational Science Award), DK 56341 (Nutrition and Obesity Research Center), DK 37948 and DK 20579 (Diabetes Center Grant), and UL1TR000450 (KL2 Award); a Central Society for Clinical and Translational Research Early Career Development Award, and by grants from the Longer Life Foundation and the Kilo Foundation.

Obesity is an important risk factor for the development of nonalcoholic fatty liver disease (NAFLD); the prevalence of NAFLD is ~15% and ~65% in lean and obese adults, respectively [224]. The mechanisms responsible for excessive accumulation of intrahepatic triglyceride (IHTG) induced by weight gain are not known [453], but likely involve an imbalance between free fatty acid (FFA) delivery to the liver and de novo fatty acid synthesis and the rate of fatty acid oxidation and export (as triglyceride [TG] within very low-density lipoprotein [VLDL]) [1].

4.2 AIMS AND OBJECTIVES

The purpose of this study was to evaluate the physiological mechanisms responsible for the accumulation of IHTG after moderate weight gain in obese people. Twenty-seven obese subjects (7 men, 20 women; age 48±10 years old) (Table 4.1) were studied before and after they gained a target of ~6% body weight by consuming an additional ~1000 kcal/d of foods containing the same macronutrient distribution (percent calories from carbohydrate, fat and protein) as their usual diet. Magnetic resonance spectroscopy and imaging were used to evaluate body composition and fat distribution. Stable isotopically-labeled glycerol, palmitate, leucine, acetate and β-hydroxybutyrate tracer infusions, in conjunction with mathematical modeling and mass isotopomer distribution analyses, were used to evaluate: i) the rate of release of FFA from

Gemma Fraterrigo

adipose tissue into the bloodstream, which is an important source of fatty acids delivered to the liver for IHTG synthesis; ii) hepatic de novo lipogenesis (DNL), which provides fatty acids synthesized from carbohydrate precursors for esterification into IHTG; iii) hepatic β-hydroxybutyrate secretion rate, which is a marker of intrahepatic fatty acid oxidation; iv) hepatic VLDL-TG secretion rate, which exports TG out of the liver, and are comprised of fatty acids derived from both systemic (plasma FFA) and non-systemic (visceral adipose tissue lipolysis, DNL, and/or hydrolysis of intrahepatic TG) sources [226]; and v) VLDL-apolipoprotein B100 (VLDL-apoB100) secretion rate, which is a marker of the number of VLDL particles secreted by the liver because each particle contains one molecule of apoB100 (see Supplemental Material for details of experimental protocol, sample analyses, calculations and statistical analyses).

4.3 RESULTS

4.3.1 Body Composition

Subjects consumed the high-calorie diet for up to 12 weeks in an effort to gain ~6% body weight. The average duration of high-calorie diet consumption was 8±3 weeks, which caused a 5.8±1.1% (range 3% to 9 %) weight gain that was mostly due to an increase in fat mass (Table 4.1). Weight gain caused a 4.0±4.1% absolute increase (55±49% relative increase) in IHTG content (Table 4.1). Basal plasma glucose did not significantly change with weight gain, whereas plasma insulin and HOMA-IR values increased by ~15% to 20%, demonstrating a deterioration in insulin sensitivity (Table 4.1).

TABLE 4.1. Body Composition and Metabolic Characteristics Before and After Weight Gain

	Before	After
Body mass index (kg/m^2)	34.6±3.5	36.6±3.7*
Body weight (kg)	96.8±13.8	102.4±14.6*
Fat-free mass (kg)	52.5±11.0	54.1±11.3*

Fat mass (kg)	43.8±6.8	47.7±7.1*
Body fat mass (%)	45.5±5.8	46.8±5.3*
Visceral adipose tissue volume (cm^3)	1175 (1002, 1377)	1314 (1125, 1535)*
Intrahepatic triglyceride content (%)	4.8 (3.3, 7.0)	7.1 (4.7, 10.7)*
Glucose (mg/dL)	97 (94, 100)	99 (96, 103)
Insulin (mU/L)	13 (10, 16)	15 (11, 19)*
HOMA-IR	3.1 (2.4, 4.0)	3.7 (2.8, 4.8)*
Free fatty acid (µmol/L)	350±80	360±90
Free fatty acid Ra (µmol/min)	300±69	305±72
Triglyceride (mg/dL)	110±48	126±51*
Apolipoprotein B100 (mg/dL)	82±22	89±25§
β-hydroxybutyrate (µmol/L)	79 (52, 121)	46 (37, 58)*
Respiratory quotient	0.73±0.03	0.76±0.05**
Fat oxidation (g/kg/d)	1.5±0.4	1.3±0.4**
Carbohydrate oxidation (g/kg/d)	0.6±0.6	0.9±1.0**

Values are mean ± SD or 95% CIs. Ra=rate of appearance. Value significantly different than corresponding value before weight gain, *P<0.001, **P<0.05, §P=0.07

Gemma Fraterrigo

4.3.2 Hepatic Fatty Acid Availability and *De Novo* Lipogenesis

We then evaluated the effect of weight gain on the metabolic pathways that influence IHTG production rate, namely hepatic fatty acid availability from plasma and DNL from carbohydrate precursors. Plasma FFA concentration and the rate of appearance (Ra) of FFA into plasma, a measure of adipose tissue lipolytic rate and fatty acid availability to the liver, did not change with weight gain (Table 4.1). In contrast, the rate of intrahepatic de novo synthesis of fatty acids from carbohydrate precursors increased by ~20% with weight gain (Figure 4.1A).

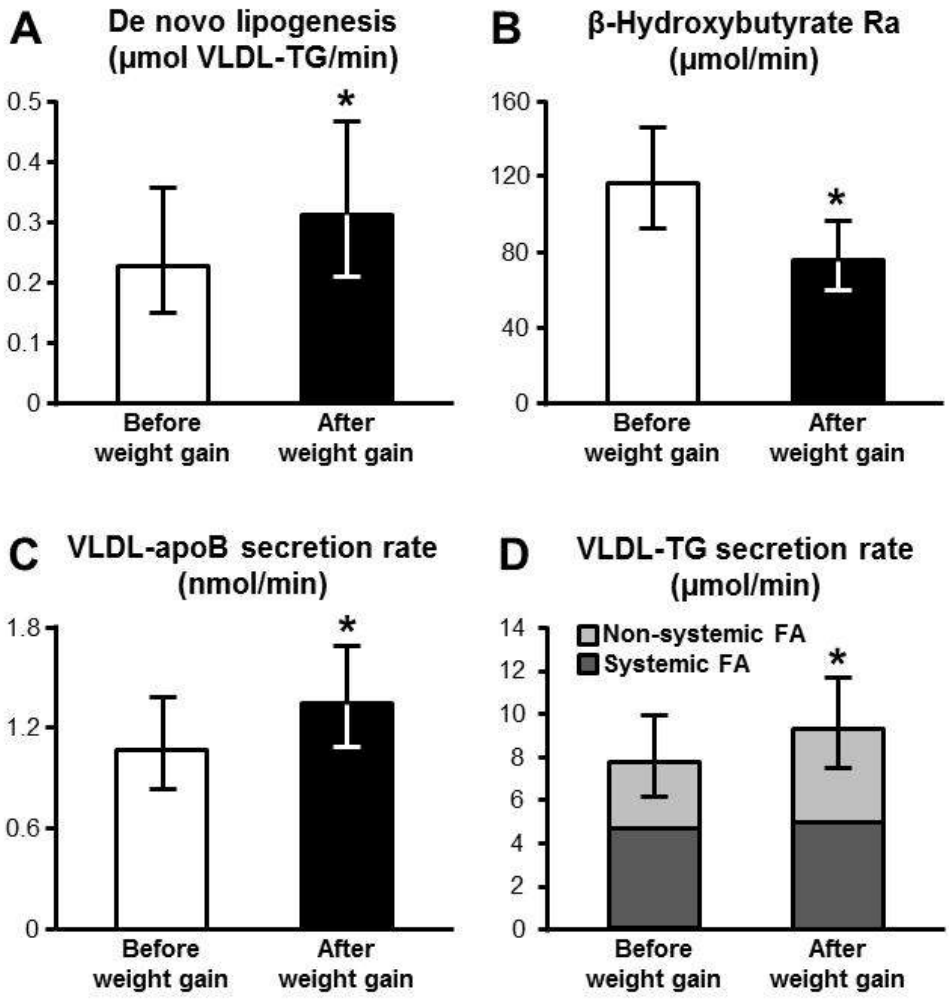

FIGURE 4.1 Effect of Moderate Weight Gain on: A) Hepatic De Novo Lipogenesis, assessed as fatty acid produced from carbohydrate precursors and incorporated into very low-density lipoprotein-triglyceride (VLDL-TG); **B) β-hydroxybutyrate Rate of Appearance (Ra); C)**

Gemma Fraterrigo

Hepatic Secretion of VLDL-apolipoprotein B100 (VLDL-apoB100); and D) Hepatic Secretion of VLDL-TG, and contribution of systemic (dark grey) and non-systemic (light grey) fatty acid sources to triglyceride secreted within VLDL. *Value different from before weight gain value, $P<0.05$. Data are back-transformed from the log and are presented as means and 95% CIs.

