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Application of genetic polymorphism & gene experience of R-SPONDIN3 as biomarker of cardiometabolic traits associated with or without obesity in sample of Sudanese patients in Khartoum state.

By

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MSc. in Medical Laboratory Sciences (Clinical Chemistry) Omdurman Islamic university (2013)

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الاية

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(قُلْ هُوَ الَّذي أَنشَأَكُمْ وجعل لَكُمُ السَّمْعَ وَالأَبْصَارَ وَالأَفْئِدَةَ قَليلا مَّا تَشْكُرُونَ)

صدق الله العظيم الملك (23)

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I'm **Ashraf Mohammed Adam Alkinain under signed; I declare and affirm that this Thesis is our own original work. We have followed all ethical and technical principles in the preparation, data collection, data analysis and compilation of this Thesis. Any scholarly matter that is included in the Thesis has been given recognition through citation. I solemnly declare that this Thesis has not been submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.**

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Application of genetic polymorphism & gene experience of R-SPONDIN3 as biomarker of cardiometabolic traits associated with or without obesity in sample of Sudanese patients in Khartoum state.

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Dedication

This work is dedicated to my

Wonderful Father,

The symbol of love and giving

To my Parents

Who gave me Light

To my Teachers

Who taught me wrong from Right

To my brother, sisters,

Who always with me

To Those Who Have,

And Always Will,

Stand Beside me

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ABSTRACT

Obesity is a major risk factor for the development of cardiovascular disease. A growing database of clinical evidence implicates intra-abdominal adiposity as a powerful driving force for elevated cardiometabolic risk. Addressing intra-abdominal adiposity should play a central role in future strategies aimed at improving cardiovascular outcomes in patients with abdominal obesity and its associated cardiometabolic risk in Sudan.This research is focusing on a particular gene called R-SPONDIN3, which is play a part in controlling fat distribution, cardiac growth through modulation of Wnt (Wnt/β-catenin signaling path way) signaling R-SPONDIN3 is essential for coronary artery formation in the developing heart.

The study was including (300) participants (156 males and 144 females) classified into (3) groups. The first group was including (100) participants with abdominal obesity (obese), the second group was including (100) participants already diagnosed with CVD entangled with obesity (Heart Group as positive control group), while the third group was including (100) healthy lean volunteers (negative control group). All the participants their age group between (27 to 63 years) old. BMI and WHR were taken for all subjects too. The study was carried out during the period from August 2016 to March 2019. For detection the mutation in RSPO3 gene, Conventional PCR was done for control, obesity and heart subjects respectively and followed by Real Time PCR for all of them to measure the amount of gene expression. The findings of this study showed Conventional PCR results were significantly different ($P < 0.001$) in heart group subjects as compared to healthy controls and obese group. Among heart group, mutation was detected in some subjects (19%) and the rest without mutation (81%) but in obese group no mutation was detected. Comparison between the different studied groups according to gene expression showed significant differences ($P < 0.001$) mean value of gene expression in healthy group subjects was (1.0 ± 0.0), Obesity group was (2.44 ± 0.50) and heart group subjects was (4.54 ± 0.87) respectively.

The result of the current study adopted that there was correlation between mutation in R-SPONDIN3 gene and abdominal obesity but this mutation was showed only in heart group so the mutation of R-SPONDIN3 has significant effect in obese patients whom having CVD and no

mutant SPONDIN3 gene in obese . Also the amount of R-SPONDIN3 gene expression among the obese and CVD patients revealed significant difference and the amount of gene expressing among the CVD patients is higher than obese which is suggested that the amount of gene expressed in obese patients with heart disease more than obese patients without cardiovascular complications .Also the results showed a weak correlation between the amount of genes expressed among obese and CVD patients with BMI, WHR and age. Also the elevated the amount of gene expressed has significant affect on the fat phenotype (Visceral fat and subcutaneous fat) and was insignificant on Gluteal Fat.

Key Words: R-SPONDIN3 gene, abdominal Obesity, CVD.

ملخص الدراسة

السمنة هي أحد عوامل الخطر الرئيسية لتطوير أمراض القلب والأوعية الدموية. قاعدة بيانات متزايدة من الأدلة السريرية تشير إلى السمنة داخل البطن كقوة دافعة قوية لارتفاع مخاطر امراض القلب. معالجة السمنةالبطنيه يجب أن تلعب دوراً مركزياً في الاستراتيجيات المستقبلية التي تهدف إلى تحسين النتائج المتعلقهبالامراض القلبية الوعائية في المرضى الذين يعانون من السمنة في منطقة البطن ومخاطر أمراض القلب المرتبطة بها في السودان. تهدف العديد من الدراسات إلى تحديد بعض العوامل التي تتحكم في حجم ووظائف المناطق المختلفة من الدهون. يركز البحث على جين معين يسمى R-SPONDIN3، والذي يلعب دوراً في التحكم في توزيع الدهون ونمو القلب من خلال تعديل طريقة مسار الإشارة Wnt (Wnt / β-catenin)، ويشير إلى أن R-SPONDIN3 ضروري لتشكيل الشريان التاجي في تطوير القلب.