4.3.3. IHTG Fatty Acid Oxidation and Export of TG within VLDL Particles

We also evaluated the effect of weight gain on the metabolic pathways that are involved in the removal of IHTG, namely intrahepatic fatty acid oxidation and export of TG within VLDL particles. Fatty acids are used by the liver to produce energy through a multi-step process that involves β-oxidation and production of β-hydroxybutyrate (the most predominant ketone body). Accordingly, the rate of release of β-hydroxybutyrate into the systemic circulation reflects the rate of hepatic fatty acid oxidation [454,455]. We found that weight gain caused a reduction in hepatic fatty acid oxidation rate, as demonstrated by a decrease in both β-hydroxybutyrate concentration (Table 4.1) and β-hydroxybutyrate Ra (Figure 4.1B). Weight gain also caused a decrease in whole-body fatty acid oxidation rate and an increase in carbohydrate oxidation rate (Table 4.1). VLDL particles produced by the liver are composed primarily of TG, a single molecule of apoB100 and some cholesterol, phospholipids and small, exchangeable lipoproteins.[456] The secretion of VLDL provides a mechanism for exporting water-insoluble lipids from the liver as a water-soluble particle into the bloodstream to peripheral tissues. The rate of VLDL-apoB100 secretion represents the number of VLDL particles secreted by the liver, and the rate of secretion of VLDL-TG is a measure of the amount of triglyceride exported from the liver. Weight gain caused an increase in both VLDL-apoB100 (Figure 4.1C) and VLDL-TG (Figure 4.1D) secretion rates, and an increase in plasma total triglyceride and total apolipoprotein B100 (Table 4.1). Moreover, the increase in VLDL-TG secretion rate was almost entirely attributable to a marked increase in the contribution of fatty acids originating from non-systemic sources (visceral adipose tissue lipolysis, DNL, and/or hydrolysis of IHTG) (Figure 4.1D).

4.4 DISCUSSION

4.4.1 Mechanisms Underlying IHTG Accumulation with Weight Gain

The results from the present study elucidate the physiological mechanisms responsible for IHTG accumulation caused by moderate weight gain in obese people. Our data demonstrate that weight gain induced by a macronutrient-balanced, high-calorie diet causes alterations in specific

metabolic pathways that contribute to an increase in steatosis. Weight gain affected both sides of intrahepatic triglyceride balance by increasing the *de novo* synthesis of fatty acids from carbohydrate, in conjunction with a decrease in the elimination of fatty acids by intrahepatic fatty acid oxidation. In fact, the decrease in fatty acid oxidation in the liver was associated with a shift in whole-body substrate oxidation from lipid to carbohydrate.

4.4.2 VLDL-TG Secretion from the Liver Increases with Weight Gain but does not Counterbalance the Increased IHTG Production

Weight gain also caused an increase in the export of TG out of the liver by secreting a greater number of TG-rich VLDL particles, manifested by the increase in VLDL-apoB100 and VLDL-TG secretion rates. The increase in VLDL-TG secretion was due entirely to a contribution from non-systemic fatty acid sources, presumably derived from lipolysis of visceral adipose tissue, *de novo* lipogenesis, and/or hydrolysis of IHTG. However, the increase in TG export was not adequate to fully compensate for the increased rate of IHTG production, because IHTG content increased. The alteration in VLDL kinetics was likely responsible for the observed increase in plasma TG and apoB100 concentrations.

4.4.3 Increased Subcutaneous Adipose Tissue Lipolytic Activity does not seem to Play an Important Factor in the Pathogenesis of NAFLD

In contrast, weight gain did not cause an increase in the delivery of fatty acids to the liver from lipolysis of subcutaneous adipose tissue triglycerides, so the notion that an increase in subcutaneous adipose tissue lipolytic activity is an important factor in the pathogenesis of NAFLD [457] is probably not true.

4.5 CONCLUSION

Weight gain is associated with the development and worsening of nonalcoholic fatty liver disease (NAFLD), but the mechanisms responsible for this association are not known. Here we combined imaging and stable isotope tracers techniques to evaluate the effect of moderate weight gain in 27 obese people on intrahepatic triglyceride (IHTG) content and hepatic lipid metabolism

in order to elucidate the mechanisms responsible for weight-gain induced IHTG accumulation. Our results demonstrate that weight gain causes an imbalance between hepatic availability and disposal of fatty acid (increase in DNL, reduction of fatty acid oxidation, inadequate increase in VLDL secretion) which are likely responsible for increased IHTG accumulation.

CHAPTER 5 Analyis of Specific Adipose Tissue CD4+ T-cell Populations and Insulin Resistance in Obese

5.1 INTRODUCTION

This chapter focuses on the characteristics of T cells in adipose tissue from metabolically abnormal insulin-resistant obese (MAO) subjects, metabolically normal insulin-sensitive obese (MNO) subjects, and lean subjects as the control group. This work was supported by NIH grants UL1RR024992 (Clinical Translation Science Award), DK 56341 (Nutrition and Obesity Research Center), DK 37948, DK60022, DK33301, a grant from Pfizer Inc., and a grant from the Longer Life Foundation.

Obesity is associated with insulin resistance, which is directly associated with increased triglyceride accumulation in the liver [4,225,229], and is an important risk factor for type 2 diabetes, the metabolic syndrome, and coronary heart disease [1,2]. However, not all obese individuals develop insulin resistance and metabolic abnormalities. About one third of obese adults are metabolically normal based on insulin sensitivity measure by using the hyperinsulinemic-euglycemic clamp technique [45,46], and about one third have normal intrahepatic triglyceride content assessed by using magnetic resonance spectroscopy [224]. It is not known why weight gain and body fat accumulation cause insulin resistance in some people but not in others. Data from a series of studies conducted in the last 15 years have demonstrated that metabolic dysfunction in obese individuals is associated with adipose tissue inflammation, suggesting a possible link between the metabolically abnormal, insulin-resistant obese phenotype and a dysregulation of adipose tissue immune functions [458-462]. Since the first observations of increased levels of tumor necrosis factor-α that adipose tissue from obese mice and people have compared with lean controls [463,464], data from subsequent studies support the notion that obesity is associated with chronic low-grade inflammation, which leads to development of metabolic dysfunction [465,466].

The major research focus in obesity-related inflammation has been on adipose tissue macrophages, which has led to the concept that obesity is associated with increased adipose tissue macrophage infiltration [286,287] in conjunction with a switch in macrophage population from an anti-inflammatory to a pro-inflammatory state [465]. Recently, data from studies conducted in

rodent models have indicated that the distribution of adipose tissue and hepatic T lymphocytes might also have an important role in obesity-related adipose tissue inflammation and metabolic dysfunction and in the development of steatosis and steatohepatitis [289,290,467-469]. However, the potential importance of alterations in adipose tissue lymphocytes in the pathogenesis of metabolic dysfunction in obese people is not known.

An increased number of macrophages in adipose tissue is associated with insulin resistance and metabolic dysfunction in obese people. However, little is known about other immune cells in adipose tissue from obese people, and whether they contribute to insulin resistance. The characteristics of T cells in adipose tissue from metabolically abnormal obese subjects, metabolically normal obese subjects, and lean subjects were investigated. Insulin sensitivity was determined by using the hyperinsulinemic euglycemic clamp procedure. Plasma cytokine concentrations and subcutaneous adipose tissue CD4+ T-cell populations were assessed in 9 lean subjects, 12 metabolically normal obese subjects, and 13 metabolically abnormal obese subjects. Skeletal muscle and liver samples were collected from 19 additional obese patients undergoing bariatric surgery to determine the presence of selected cytokine receptors.

5.2 AIMS AND OBJECTIVES

The purpose of the present study was to determine if metabolically abnormal (insulin-resistant) obesity (MAO) is associated with an altered polarization of adipose tissue CD4 T lymphocytes compared with lean and metabolically normal (insulin-sensitive) obese (MNO) subjects, and if this specific polarization could be mechanistically related with insulin resistance in the liver and skeletal muscle.

5.3 RESULTS

5.3.1 Metabolic Variables and Body Composition of the Study Subjects

The characteristics of the study subjects are shown in Table 5.1. Although percent body fat was similar among obese subjects, fat distribution differed between the MNO and MAO groups. MAO subjects had greater intrahepatic triglyceride content than both lean and MNO subjects. Intra-abdominal fat volume was also greater in the MAO than in the lean group, and tended to be greater in MAO than MNO subjects. Fasting plasma glucose concentration was not different

among groups, but MAO subjects had greater plasma insulin concentration and homeostasis model of assessment—insulin resistance values compared to both MNO and lean subjects. Basal hepatic glucose production rate was not different between groups (MAO = 16.3 ± 3.2 μmol/kgFFM/min; MNO = 15.7 ± 2.1μmol/kgFFM/min; lean = 12.5 ± 2.6 μmol/kgFFM/min; $P > 0.05$); however, hepatic insulin sensitivity (assessed by using the Hepatic Insulin Sensitivity Index) and skeletal muscle insulin sensitivity (assessed by insulin-stimulated increase in skeletal muscle glucose uptake) were much lower in the MAO than in the MNO and lean groups (Figure 5.1A and B).

TABLE 5.1 Characteristics of Study Subjects

	Lean	Metabolically normal obese	Metabolically abnormal obese	ANOVA
Sex, n (*male/female*)	9 (1/8)	12 (2/10)	13 (2/11)	
Age, *y*, mean ± SD	47 ± 15	48 ± 9	38 ± 12	.092
Body mass index, mean ± SD	22.7 ± 1.9	34.9 ± 4.5[a]	43.8 ± 9.0[ab]	<.001
Body fat mass, %, mean ± SD	36 ± 4.7	44 ± 6[a]	48 ± 7[a]	<.001
IHTG content, %, mean ± SD	1.8 ± 1.3	4.4 ± 3.6	11.3 ± 8.7[ab]	.002
IAAT volume, cm^3, mean ± SD	594 ± 262	1386 ± 604	1630 ± 450[a]	<.001
Glucose, *mg/dL*, mean ± SD	93 ± 7	94 ± 8	99 ± 8	.145
Insulin, *mU/L*, mean ± SD	3.1 ± 1.3	7.3 ± 2.8	21.0 ± 8.5[ab]	<.001
HOMA-IR, mean ± SD	0.7 ± 0.3	1.7 ± 0.7	5.2 ± 2.3[ab]	<.001
Total-cholesterol, *mg/dL*, mean ± SD	192 ± 36	174 ± 40	164 ± 25	.173
LDL-cholesterol, *mg/dL*, mean ± SD	105 ± 32	103 ± 28	103 ± 18	.982
HDL-cholesterol, *mg/dL*, mean ± SD	65 ± 18	52 ± 12	37 ± 6[ab]	<.001
Triglyceride, *mg/dL*, mean ± SD	114 ± 48	98 ± 53	120 ± 50	.535

ANOVA, analysis of variance; HDL, high-density lipoprotein; IAAT, intra-abdominal adipose tissue; HOMA-IR, homeostasis model assessment of insulin resistance; IHTG, intrahepatic triglyceride; LDL, low-density lipoprotein.
[a]Significantly different from lean group.
[b]Significantly different from metabolically normal group, $P < .01$.

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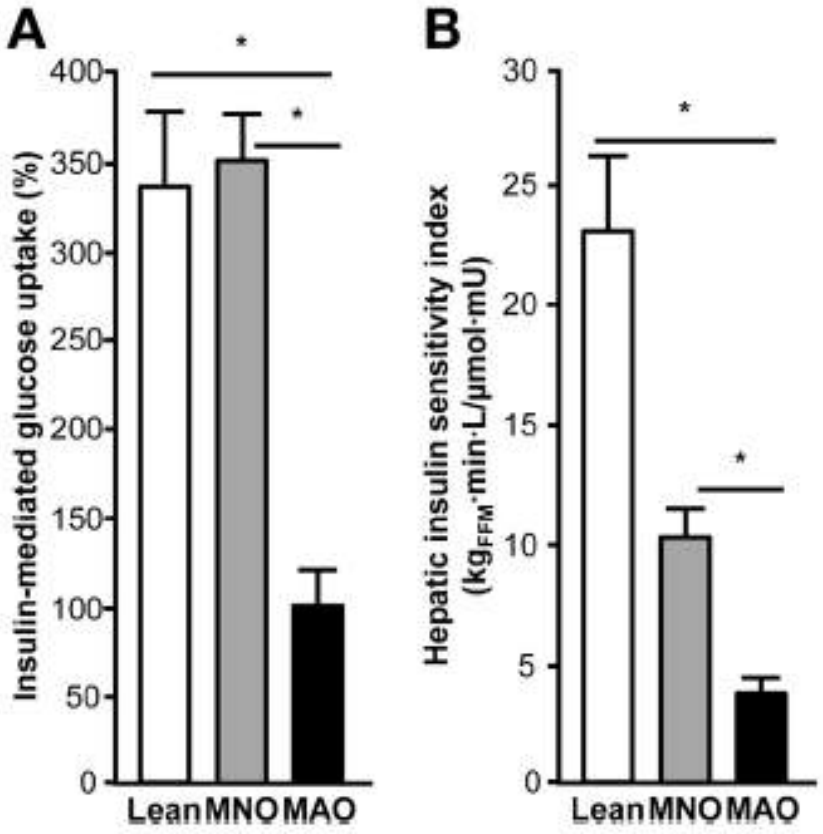

FIGURE 5.1 Hepatic and Skeletal Muscle Insulin Sensitivity in Study Participants. Hepatic insulin sensitivity (**A**), assessed by using the Hepatic Insulin Sensitivity Index as a measure of endogenous glucose production in relation to plasma insulin concentration, and skeletal muscle insulin sensitivity (**B**), assessed as the stimulation of skeletal muscle glucose uptake during insulin infusion, are imapaired in MAO subjects compared with MNO and lean subjects. One-way analysis of variance with planned contrasts was used to compare the differences between MAO and the other 2 groups. Values significantly different from the MAO group, **P* < 0.001. Values are mean ± SEM.

5.3.2 MAO Subjects have a Specific CD4 T-Cell Signature in Adipose Tissue

Adipose tissue CD4+ T-cell content was evaluated along with the potential differences in the distribution of CD4+ T-cell populations among MAO, MNO, and lean subjects. Total CD4+ T-cell content, measured by gene expression, increased progressively from lean, to MNO, to MAO subjects ($P < 0.05$; Figure 5.2A). The CD4+ T cells were analyzed for the production of cytokines associated with the T helper (Th) 1 (interferon [IFN] γ), Th2 (IL-13), Th17 (IL-17), and Th22 (IL-22) subsets of Th cells. We found that CD4+ T lymphocytes in adipose tissue of MAO subjects were skewed toward a Th17 and Th22 phenotype; MAO subjects had a greater

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percent of adipose tissue lymphocytes producing IL-17 and IL-22 cytokines compared with MNO and lean subjects (Figure 5.3.A and B). There were no differences among groups in the percent of lymphocytes polarized toward producing IL-13 (MAO = 13.0% ± 9.7%, MNO = 14.1% ± 10.2%, lean = 13.0% ± 9.7%; $P > 0.05$) or IFN-γ (MAO = 68% ± 13%, MNO = 66% ± 22%, lean = 57% ± 24%; $P > 0.05$). However, adipose tissue gene expression of CCL5 and IL-7, which are cytokines involved in T-cell proliferation, survival, and recruitment [470-472], increased progressively from lean to MNO to MAO subjects ($P < 0.05$; Figure 5.2.B and C).

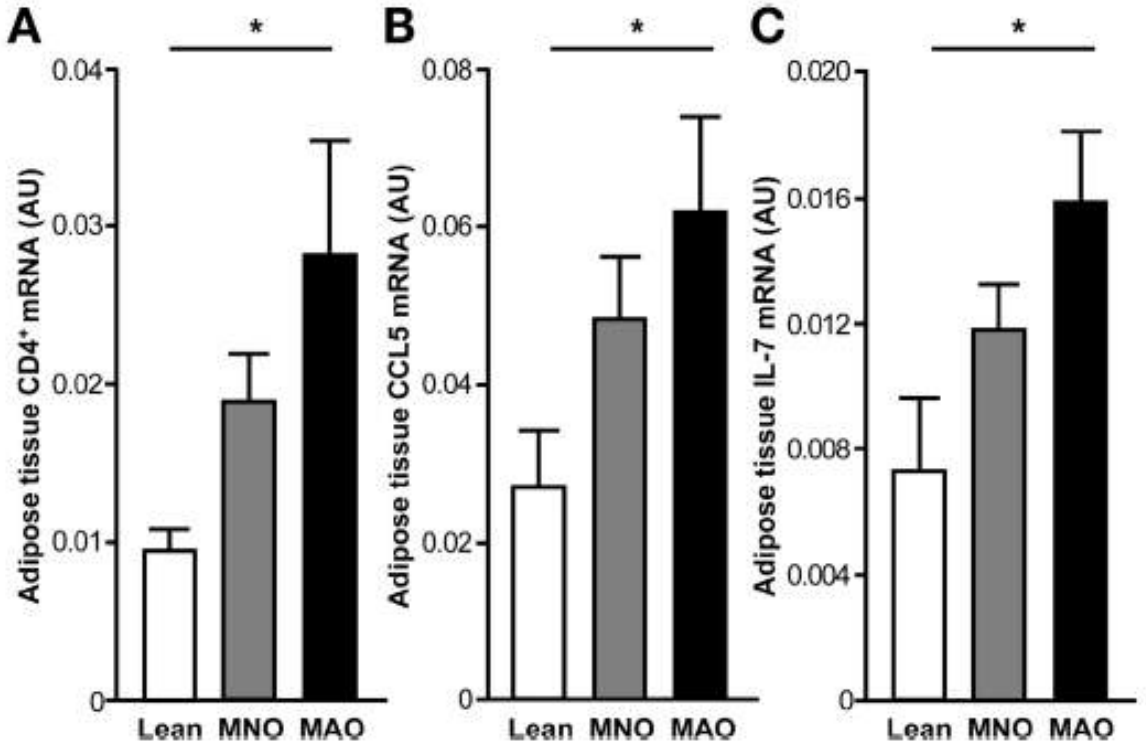

FIGURE 5.2 Adipose Tissue Gene Expression of CD4+ (A), CCL5 (B), and IL-7 (C) in Lean, MNO, and MAO Participants. Adipose tissue messenger RNA values increased progressively from lean to MNO to MAO subjects (*P value for linear trend < 0.05, by one-way analysis of variance on log-transformed data). Values are mean ± SEM.