شملت الدراسة 300 مشارك (156 ذكور و144 إناث) مصنفة ضمن ثلاث مجموعات. كانت المجموعة الأولى تضم مائة من المشاركين الذين يعانون من السمنة في منطقة البطن (السمنة)، وكانت المجموعة الثانية تضم مائة من المشاركين الذين تم تشخيصهم بالفعل بأنهم مرضى الأمراض القلبية الوعائية متشابكين مع السمنة (مجموعة القلب كمجموعة سيطرة إيجابية) ، في حين كانت المجموعة الثالثة تضم مائة من المتطوعين الأصحاء. (مجموعة مراقبة سلبية). جميع المشاركين تتراوح أعمارهم بين 27 إلى 63 سنة. تم أخذ مؤشر كتلة الجسم وWHR لجميع المواد أيضا. أجريت الدراسة خلال الفترة من أغسطس 2016 إلى مارس 2019.للكشف عن الطفرة في الجين RSPO3 ، تم إجراء PCR التقليدية للسيطرة ، والسمنة والقلب على التوالي وعلى التوالي تليها PCR الوقت الحقيقي لجميعهم لقياس كمية التعبير الجيني.

أظهرت نتائج هذه الدراسة أن نتائج PCR التقليدية كانت مختلفة بشكل كبير (P<0.001) في موضوعات مجموعة القلب بالمقارنة مع الضوابط الصحية والمجموعة السمينة. تم الكشف عن طفرة مجموعة القلب في بعض المواد (19 ٪) والباقي دون طفرة (81 ٪) . أظهرت المقارنة بين مختلف المجموعات المدروسة وفقا للتعبير الجيني اختلافات معنوية (P<0.001) متوسط قيمة التعبير الجيني في مجموعة صحية كانت 1.0 ± 0.0، وكانت مجموعة السمنة 2.44 ± 0.50 وكان عدد أفراد مجموعة القلب 4.54 ± 0.87 على التوالي.

أثبتت نتائج الدراسة الحالية أن هناك علاقة بين الطفرة في جين R-SPONDIN3 والسمنة في منطقة البطن، ولكن هذه الطفرة لم تظهر إلا في مجموعة القلب ،

وبالتالي فإن طفرة R-SPONDIN3 لها تأثير كبير في المرضى البدناء الذين يعانون من الأمراض القلبية الوعائية. لا يوجد جينات R-SPONDIN3 متحولة في السمنة. كما أن كمية التعبير الجيني R-SPONDIN3 بين مرضى السمنة والمرض القلبي الوعائي تظهر بشكل كبير ومقدار كمية الجين المعبر بين مرضى الأمراض القلبية الوعائية أعلى من البدانة التي تشير إلى أن كمية الجين المعبر عنها في البدينات المصابة بأمراض القلب أكثر من البيناتين البدينين دون مضاعفات القلب والأوعية الدموية. كما أظهرت النتائج وجود ارتباط ضعيف بين كمية الجين المعبر عنها بين مرضى السمنة والمرض القلبي الوراثي مع BMIو WHR والعمر. كما أظهرت النتائج أن مقدار الجين المعبر له تأثير كبير على النمط الظاهري من الدهون (الدهون الحشوية والدهون تحت الجلد) له تأثير ضئيل على الدهون الألوية.

Table of contents

List of Tables

List of Figures

List of Abbreviations

ACE	Angiotensin-Converting Enzyme
AGN	Angiotensinogen
APC	Adenomatosis Polyposis Coli
ApoB	Apolipoprotein B
ATR-1	Angiotensin II Type 1 Receptor
BIA	Bioelectric Impedance Analysis
BMI	Body Mass Index
CABG	Coronary Artery Bypass Surgery
CAD	Coronary Artery Disease
CCK	Cholecystokinin
CE	Cholesterol Esterase
CHF	Congestive Heart Failure
CNS	Central Nervous System
CRD	Cysteine-Rich Domain
CRP	C-Reactive Protein
CRSTDN1	Cysteine-Rich And Single Thrombospondin Domain Containing-1
CRT	Cardiac Resynchronization Therapy
CT	Computed Tomography
CTD	Carboxy-Terminal Domains
CVD	Cardiovascular Disease

DAAM	Dishevelled-Associated Activator of Morphogenesis
DEXA	Dual-Energy X-Ray Absorptiometry
ECG	Electrocardiogram
EDTA	ethylene di-amine tetra-acetic acid
ER	Endoplasmic Reticulum
EWAT	Epididymal White Adipose Tissue
FC	Functional Connectivity
FFA	Free Fatty Acid
Fz	Frizzled
GF	gluteal Fat
GH	Growth Hormones
GLP-1	Glucagon-Like Peptide 1
GPO	glycerol phosphate oxidase
GSK3	Glycogen Synthase Kinase 3
GWAS	Genome-Wide Association Studies
HDL	High-Density Lipoprotein
HF	Heart Failure
HPA	Hypothalamic-Pituitary-Adrenal
HPAL	Hypothalamus
HPSCs	Hematopoietic Stem Cells
hs-CRP	High Sensitivity C-Reactive Protein
IDKK1	Inhibitor Dickkopf-1
IGFBP3	Insulin Like Growth Factor+Insulin-Binding Protein 3

IHD	Ischemic Heart Disease
IPC	Ischemic Preconditioning
LAD	Left Anterior Descending
LDL	Low-Density Lipoproteins
LRP	Receptor-Related Protein
LRRCGCR	Leucine-Rich Repeat-Containing G-Protein-Coupled Receptor
LVH	Left Ventricular Hypertrophy
MD	Mesolimbic Dopamine
MetS	Metabolic Syndrome
MI	Myocardial Infraction
MPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
NAc	Nucleus Accumbens
NFQ	non-fluorescent quencher
NIDDK	National Institute Of Diabetes, Digestive And Kidney Diseases
NO	Nitric Oxide
NOMO	Non-Syndromic Obesity
NTD	Amino-Terminal Domains
NT-proBNP	N-Terminal Pro B-Type Natriuretic Peptide
ONC	One Negative Control
PAD	Peripheral Artery Disease
PCI	Percutaneous Coronary Intervention
PCP	Planar Cell Polarity

PDsh	Phosphoprotein Dishevelled
PET	Positron Emission Tomography
POMC	Proopiomelanocortin
PUFAs	Polyunsaturated Fatty Acids
PWS	Prader-Willi Syndrome
PYY	Peptide YY
QA	Quality Assurance
ROCK	Rho Activates Rho-Associated Kinase
RSPO3	Roof Plate Spondin 3
RTK	Receptor Tyrosine Kinase
S MDSCs	Skeletal Muscle-Derived Stem Cells
SAD	Sagittal Abdominal Diameter
SAH	Supine Abdominal Height
SDS	Sequence Detection System
SF	Subcutaneous Fat
SNP	Single Nucleotide Polymorphisms
SSG	Serum Separator Gel
T2DM	Type 2 Diabetes Mellitus
TGs	Triglycerides
TSR-1	Thrombospondin Type 1 Repeat
VAT	Visceral Adipose Tissue
VLDLs	Very Low-Density Lipoproteins
VMPFC	Ventromedial Prefrontal Cortex

Wg	Wingless
WHO	World Health Organization
WHR	Waist–Hip Ratio
WIF	Wnt Inhibitory Factor

Chapter one

Introduction

1.1 Introduction

Obesity is defined as a body fat content of more than (20 %) in average adult males and over (30%) in females. [(1)] However, obese individuals vary in the amount of excess fat that they store, the regional distribution of that fat within the body, and the related health consequences differ noticeable amongst these obese persons. It is therefore essential to make a distinction between those at augmented risk as a result of abdominal obesity from those with general obesity. Even thought most epidemiological studies have only used BMI as a predictor of disease, as assessed by measurement of waist circumference or waist – hip ratio, are at a greater risk of cardiometabolic risk.

Abdominal obesity also known as central or visceral obesity is one of the essential characteristics of metabolic syndrome. There is a strong relationship between visceral fat (android obesity phenotype) and CVD. [(2)] Visceral fat is technically excess intra-abdominal adipose tissue accumulation. In other words, it's known as a "deep" fat that's stored further underneath the skin than "subcutaneous" belly fat. It's a form of gel-like fat that's wrapped around major organs, including the liver, pancreas and kidneys.

If having a protruding belly and large waist, that's a clear sign, you're storing dangerous visceral fat. While it is most noticeable and pronounced in obese individuals, anyone can have visceral fat, many without even knowing it.

Visceral fat is specially dangerous because, as it will be found out, these fat cells do more than just sit there and cause the body in pants to feel tight — they also change the human your body operates.[(3)]

Carrying around excess visceral fat is linked with an increased risk for Coronary heart disease. Visceral fat is considered toxic and spells double-trouble in the body because it's capable of provoking inflammatory pathways, plus signaling molecules that can interfere with the body's normal hormonal functions. In fact, it acts almost like its very organ since it's capable of having such a large impact on the body. [(4)]

Fat cells do more than simply store extra calories — but have proved to be much more involved in human physiology than in previously thought. It is well know that fat tissue itself acts like its own

organ by pumping out hormones and inflammatory substances. Storing excess fat around the organs increases production of pro-inflammatory chemicals, also called cytokines, which leads to inflammation; at the same time, it interferes with hormones that regulate appetite, weight, mood and brain function.[5]

Genetic study is needed for overweight person. Discovery of genes associated with obesity are all arguments reinforcing the genetic dimension of abdominal obesity. There is also evidence that a high level of cardiorespiratory fitness is predictive of a reduced cardiovascular disease (CVD) risk, independently from its association with a more favorable cardiometabolic risk profile. There is now considerable evidence supporting the notion that obesity is a heterogeneous condition. Such heterogeneity appears to be explained, to a very significant extent, by individual differences in regional body fat distribution, particularly in visceral adipose tissue accumulation. In addition to visceral adiposity as key drivers of the cardiometabolic risk associated with overweight/obesity also contribute to the risk of various cardiovascular outcomes, and further work should clarify their specific functions. Studying these relationships between mutation of RSPO3 gene and CVD lead to lose weight by understand the biology underlying body weight regulation and hope that these strategies contribute to intervene more efficiently in the development of prescription drugs are better able to reduce the weight. The RSPO3 gene may not affect the overall weight or body mass index, but also affects the distribution of fat and reduce or raise the risk of cardiovascular disease, suggesting that different types of measurements can provide insight into the process of losing weight.