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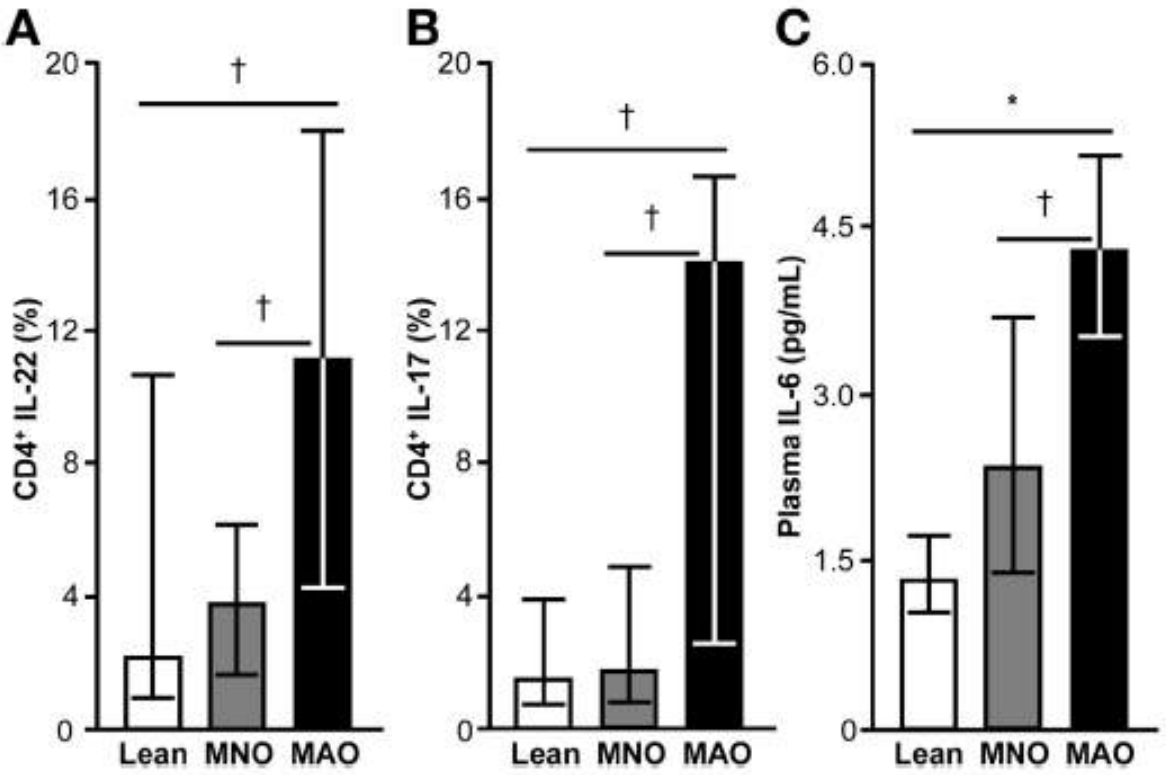

FIGURE 5.3 Adipose Tissue T-cell Polarization and Plasma IL-6 Concentrations in Lean, MNO, and MAO Participants. MAO subjects show a distinctive polarization of CD4+ T cells expanded from subcutaneous adipose tissue towards IL-22 (A) and IL-17 (B) producing cells. Plasma IL-6 concentration, known to stimulate lymphocyte polarization toward the Th17 and Th22 phyenotype, is greater in MAO than both MNO and lean subjects (C). One-way analysis of variance with planned contrasts was used to compare the differences between MAO and the other 2 groups. Values significantly different from the MAO group; $*P < 0.001$, $^{+}P < 0.05$. Values are median and quartiles.

5.3.3. Plasma IL-22 and IL-6 Concentations are Increased in MAO Subjects

Plasma IL-22 concentration was greater in MAO (7.0 ± 4.0 pg/ml) than MNO (2.9 ± 2.2 pg/ml) and lean (3.1 ± 1.9 pg/ml) subjects ($P < 0.05$). We were unable to detect IL-17 in plasma, presumably because of inadequate sensitivty of the assay to detect very low plasma concentrations. Circulating IL-6 stimulates lymphocyte polarization toward the Th17 and Th22 phenotype [473]. Therefore, we measured IL-6 concentration in plasma and found that MAO subjects had greater plasma IL-6 concentrations than both MNO and lean groups, suggesting that increased circulating IL-6 contributes to the characteristic Th17/Th22 phenotype observed in our MAO subjects (Figure 5.3.C). There were no differences between groups in plasma

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concentrations of CCL5 (lean: 47 ± 28 ng/ml, MNO: 56 ± 26 ng/ml, MAO: 55 ± 29 ng/ml; $P > 0.05$) and IL-7 (lean: 10.8 ± 3.2 ng/ml, MNO: 11.8 ± 3.8 ng/ml, MAO: 11.6 ± 4.1 ng/ml; $P > 0.05$).

5.3.4 Human Skeletal Muscle and Liver Express IL-17 and IL-22 Receptors

We evaluated whether the receptors for IL-17 and IL-22 are present in liver and skeletal muscle in human tissue samples because these are the key organs involved in obesity-associated metabolic dysfunction. Skeletal muscle and liver biopsies for this purpose were obtained from a separate cohort of obese subjects undergoing bariatric surgery procedures (Table 5.2). We found that receptors for IL-17 (both IL-17RA and IL-17RC subunits of the heterodimeric IL-17 receptor) and IL-22 (IL-22RA, which is the receptor subunit specific for IL-22) were expressed in both tissues (Figure 5.4).

TABLE 5.2 Characteristics of Subjects From Whom Skeletal Muscle and Liver Biopsies Were Obtained

		Range
Sex, n (*male/female*)	19 (2/17)	
Age, *y*, mean ± SD	43 ± 11	22–66
Body mass index, mean ± SD	47.4 ± 9.0	35.9–68.6
Body fat mass, %, mean ± SD	52 ± 4	44–59
Glucose, *mg/dL*, mean ± SD	95 ± 8	80–108
Insulin, *mU/L*, mean ± SD	25 ± 9	12–46
HOMA-IR, mean ± SD	6.1 ± 2.4	2.6–11.6
Total cholesterol, *mg/dL*, mean ± SD	169 ± 31	114–210
LDL-cholesterol, *mg/dL*, mean ± SD	98 ± 21	59–130
HDL-cholesterol, *mg/dL*, mean ± SD	40 ± 9	26–64
Triglyceride, *mg/dL*, mean ± SD	156 ± 60	71–258

HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein.

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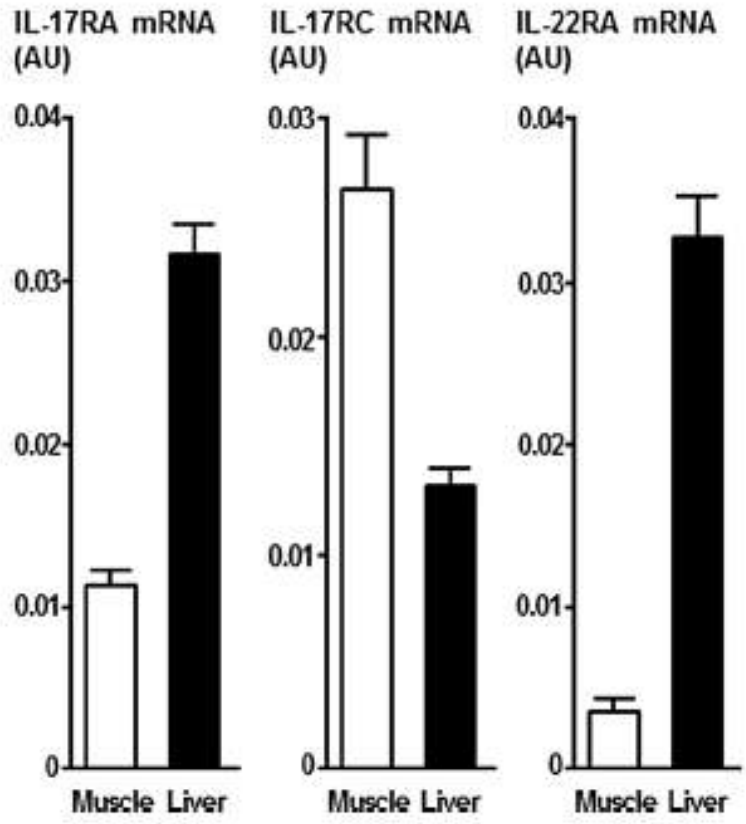

FIGURE 5.4 Gene Expression of Receptors for IL-17 and IL-22 in Liver and Skeletal Muscle Obtained from Human Subjects. Expression of the interleukin receptors IL-17RA, IL-17RC, and IL-22RA was detected and identified in human liver (black bars) and skeletal muscle (white bars) from obese subjects by using quantitative reverse transcription polymerase chain reaction. Results were analyzed by comparing the threshold crossing of each sample after normalization to the housekeeping 36B4 gene.