R-Spondin 3 (RSPO3) also called cysteine-rich and single thrombospondin domain containing-1 (CRISTIN1), Protein with TSP type-1 repeat (PWTSR), is a member of the R-Spondin protein family. R-spondins (RSPO) are a recently discovered secretory protein family with (4) members in human. RSPO3 is the activator of the β-catenin signaling cascade, leading to TCF-dependent gene activation. RSPO3 acts both in the canonical Wnt/beta-catenin-dependent pathway and in non-canonical Wnt signaling pathway, probably by acting as an inhibitor of ZNRF3, an important regulator of the Wnt signaling pathway. RSPO3 also acts as a ligand for frizzled FZD8 and LRP6 and may negatively regulate the TGF- β pathway. This gene belongs to the R-spondin family. The encoded protein plays a role in the regulation of Wnt (wingless-type MMTV integration site family)/beta-catenin and Wnt/planar cell polarity PCP signaling pathways, which are involved in

development, cell growth and disease pathogenesis RSPO3: Activator of the β-catenin signaling cascade, leading to TCF-dependent gene activation. Acts both in the canonical Wnt/β-catenin-dependent pathway and in non-canonical Wnt signaling pathway, probably by acting as an inhibitor of ZNRF3, an important regulator of the Wnt signaling pathway. Acts as a ligand for frizzled FZD8 and LRP6. May negatively regulate the TGF-beta pathway. Belongs to the R-spondin family. (2) Isoforms of the human protein are produced by alternative splicing. [6]

Coronary arteries are essential to support the heart with oxygen and coronary diseases are the leading cause of death worldwide. Identifying the signaling pathways involved in the formation and specification of coronaries is therefore essential, as it could inspire novel regenerative treatments for cardiac diseases. The coronary arteries are derived from the vascular plexus of the heart that arises at E (11.5) and is remodeled until the postnatal period. An integral part in this remodeling process is arterial venous differentiation. Arterial specification in the embryonic vasculature and postnatal vessels of the retina appears to require specific activation of the transcription factor SOX17. How coronary specification is acheived and – more importantly – which signaling molecules drive this process remains elusive. Here we identify R-spondin3 (Rspo3), a secreted activator of β – catenin signaling, as a crucial regulator of coronary artery differentiation. RSPO3 deficient embryos die early in development due to vascular defects in the Heart and conditional deletion of RSPO3 in the heart with the Islet1-Cre cause's impaired development of the secondary heart field. However, RSPO3 expression persists in angiogenic regions with high Wnt/β-catenin signaling in the heart. Temporal deletion of RSPO3 (11.5) days post coitum with the ubiquitously expressed cCAGCreERT2 line leads to a complete absence of the coronary arteries and a drastic reduction in proliferation of the compact myocardium. Closer inspection of Rspo3 expression reveals it is specifically expressed in the cardiomyoblasts surrounding the first order branch vessels of the left and right coronary arteries, and that Sox17 is highly expressed in the endothelial cells of these vessels. Ablation of RSPO3 leads to decreased Wnt/Bcatenin signaling and, consequently, a significant reduction of Sox17 in these vessels. These results identify RSPO3 as a key regulator of arterial/venous differentiation in the first order branch vessels of the heart by controlling the expression of Sox17 in a Wnt/B-catenin-dependentfashion. Body fat distribution is a heritable trait that independently predicts type 2 diabetes and cardiovascular risk. Genome-wide association studies (GWAS) meta-analyses have identified sexually dimorphic associations, with greater effect in women, between loci within RSPO3 (e.g.

rs9491696) and BMI-adjusted waist-to-hip ratio (WHR). RSPO3 is a LGR4 receptor ligand and a Wnt/β-catenin signalling agonist. Consistent with a role in modulating regional adiposity, RSPO3 expression was higher in abdominal vs gluteal the WHR-increasing allele (G) at rs9491696 was associated with ~2-fold higher RSPO3 expression in both abdominal Tissue, despite being associated with increased android and reduced leg fat mass. Accordingly, RSPO3 had distinct effects on abdominal and gluteal biology due, in part, to differential modulation of Wnt/β-catenin signalling. Specifically, ectopic RSPO3 expression led to increased proliferation selectively in abdominal APs and adipogenesis was impaired in both abdominal and gluteal RSPO3 over-expressing cells. RSPO3 signals through LGR4 to differentially regulate abdominal and gluteal adipose progenitor cell proliferation and adipogenesis, thereby modulating body fat distribution. [7]

1.2 Rationale

Obesity is a major risk factor for the development of CVD. A growing database of clinical evidence implicates intra-abdominal adiposity as a powerful driving force for elevated cardiometabolic risk. Addressing intra-abdominal adiposity should play a central role in future strategies aimed at improving cardiovascular outcomes in patients with abdominal obesity and its associated cardiometabolic risk in Sudan.