5.3.5 IL-17 and IL-22 Inhibit Skeletal Muscle Glucose Uptake

We then sought to determine whether IL-17 and IL-22 could affect skeletal muscle glucose metabolism by using an isolated rat muscle system. We found that both IL-17 and IL-22 receptors were expressed in soleus, but not in epitrochlearis muscle strips with either IL-17 or IL-22, and found a marked inhibition of insulin-mediated glucose uptake in soleus (Figure 5.5.A), but no effect on epitrochlearis muscle (Figure 5.5.B), consistent with the presence or absence of IL-17 and IL-22 receptors.

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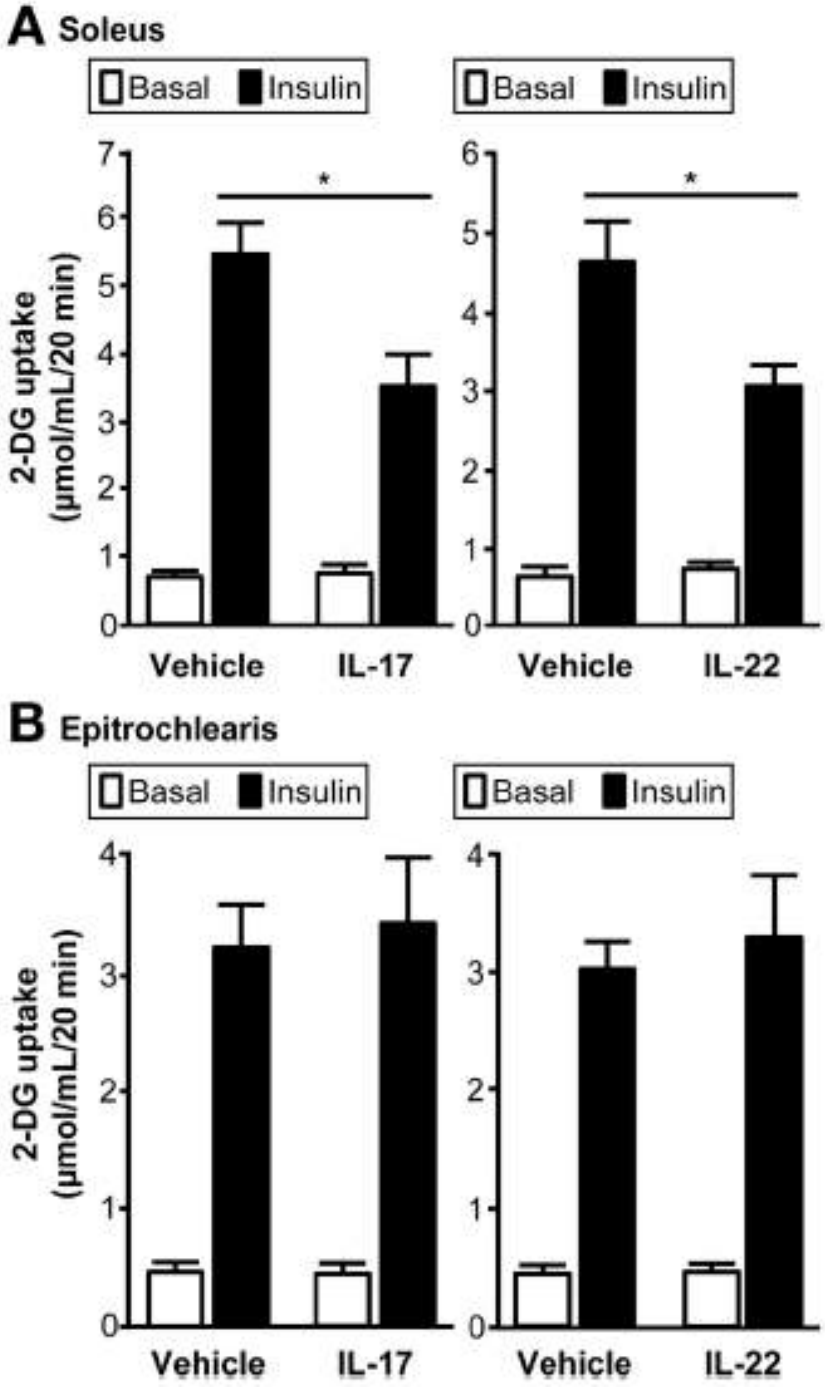

FIGURE 5.5 Effects of IL-17 and IL-22 on Rat Skeletal Muscle 2-deoxyglucose (2-DG) Uptake. Incubation of rat muscle strips with IL-17 or IL-22 inhibits insulin-stimulated increase in glucose uptake in soleus muscle (A), but not in epitroclearis muscle (B). Value for muscle incubated with IL-17 and IL-22 is significantly different than value for muscle incubated with vehicle; $*P < 0.01$. Values are mean ± SEM.

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5.3.6 IL-17 and IL-22 Reduce Insulin Sensitivity in Human Hepatocytes

To further determine if IL-17 and IL-22 are involved in the regulation of insulin action in the liver, the effects of these cytokines on insulin signaling in primary human hepatocytes were evaluated. Treatment with either IL-17 or IL-22 led to diminished phosphorylation of Akt Ser473 in response to insulin stimulation (Figure 5.6.A), demonstrating the inhibitory effect of these cytokines on hepatic insulin signaling. We then determined whether IL-17 or IL-22—mediated impairment in insulin action could cause alterations in hepatocyte glucose metabolism. Two key effects of insulin on hepatic glucose metabolism were examined: insulin-mediated suppression of glucose production and insulin-stimulated increase in anaerobic glucose utilization (glycolysis). Consistent with the observed effects on insulin signaling, the inhibitory effect of insulin on glucose production rate (Figure 5.6.B) and stimulatory effect on glycolytic rate (Figure 5.6.C) were significantly attenuated by pretreatment with either IL-17 of IL-22.