Although many risk factors may appear to be as an earlier predictor for cardiac disease — such as family history, sex or age — but the laboratory and clinical evaluations are very important to have the final word. This research is focusing on a particular gene called RSPO3, which is play a part in controlling fat distribution, cardiac growth through modulation of Wnt (Wnt/β-catenin signaling path way) signaling RSPO3 is essential for coronary artery formation in the developing heart. [8]

The identification of such factors is of great clinical, as well as theoretical importance for several reasons. Firstly, genetic influences are likely to be particularly powerful in people with severe and early-onset obesity; the group is the most likely to suffer adverse clinical consequences. Secondly, the use of biogenetics to identify critical molecular components of the human control system for energy homoeostasis may help to target safe and specific drug development. Finally, it is known that diet and exercise programs, while frequently effective in inducing weight loss, rarely maintain this. It is very likely that the genetic makeup of an individual may influence his/her response to particular measures.

Genetic study is needed for overweight person. Discovery of genes associated with obesity are all arguments reinforcing the genetic dimension of abdominal obesity. There is also evidence that a high level of cardiorespiratory fitness is predictive of a reduced cardiovascular disease (CVD) risk, independently from its association with a more favorable cardiometabolic risk profile. There is now considerable evidence supporting the notion that obesity is a heterogeneous condition. Such heterogeneity appears to be explained, to a very significant extent, by individual differences in regional body fat distribution, particularly in visceral adipose tissue accumulation. In addition to visceral adiposity as key drivers of the cardiometabolic risk associated with overweight/obesity also contribute to the risk of various cardiovascular outcomes, and further work should clarify their specific functions. Studying these relationships between mutation of RSPO3 gene and CVD lead to lose weight in order to understand the biology underlying body weight regulation and hope that

these strategies contribute to intervene more efficiently in the development of prescription drugs are better able to reduce the weight. The researchers found the human gene may not affect the overall weight or BMI, but also affects the distribution of fat and reduce or raise the risk of CVD, suggesting that different types of measurements can provide insight into the process of losing weight.

1.4 Objectives: -

1.3.1 Main Objective: -

The aim of the present study is to apply of genetic polymorphism & gene experience of RSPO3 as biomarker of cardiometabolic traits associated with or without obesity in sample ofsudaneese pateins in Khartoum state.

1.3.2 Specific Objectives: -

- To detect the presence of gene mutation.
- To investigate the possible correlation between mutation in RSPO3 gene and abdominal obesity.
- To determine the possible correlation between mutation in RSPO3 gene and fat phenotype.
- To determine possible correlation between mutation in RSPO3 gene and BMI, WHR, age and sex.
- To investigate the possible correlation between RSPO3 gene expression and abdominal obesity.
- Possible correlation between RSPO3 gene expression and fat phenotype.
- Possible correlation between RSPO3 gene expression and BMI, WHR, age and sex.
- Correlation between fat phenotype (GF, VF, SF) and complication of obesity (CVD risk factors).
- Relation between the BMI and duration of obesity.
- To measure lipid profile comparing to waist to hip ratio WHR and BMI.

Chapter Two

Literature Review

2.1Obesity

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health. People are generally considered obese when their BMI is increase, a measurement obtained by dividing a person's weight by the square of the person's height, is over ($30\ kg/m^2$), with the range ($25–30\ kg/m^2$) defined as overweight. Some East Asian countries use lower values. Obesity increases the likelihood of various diseases, particularly heart disease, T2DM, obstructive sleep apnea, certaintypes of cancer, osteoarthritis and depression.[9]

Obesity is most commonly caused by a combination of excessive food intake, lack of physical activity, and genetic susceptibility. A few cases are caused primarily by genes, endocrine disorders, medications, or mental illness.The view that obese people eat little yet gain weight due to a slow metabolism is not generally supported. On average, obese people have a greater energy expenditure than their thin counterparts due to the energy required to maintain an increased body mass. [10]

Obesity is mostly preventable through a combination of social changes and personal choices.Changes to diet and exercising are the main treatments. Diet quality can be improved by reducing the consumption of energy-dense foods, such as those high in fat and sugars, and by increasing the intake of dietary fiber. Medications may be taken, along with a suitable diet, to reduce appetite or decrease fat absorption. If diet, exercise, and medication are not effective, a gastric balloon or surgery may be performed to reduce stomach volume or bowel length, leading to feeling full earlier or a reduced ability to absorb nutrients from food. [11, 12]

Obesity is a leading preventable cause of death worldwide, with increasing rates in adults and children. In 2014, (600) million adults (13%) and (42) million children under the age of (5) were obese.Obesity is more common in women than men. Authorities view it as one of the most serious public health problems of the 21st century. Obesity is stigmatized in much of the modern world (particularly in the Western world), though it was seen as a symbol of wealth and fertility at other times in history and still is in some parts of the world. In 2013, the American Medical Association classified obesity as a disease. [13]

2.2 Classification of Obesity

Obesityis defined by BMI and further evaluated in terms of fat distribution via the waist–hip ratio and total cardiovascular risk factors. BMI is closely related to both percentage body fat and total body fat. In children, a healthy weight varies with age and sex. Obesity in children and adolescents is defined not as an absolute number but in relation to a historical normal group, such that obesity is a BMI greater than the 95^{th} percentile.The reference data on which these percentiles were based date from 1963 to 1994, and thus have not been affected by the recent increases in weight.BMI is defined as the subject's weight divided by the square of their height and is calculated as follows.[14]

Table (2-1): - BMI Categories. [13]

BMI (kg/m^2)		Classification
From	**Up To**	
-	18.5	***Underweight***
18.5	25.0	***Normal Weight***
25.0	30.0	***Overweight***
30.0	35.0	***Class I Obesity***
35.0	40.0	***Class II Obesity***
40.0	**-**	***Class III Obesity***

$$\mathrm{BMI} = \frac{\mathrm{mass(kg)}}{(\mathrm{height(m)})^2}$$

Where ***m*** and ***h*** are the subject's weight and height respectively. BMI is usually expressed in (kg/m^2), resulting when weight is measured in kilograms and height in metres. To convert from pounds per square inch multiply by 703 (kg/m^2)/ (lb/sq in). [4]

The most commonly used definitions, established by the World Health Organization (WHO) in 1997 and published in 2000; provide the values listed in the table above.

Some modifications to the WHO definitions have been made by particular organizations.The surgical literature breaks down class II and III obesity into further categories whose exact values are still disputed.

- Any BMI (≥ 35 or 40 kg/m^2) is severe obesity.
- A BMI of (≥ 35 kg/m^2) and experiencing obesity-related health conditions or (≥40–44.9 kg/m^2) is morbid obesity.
- A BMI of (≥ 45 or 50 kg/m^2) is super obesity. [5]

As Asian populations develop negative health consequences at a lower BMI than Caucasians, some nations have redefined obesity; Japan have defined obesity as any BMI greater than 25 kg/m^2 while China uses a BMI of greater than (28 kg/m^2).

2.3 Effects on Health

Excessive body weight is associated with various diseases, particularly CVD,T2DM, obstructive sleep apnea, certain types of cancer, osteoarthritis and asthma. As a result, obesity has been found to reduce life expectancy. [6]

2.4 Mortality

Obesity is one of the leading preventable causes of death worldwide. A number of reviews have found that mortality risk is lowest at a BMI of (20–25 kg/m^2) in non-smokers and at (24–27 kg/m^2) in current smokers, with risk increasing along with changes in either direction.This appears to apply in at least (4) continents. Other evidence suggests that the association of BMI and waist circumference with mortality is U- or J-shaped, while the association between waist-to-hip ratio

and waist-to-height ratio with mortality is more positive. InAsians the risk of negative health effects begins to increase between (22–25 kg/m^2). A BMI above (32 kg/m$^{2)}$ has been associated with a doubled mortality rate among women over a (16 yrs) period. In the United States obesity is estimated to cause (111,909 to 365,000) deaths per year, while 1 million (7.7%) of deaths in Europe are attributed to excess weight. On average, obesity reduces life expectancy by (6) to (7) years, a BMI of (30–35 kg/m^2) reduces life expectancy by (2) to (4) years, while severe obesity (BMI > 40 kg/m^2) reduces life expectancy by (10) years.[7]

2.5 Morbidity

Obesity increases the risk of many physical and mental conditions. These comorbidities are most commonly shown in metabolic syndrome, a combination of medical disorders which includes: T2DM, high blood pressure, high blood cholesterol, and high triglyceride levels.

Complications are either directly caused by obesity or indirectly related through mechanisms sharing a common cause such as a poor diet or a sedentary lifestyle. The strength of the link between obesity and specific conditions varies. One of the strongest is the link with T2DM. Excess body fat underlies (64%) of cases of DM in men and (77%) of cases in women. [8, 9]

Health consequences fall into (2) broad categories: those attributable to the effects of increased fat mass (such as osteoarthritis, obstructive sleep apnea, social stigmatization) and those due to the increased number of fat cells (DM, cancer, CVD, non-alcoholic fatty liver disease).BIncreases in body fat alter the body's response to insulin, potentially leading to insulin resistance. Increased fat also creates a proinflammatory state, and a prothrombotic state. [9]