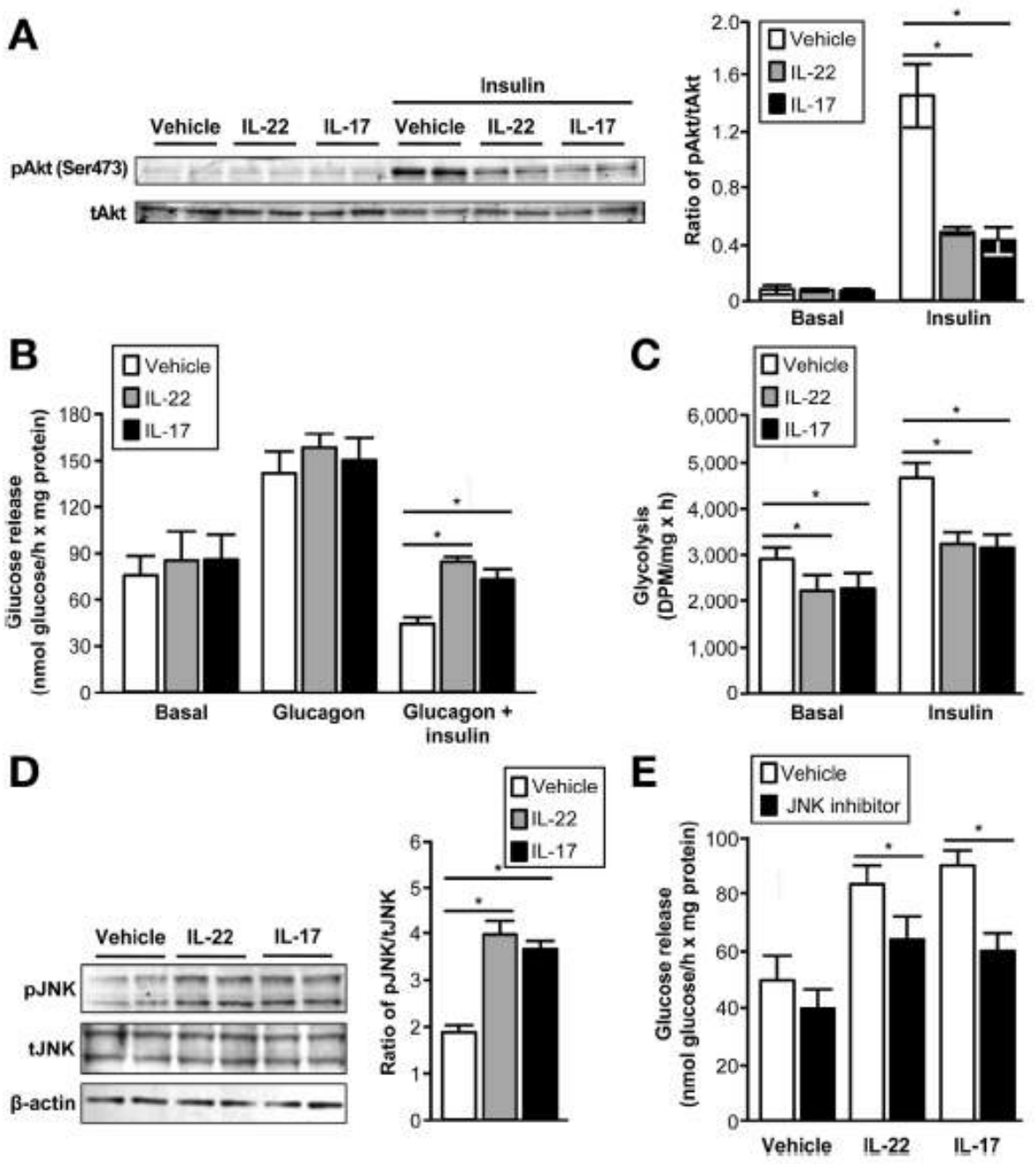

FIGURE 5.6 Effects of IL-17 and IL-22 on Insulin-mediated Glucose Metabolism in Primary Human Hepatocytes. Both IL-17 and IL-22 inhibit insulin-stimulated phosphorylation of Akt (A), insulin-mediated suppression of hepatocyte glucose production (release into the media) (B), and hepatocyte anaerobic glucose metabolism (C). Western blotting analyses demonstrate that both IL-22 and IL-17 increase the phosphorylation of JNK (pJNK); total JNK (tJNK) is not affected by treatment with either cytokine (D). Pretreating human hepatocytes with

a JNK inhibitor (PD098059) attenuated the increase in hepatocyte glucose production caused by IL-17 or IL-22 treatment in the presence of insulin and glucagon (E). Values for hepatocytes, incubated with IL-17, IL-22 or JNK inhibitor are significantly different from vehicle values; $*P < 0.01$. Vales are mean ± SEM.

We next sought to determine whether activation of c-Jun (JNK), which is known to inhibit insulin action in skeletal muscle and liver (34), might explain the observed effects of IL-17 and IL-22 on glucose production. We found that treatment of human hepatocytes with either IL-17 or IL-22 increased the phosphorylation of JNK without affecting totale JNK content (Figure 5.6.D). In addition, pretreating human hepatocytes with a JNK inhibitor (PD098059) attenuated the increase in hepatocyte glucose production caused by IL-17 or IL-22 treatment in the presence of insulin and glucagon (Figure 5.6.E).

5.4 DISCUSSION

5.4.1 Different T-lymphocyte Polarization in MAO Adipose Tissue

Chronic low-grade inflammation is associated with obesity-related metabolic complications, including nonalcoholic fatty liver disease (NAFLD), type 2 diabetes, the metabolic syndrome, and coronary heart disease. The discovery that adipose tissue from both obese mice and human subjects is infiltrated with inflammatory macrophages provided a major breakthrough into understanding how obesity and inflammation are inter-related [286,292]. However, the focus of research on adipose tissue macrophages largely ignored the potential role of other immune cells in obesity-related inflammation. In the present study, we evaluated subcutaneous adipose tissue T-lymphocyte polarization in lean and obese human subjects who were separted into distinct groups based on skeletal muscle insulin sensitivity determined by using the hyperinsulinemic-euglycemic clamp procedure in conjunction with stable-isotopically labeled glucose tracer infusion, and who had differences in intrahepatic triglyceride content. Our data demonstrate greater total CD4+ T lymphocytes toward IL-17 and IL-22—producing cells in subcutaneous adipose tissue of MAO subjects compared with MNO and lean subjects. This specific adipose tissue polarization was partially mirrored by greater plasma IL-22 concentrations in MAO than MNO and lean subjects.

5.4.2 Novel Putative Link between Adipose Tissue Lymphocyte Function

Alterations and Insulin-mediated Glucose Metabolism Inhibition

In addition, we found that both IL-17 and IL-22 receptors are expressed in human liver and skeletal muscle and that both cytokines inhibit insulin-mediated glucose metabolism in these tissues in vitro, thereby providing a novel putative link between alterations in adipose tissue lymphocyte function and metabolic disease. The observation that lymphocytes producing IL-17 and IL-22 are increased in adipose tissue of MAO, but not MNO and lean subjects, supports the potential importance of this specific lymphocyte signature in obesity-related insulin resistance and could help explain why some obese individuals develop metabolic abnormalities and others do not. However, additional studies are needed to determine whether this is a simple association or a true cause-and-effect relationship.

5.4.3 Involvement of JNK in the Pathogenesis of Insulin Resistance

Our data suggest that the mechanism responsible for IL-17 and IL-22—induced insulin resistance is mediated, at least in hepatocytes, by activation of JNK. Data from previous studies have shown that JNK phosphorylates Ser307 on IRS-1, thereby affecting down-stream insulin signaling [474]. Our findings are consistent with data from studies showing that JNK knockout mice are protected from obesity-related insulin resistance and that JNK inhibitors can act as insulin-sensitizing agents [475]. JNK is also a component of the general inflammatory response and is linked to activation of other inflammatory pathways involved in the pathogenesis of insulin resistance and the metabolic dysfunction associated with obesity [475]. Therefore, these data collectively suggest that IL-17 and IL-22 in MAO subjects are involved in the pathogenesis of insulin resistance through increased JNK activity in insulin target tissues.

5.4.4 Increase in Intrahepatic Lymphocyte Production of IL-17 and IL-22 could be Involved in the Development of NAFLD

The effect of IL-17 and IL-22 on JNK signaling [476-478] suggests the existence of an association between Th17 and Th22 polarization and NAFLD. Although the mechanisms responsible for developing steatosis and chronic liver injury in NAFLD are not clear, data from a series of studies suggest that JNK activation is involved in this process. Hepatic lipid accumulation and development of liver-injury in mouse models of diet-induced NAFLD is mediated by JNK [479],

and mice lacking JNK are protected from developing liver steatosis and steatohepatitis [479]. In addition, both mice fed a high-fat and high-calorie diet and patients with nonalcoholic steatohepatitis have an increase in hepatic Th17 lymphocytes, and neutralization of IL-17 reduces hepatic inflammation and injury [469]. The summation of data from the present study and these previous reports support the notion that an increase in intrahepatic lymphocyte production of IL-17 and IL-22 could be involved in the development of NAFLD.

5.4.5 CD4+ Th cell Polarization Observed in Obese Mice is distinct from that Observed in Obese Humans

The results from recent studies conducted in animal models found the distribution of adipose tissue T-lymphocyte subsets is altered by obesity [289,290,467,468]. In obese mice, CD4+ Th cells in adipose tissue are skewed towards a Th1 phenotype. These cells secrete IFN-γ, which stimulates adipose tissue macrophages to produce inflammatory cytokines (tumor necrosis factor—α and IL-6), which can induce insulin resistance. In lean mice, CD4+ Th cells in adipose tissue are skewed toward IL-4—secreting Th2 cells and regulatory T cells, which counteract inflammation and protect against insulin resistance. In contrast, there was no significant difference among our 3 groups of subjects (lean, MNO, and MAO) in the polarization of lymphocytes toward those producing IFN-γ, which would reflect Th1 cells, or those producing IL-13, which would reflect Th2 cells. Similarly, data from a recent study also found no differences in peripheral blood Th1 polarization between obese and lean subjects [480], but found blood lymphocytes were skewed toward a more anti-inflammatory (T regulatory and Th2) phenotype in obese compared with lean people [480]. In addition, data from another study, which evaluated gene expression of markers for T cell subsets from visceral and subcutaneous adipose tissue, found both pro-inflammatory (T cytotoxic and Th1) and protective (T regulatory and Th2) lymphocyte populations were greater in adipose tissue from extremely obese than from lean and overweight subjects, and that the ratio of pro-inflammatory to anti-inflammatory T-cell subsets in visceral adipose tissue favored a protective anti-inflammatory T-cell profile [481]. These results, in conjunction with the data from our study, suggest that the polarization of CD4+ Th cells observed in obese mice (increase in Th1 and reduction in Th2) is distinct from the polarization seen in obese humans.