5.6 Survival Paradox

Although the negative health consequences of obesity in the general population are well supported by the available evidence, health outcomes in certain subgroups seem to be improved at an increased BMI, a phenomenon known as the obesity survival paradox.The paradox was first described in 1999 in overweight and obese people undergoing hemodialysis, and has subsequently been found in those with HF and peripheral artery disease (PAD).[9][10]

In people with HF, those with a BMI between (30.0 and 34.9) had lower mortality than those with a normal weight. This has been attributed to the fact that people often lose weight as they become progressively more ill. [10][11]

2.7 Causes of Obesity

At an individual level, a combination of excessive food energy intake and a lack of physical activity are thought to explain most cases of obesity. A limited number of cases are due primarily to genetics, medical reasons, or psychiatric illness. In contrast, increasing rates of obesity at a societal level are felt to be due to an easily accessible and palatable diet, increased reliance on cars, and mechanized manufacturing. [11]

A 2006 review identified (10) other possible contributors to the recent increase of obesity: (1) insufficient sleep, (2) endocrine disruptors (environmental pollutants that interfere with lipid metabolism), (3) decreased variability in ambient temperature, (4) decreased rates of smoking, because smoking suppresses appetite, (5) increased use of medications that can cause weight gain (e.g., atypical antipsychotics), (6) proportional increases in ethnic and age groups that tend to be heavier, (7) pregnancy at a later age (which may cause susceptibility to obesity in children), (8) epigenetic risk factors passed on generationally, (9) natural selection for higher BMI, and (10) assortative mating leading to increased concentration of obesity risk factors (this would increase the number of obese people by increasing population variance in weight). While there is substantial evidence supporting the influence of these mechanisms on the increased prevalence of obesity, the evidence is still inconclusive. [12]

2.7.1 Diet

Dietary energy supply per capita varies markedly between different regions and countries. It has also changed significantly over time.From the early 1970s to the late 1990s the average food energy available per person per day (the amount of food bought) increased in all parts of the world except Eastern Europe. The United States had the highest availability with (3,654) calories (15,290 kJ) per person in 1996. [12] This increased further in 2003 to (3,754) calories (15,710 kJ). During the late 1990s Europeans had 3,394 calories (14,200 kJ) per person, in the developing areas of Asia there were 2,648 calories (11,080 kJ) per person, and in sub-Saharan Africa people had (2,176) calories (9,100 kJ) per person. Total food energy consumption has been found to be related to obesity. [13]

The widespread availability of nutritional guidelines has done little to address the problems of overeating and poor dietary choice. From 1971 to 2000, obesity rates in the US increased from

(14.5%) to (30.9%). During the same period, an increase occurred in the average amount of food energy consumed. For women, the average increase was (335) calories (1,400 kJ) per day (1,542 calories (6,450 kJ) in 1971 and (1,877) calories (7,850 kJ) in 2004), while for men the average increase was 168 calories (700 kJ) per day (2,450 calories (10,300 kJ) in 1971 and (2,618) calories (10,950 kJ) in 2004). Most of this extra food energy came from an increase in carbohydrate consumption rather than fat consumption (13). The primary sources of these extra carbohydrates are sweetened beverages, which now account for almost (25 %) of daily food energy in young adults in America, and potato chips. Consumption of sweetened drinks such as soft drinks, fruit drinks, iced tea, and energy and vitamin water drinks is believed to be contributing to the rising rates of obesity and to an increased risk of metabolic syndrome and T2DM. Vitamin D deficiency is related to diseases associated with obesity. [14]

As societies become increasingly reliant on energy-dense, big-portions, and fast-food meals, the association between fast-food consumption and obesity becomes more concerning. In the US consumption of fast-food meals tripled and food energy intake from these meals quadrupled between 1977 and 1995.

Agricultural policy and techniques in the US and Europe have led to lower food prices. In the United States, subsidization of corn, soy, wheat, and rice through the U.S. farm bill has made the main sources of processed food cheap compared to fruits and vegetables.[15] Calorie count laws and nutrition facts labels attempt to steer people toward making healthier food choices, including awareness of how much food energy is being consumed.

Obese people consistently under-report their food consumption as compared to people of normal weight. This is supported both by tests of people carried out in a calorimeter roomand by direct observation.

2.7.2 Sedentary Lifestyle

A sedentary lifestyle plays a significant role in obesity.Worldwide there has been a large shift towards less physically demanding work, and currently at least (30%) of the world's population gets insufficient exercise. This is primarily due to increasing use of mechanized transportation and a greater prevalence of labor-saving technology in the home. In children, there appear to be declines in levels of physical activity due to less walking and physical education. World trends in

active leisure time physical activity are less clear. The WHO indicates people worldwide are taking up less active recreational pursuits. [16]

In both children and adults, there is an association between television viewing time and the risk of obesity.A review found (63 of 73) studies (86%) showed an increased rate of childhood obesity with increased media exposure, with rates increasing proportionally to time spent watching television.[17]

2.7.3 Genetics

Like many other medical conditions, obesity is the result of interplay between genetic and environmental factors.