5.4.6 Expansion of Th17/Th22 Cells in Adipose Tissue of MAO Subjects could

be a Consequence of Increased IL-6 and Adipose Tissue Cytokine Expression

The mechanism(s) responsible for the polarization of adipose tissue CD4+ T cells toward Th17/Th22 is not clear, but could be related to an increase in circulating or adipose tissue cytokines, or both. We found plasma IL-6, which promotes the differentiation of CD4+-naïve T cells into Th17 cells [482], was greater in our MAO than MNO and lean subjects. We also found that adipose tissue expression of CCL5 and IL-17, which stimulate T-cell recruitment and proliferation [470-472], was greater in our MAO than MNO and lean subjects. Therefore, the expansion of Th17/Th22 cells in the adipose tissue of our MAO subjects could be a direct consequence of increased circulating IL-6 and adipose tissue cytokine expression.

5.4.7 Limitations of this Study

This study has several important limitations. First, our data show an association, but do not demonstrate a direct cause-and-effect relationship, between adipose tissue lymphocyte polarization and skeletal muscle or hepatic insulin resistance. Second, the study subjects were primarily comprised of women, so these results might not necessarily apply to men. Third, although we found both liver and skeletal muscle in obese people express IL-17 and IL-22 receptors, we did not determine whether IL-17 and IL-22 receptor expressions differ between lean and obese subjects, and if differences in receptor abundance affect metabolic function. Finally, this study cannot determine if the major ligands for these receptors are derived from circulating IL-17 and IL-22 produced by adipose tissue lymphocytes, or from IL-17 and IL-22 produced locally by specific T-cell subsets that have infiltrated or proliferated in these organs. For example, it has been shown that T regulatory or Th17 cells can infiltrate the liver and either promote [483] or prevent [484] the development of viral hepatitis—induced fibrosis formation. These limitations need to be addressed in future studies.

5.5 CONCLUSION

In conclusion, the accumulation of excessive adipose tissue mass has been considered an important source of pro-inflammatory adipocytokines that contribute to metabolic dysfunction [485,486]. However, not all obese people exhibit metabolic abnormalities, demonstrating that increased adipose tissue alone is not adequate to cause adipose tissue inflammation or metabolic

dysfunction. The results from this study demonstrate that obese people who are metabolically abnormal (characterized by impaired insulin-mediated glucose metabolism) have a characteristic polarization of CD4+ T cells in adipose tissue, which is different from both lean subjects and obese subjects who are metabolically normal (characterized by impaired insulin-mediated glucose metabolism). In addition, the cytokines produced by these lymphocyte subsets cause metabolic dysfunction in vitro in hepatocytes and muscle tissue. Additional studies are needed to determine whether alterations in lymphocyte populations in adipose tissue and possibily other organs are directly involved in the pathogenesis of hepatic and skeletal muscle insulin resistance in obese people.

CHAPTER 6 Further Work and Conclusion

Obesity is a worldwide problem of the modern society. The characterization of the differences in MNO, MAO, and lean subjects is fundamental for a better understanding of this health public issue. The accumulation of excessive adipose tissue mass in obese subjects has been considered an important source of pro-inflammatory adipocytokines that contribute to metabolic dysfunction. But not all obese people are metabolically abnormal, which goes to show that increased adipose tissue alone is not adequate to cause adipose tissue inflammation or metabolic dysfunction. The results from this study showed that obese people who are metabolically abnormal (characterized by impaired insulin-mediated glucose metabolism) have a characteristic polarization of CD4+ T cells in adipose tissue, which is different from both lean subjects and obese subjects who are metabolically normal (characterized by non-impaired insulin-mediated glucose metabolism). Also, this study has shown that the cytokines produced by these lymphocyte subsets cause metabolic dysfunction in vitro in hepatocytes and muscle tissue. This novel putative link is very promising, though additional studies are necessary in order to determine whether alterations in lymphocyte populations in adipose tissue and possibily other organs are directly involved in the pathogenesis of hepatic and skeletal muscle insulin resistance in obese people.

The real-life application of the findings of such complex studies is what truly is of significance. Being able to characterize different types of obese subjects helps us to better understand them and by doing so, leads to better treatment and follow-up, targeted and personalized. In order to further apply such findings in the real life clinical setting, we are in the process of conducting an in-depth short-term (2 to 12 wks) intervention trials, which involve sensitive metabolic characterization (e.g. hyperinsulinemic clamp procedure) in order to effectively test the potential clinical efficacy of novel drug therapy that target specific immune and inflammatory pathways involved in the pathophysiology of obesity.

By studying the effect of moderate weight gain in MAO and MNO, we have demonstrated that moderate weight gain elicits very different physiological responses in MNO and MAO people and that IHTG content can be used to identify obese people who are prone to, or protected from, the development of metabolic disease. Weight gain is associated with the development and worsening of nonalcoholic fatty liver disease (NAFLD), but the mechanisms responsible for this

association are not known. By combining imaging and stable isotope tracers techniques to evaluate the effect of moderate weight gain in obese people on intrahepatic triglyceride (IHTG) content and hepatic lipid metabolism in order to elucidate the mechanisms responsible for weight-gain induced IHTG accumulation, we demonstrated that weight gain causes an imbalance between hepatic availability and disposal of fatty acid (increase in DNL, reduction of fatty acid oxidation, inadequate increase in VLDL secretion) which are likely responsible for increased IHTG accumulation.

The findings from this study support the need for more aggressive weight-management therapy and stricter follow-up in the subset of obese people who have NAFLD and are at high risk for continued deterioration of metabolic function with additional weight gain.

In order to continue on this path, we are currently in the process of completing a clinical research study entitled "Progressive Weight Loss". It is well known that obesity is associated with alterations in cardiometabolic functions that are risk factors for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD), including: insulin resistance, dyslipidemia, ectopic fat accumulation, adipose tissue and systemic inflammation, increased blood pressure, altered cardiac structure and function, impaired endothelial function, arterial stiffness, and atherosclerosis. Furthermore, obesity is associated with changes in the gut microflora, which may be mechanistically linked to the development of obesity-related metabolic alterations. In addition, obesity is associated with alterations in brain structure and functioning, which may adversely affect quality of life. A widely accepted principle of obesity therapy is that moderate weight loss of ~5% has important metabolic benefits and may lead to improvements in brain functions such as working memory, processing speed, and declarative memory. However, there is some evidence that it may not be enough for improving all metabolic abnormalities. The specific amount of weight loss necessary to improve each metabolic variable and the exact mechanisms by which weight loss improves metabolic function, cardiovascular health and mental health are not known. This gap in our knowledge has important clinical implications because this information is necessary to establish appropriate specific weight loss targets for obese patients. Furthermore, it will improve our understanding of the mechanisms responsible for the pathophysiology of obesity. The hypothesis of this study is that there will be a dose-response relationship between the amount of weight loss and improvement for each variable, but

that the minimum weight loss threshold for achieving a therapeutic effect will vary among organ systems. Accordingly, the overall aim of this study is to conduct a randomized, controlled trial in metabolically abnormal obese men and women (defined as men and women with insulin resistance - assessed by using the Homeostatic Model Assessment of Insulin Resistance [HOMA-IR]) to determine the effect of progressive amounts of diet-induced weight loss on cardiometabolic risk factors and brain functioning. Participants will be randomized to either: 1) a weight loss group and undergo progressive calorie restriction therapy to achieve 5%, 10%, and 15% weight loss or 2) a control group (usual diet and weight maintenance).

In terms of the body composition the hypothesis of this study is that weight loss will result in decreased total body fat mass (FM), fat-free mass (FFM), abdominal adipose tissue (intra-abdominal and subcutaneous) volume, and intrahepatic and intramyocellular triglyceride (TG) content; and that the magnitude of these effects will be directly related to the amount of weight loss. Total body FM and FFM will be measured by using dual-energy X-ray absorptiometry (DEXA), subcutaneous and intra-abdominal adipose tissue volume by using magnetic resonance imaging (MRI), and intrahepatic and intramyocellular TG contents by using proton magnetic resonance spectroscopy (MRS).

In terms of β-cell function, insulin sensitivity and factors associated with insulin resistance in key metabolic organs, the hypothesis of this study is that weight loss will improve β-cell function, insulin sensitivity in liver (suppression of glucose output), skeletal muscle (stimulation of glucose uptake), and adipose tissue (suppression of lipolysis), increase skeletal muscle ATP production, and decrease body core temperature; that the magnitude of the improvements will be directly related to the amount of weight loss; and that the threshold for improvements will be different for different variables. We will test these hypotheses by using a 2 hour oral glucose tolerance test and the euglycemic-hyperinsulinemic clamp technique in conjunction with stable isotope labelled tracer infusions and muscle and adipose tissue biopsies (to assess the activity of cellular signalling pathways and tissue factors involved in insulin action), 24 hour body core temperature monitoring, and MRS to quantitate skeletal muscle ATP production.

In terms of cardiovascular health, the hypothesis of this study is that weight loss will have beneficial effects on the plasma lipid profile (i.e., reduce TG and total and LDL-cholesterol concentrations and increase HDL-cholesterol concentration), lower systolic and diastolic blood

Gemma Fraterrigo

pressure, increase heart rate variability, decrease carotid artery intima-media thickness, and improve left ventricular structure and function, arterial elasticity and endothelial function; that the magnitude of these effects will be directly related to the amount of weight loss; and that the threshold for improvements will be different for different variables. These hypotheses will be tested by measuring fasting plasma lipid concentrations, evaluating left ventricular structure and function by using transthoracic echocardiography, measuring central blood pressures and central aortic compliance by using the non-invasive SphygmoCor system, determine carotid artery intima-media thickness by using ultrasound, brachial artery reactivity testing (BART) to evaluate endothelial function, and 24-h Holter (blood pressure and ECG) monitoring.

In terms of brain structure and function**,** the hypothesis os this study is that weight loss will, in a dose-dependent manner increase cortical thickness and volume in frontal and temporal regions and improve cognitive performance (including memory, processing speed, and cognitive control) and neuronal functioning (as evidenced by an increase in cerebral perfusion, a reduction of compensatory blood oxygen level dependent [BOLD] activity, and an increase in functional connectivity of the default network). These hypotheses will be tested by using a comprehensive battery of traditional neuropsychological tests in combination with functional magnetic resonance imaging (fMRI).

The background and significance of this study lies in the fact that the most common serious complications associated with obesity involve alterations in metabolic function and inflammation that are risk factors for T2DM, CVD and mortality (i.e., impaired β-cell function, insulin resistance, dyslipidemia - characterized by increased serum TG and decreased serum HDL-cholesterol concentrations, non-alcoholic fatty liver disease [NAFLD], adipose tissue and systemic inflammation, increased blood pressure and heart-rate variability, and decreased vascular health) [487-495]. In addition, obesity is associated with alterations in brain structure and functioning (including decreased global brain volumes[496,497], hypometabolism in prefrontal regions[498], and alterations in behavioral and blood oxygen level dependent activity on cognitive tasks and eating-related paradigms[499,500]) which may adversely affect health outcomes, quality of life, and functional ability[501-503].

Diet-induced weight loss is the cornerstone of treatment for obese persons, and can improve cardiometabolic and inflammatory abnormalities in most patients [237,401,504-511]. However, the minimum amount of weight loss necessary to achieve improvements in each cardiometabolic risk factor is not known. Furthermore, it is not known whether there is a dose-response relationship between the amount of weight loss and improvements in metabolic function. Understanding the effect of weight loss on metabolic function is further complicated because of conflicting results from different studies. For example, we have found that short-term (2 days) calorie restriction improves hepatic insulin sensitivity whereas skeletal muscle insulin sensitivity does not improve until greater weight loss (~7%) is achieved [237]. However, the results from another study indicate that even ~8% weight loss results in no change in whole-body insulin-mediated glucose uptake[512] whereas other investigators reported a two-fold improvement with similar amounts of weight loss[513] but only a relatively small improvement (~25%) after ~15% weight loss[514]. The reason for the differences between studies is not clear, but might be due to differences in participant characteristics and methods used to assess insulin sensitivity. Few studies have evaluated the effect of progressive amounts of weight loss on metabolic function and inflammation and the results are conflicting. Cross-sectional comparison of the weight loss induced changes in insulin sensitivity in participants who lost different amounts of body weight during weight loss therapy indicates that there is a dose-response relationship between the amount of body weight lost and changes in insulin sensitivity (judged by basal plasma glucose and insulin concentrations)[515] and blood pressure [516] whereas the results from a longitudinal study indicate that whole-body insulin sensitivity, judged by basal plasma glucose and insulin concentrations, changes with ~5% weight loss and further weight loss up to ~10% resulted in no additional benefit[517].

The relationship between obesity and cardiovascular health is well known [495,518-523], but the effect of weight loss on cardiovascular structure and function are unclear[511,524-529]. Some studies indicate improvements in blood pressure and flow-mediated dilation (FMD) of the brachial artery, an index of endothelial function, and the Augmentation Index, which reflects arterial stiffness [524,525,527] whereas other studies report no changes in these parameters [511,526-529]. The reason for the discrepancies in results is unclear but might be related to subject characteristics and the degree of weight loss.

It is also not known whether there is a dose-response relationship between the amount of weight loss and parameters that are thought to be involved in mediating the weight loss-induced changes in metabolic function (e.g., ectopic fat accumulation) and inflammation. For example, liver volume [530] and intrahepatic triglyceride content [237] change rapidly (nearly all of the effect occurs within days of the initiation of calorie restriction) whereas visceral adipose tissue mass declines progressively with increasing amounts of weight loss[530]. On the other hand, it has been suggested that weight loss in excess of 10% is needed to induce changes in markers of inflammation[531]. A comprehensive and simultaneous evaluation of the effect of progressive amounts of weight loss on cardiometabolic function has not been performed but is necessary to determine ideal therapeutic effects and to evaluate the relationship between factors purported to be responsible for the pathophysiology of obesity and weight-loss induced improvements in metabolic function. Our study will fill this gap in our knowledge.

Alterations in free fatty acid (FFA) metabolism, and possibly impaired muscle mitochondrial oxidative capacity and mitochondrial function [532-537], are likely a major factor in the pathogenesis of insulin resistant glucose metabolism, dyslipidemia and ectopic fat accumulation (increased intracellular TG present in "non-adipose tissues" such as liver and muscle) in obesity [245,246,493]. Excessive release of FFA from adipose tissue into plasma and increased plasma FFA concentration can impair the ability of insulin to stimulate muscle glucose uptake[247], and suppress hepatic glucose production[248]. It has been proposed that the cellular mechanisms responsible for FFA-induced insulin resistance in skeletal muscle likely involves an increase in intramyocellular fatty acid metabolites, including diacylglycerol (DAG) and ceramide, which interfere with insulin action by activating protein kinase C and mTOR [249], inhibiting Akt, and ultimately preventing the translocation of GLUT-4 from the cytoplasm to the cell membrane for glucose transport[245,250-254]. In addition, activation of the nuclear factor kappa B (IκB) pathway, a major pro-inflammatory pathway [255], has also been implicated in mediating the FFA- induced insulin resistance[251]. This effect is mediated by PKC[256-259], via direct phosphorylation of IκκB[260] or by increased production of reactive oxygen species (as a result of excessive intracellular fatty acid availability), which activate IκB-kinase (IKK-β)[251]. IκB activation has also been shown to impair insulin-induced glucose uptake via mTOR mediated serine phosphorylation of IRS1[261]. Phosphorylation by IKK-β is considered the main pathway by which IκB-α is released from NFκB and subsequent movement of NFκB from the cytosol to the nucleus. Furthermore,

excessive ectopic fat accumulation, due to redirecting FFA uptake and TG synthesis away from adipose tissue and toward liver and muscle[4], is associated with insulin resistance in those tissues[153,225-228,230]. Moreover, excessive release of FFA from adipose tissue into plasma and redirecting FFA uptake away from adipose tissue and toward liver and muscle is thought to be largely responsible for excessive ectopic fat accumulation (i.e., increased intracellular TG present in "non-adipose tissues" such as liver and muscle), stimulates hepatic very low-density lipoprotein TG production, leading to dyslipidemia [110,228,237,538]. Adipose tissue remodeling is also likely involved in the pathogenesis of some of the metabolic abnormalities associated with obesity[292]. Adipocyte hypertrophy is associated with adipose tissue inflammation and increased release of pro-inflammatory cytokines (adipokines) that can induce insulin resistance, dyslipidemia and steatosis[285-287,292,293]. Lastly, there is some evidence that the changes in gut microflora associated with obesity, e.g. a greater abundance of firmicutes and fewer bacteroidetes [539]) may be mechanistically linked to host energy metabolism by increasing energy uptake from the diet, regulating tissue FFA composition, uptake, storage and oxidation, and activating innate immunity and hepatic fibrogenesis [540].

Weight loss improves working memory, processing speed, and declarative memory [541-543]. However, the effect of weight loss on neuroimaging indicators of brain health (such as BOLD activity during challenging cognitive tasks etc.) has not been evaluated. A better understanding of the mechanisms responsible for improved brain functioning will provide critical information regarding the pathophysiology of obesity-related alterations in brain functioning and potential prevention strategies that reduce the risk of more serious dysfunction as patients advance in age [544].

Therefore this ambitious multidisciplinary study will provide a comprehensive evaluation of the effect of progressive amounts of diet-induced weight loss on cardiometabolic function and inflammation. This information will hopefully be important for developing realistic and relevant individualized therapeutic weight loss targets for obese patients, and help clinicians appreciate what can be expected from different weight loss outcomes. In addition, determining the minimal weight loss threshold for achieving a therapeutic effect in different organ systems, and evaluating the temporal relationship between changes in factors purported to be responsible for the pathophysiology of obesity and observed improvements *in vivo* in metabolic function will

potentially provide new insights into the mechanisms responsible for the pathophysiology of obesity.

CHAPTER 7 References

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Gemma Fraterrigo

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Gemma Fraterrigo

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