Polymorphisms in various genes controlling appetite and metabolism predispose to obesity when sufficient food energy is present. People with (2) copies of the FTO gene (fat mass and obesity associated gene) have been found on average to weigh (3–4 kg) more and have a (1.67-fold) greater risk of obesity compared with those without the risk allele.The differences in BMI between people that are due to genetics varies depending on the population examined from (6% to 85%).[18]

Obesity is a major feature in several syndromes, such as Prader–Willi syndrome, Bardet–Biedl syndrome, Cohen syndrome, and MOMO syndrome. (The term "non-syndromic obesity" is sometimes used to exclude these conditions.). In people with early-onset severe obesity (defined by an onset before (10 years) of age and body mass index over (3) standard deviations above normal), (7%) harbor a single point DNA mutation. [18] [19]

Studies that have focused on inheritance patterns rather than on specific genes have found that (80%) of the offspring of (2) obese parents were also obese; in contrast to less than 10% of the offspring of (2) parents who were of normal weight.Different people exposed to the same environment have different risks of obesity due to their underlying genetics. [19]

The gene hypothesis postulates that, due to dietary scarcity during human evolution, people are prone to obesity. Their ability to take advantage of rare periods of abundance by storing energy as fat would be advantageous during times of varying food availability, and individuals with greater adipose reserves would be more likely to survive famine. This tendency to store fat, however, would be maladaptive in societies with stable food supplies. This theory has received various

criticisms, and other evolutionarily-based theories such as the thrifty gene hypothesis and the thrifty phenotype hypothesis have also been proposed. [20]

2.7.3.1 Prader-Willi Syndrome (PWS)

Prader-Willi syndrome (PWS) is a genetic imprinting disorder that results in profound hyperphagia and early childhood onset obesity. PWS patients display many addictive eating behaviors. Neuroimaging studies in this naturally occurring human eating disorder model may uncover neurophysiological mechanisms governing food addiction or loss of control of eating in general. One characteristic of the disease is a marked obsessive drive to overeat not only food but also neutral non-food objects. Excessive and pathologic reinforcement produced by the ingested items themselves might contribute to this phenomenon [21]. Increased activation in the HPAL, OFC, VMPFC, bilateral middle frontal, right inferior frontal, left superior frontal and bilateral ACC regions was also observed [22]. Investigation of the neurophysiological underpinning of PWS and its association with substance dependence may aid better understanding of appetite control and food addiction.

2.7.4 Hormones and Gut Peptides

Many peripheral hormones participate in central nervous system (CNS) control of appetite and food intake, food reward, or addiction. Both palatable foods and drugs are able to activate the mesolimbic dopamine (DA) reward system essential for addiction regulation in humans and animals. [23] Hunger and satiety signals from adipose tissue (leptin), the pancreas (insulin), and the gastrointestinal tract (cholecystokinin (CCK), glucagon-like peptide-l (GLP-1), peptide YY3-36 (PYY3-36), and ghrelin) are involved in relaying information about energy status through the neural hormonal gut-brainaxis primarily targeting the hypothalamus (HPAL) and brainstem, [24] and may directly or indirectly interact with the midbrain DA pathways to impact feeding. [25]

2.7.4.1 Leptin

An anorexigenic hormone synthesized from adipose tissue, leptin regulates lipid metabolism by stimulating lipolysis and inhibiting lipogenesis. [26]

2.7.4.2 Insulin

Insulin is a pancreatic hormone critical for maintenance of glucose homeostasis. Insulin levels rise after a meal to keep blood glucose in check.

2.7.4.3 Ghrelin

Mainly secreted by the stomach, ghrelin is an orexigenic peptide that acts on hypothalamic neurons containing ghrelin receptors to exert central metabolic effects. Ghrelin increases food intake in humans by both peripheral and central mechanisms involving interplay between the stomach, the HPAL, and the hypophysis. [26]

2.7.4.4 Peptide YY (PYY)

PYY is a short, 36-amino acid peptide made in the ileum and colon in response to feeding. Following food ingestion, PYY is released from the L-cells in the distal segment of the small gut. It reduces the rate of intestinal motility, gallbladder, gastric emptying, therefore decreases appetite and augments satiety. [33]

2.7.4.5 Glucagon-Like Peptide 1 (GLP-1)

GLP-1 is a key hormone co-released with PYY from the distal intestinal L-cells of the gut after a meal. It is secreted in two equally potent forms, GLP-1 (7–37) and GLP-1 (7–36). [36] GLP-1 primarily functions to stimulate glucose-dependent insulin secretion, enhance β-cell growth and survival, inhibit glucagon release, and suppress food intake. [37]

2.7.4.6 Cholecystokinin (CCK)

Cholecystokinin (CCK), an endogenous peptide hormone present in the gut and the brain, helps control appetite, ingestive behavior, and gastric emptying via both peripheral and central mechanisms. CCK also impacts physiological processes related to anxiety, sexual behavior, sleep, memory, and intestinal inflammation. [37]

2.7.5 Other Illnesses

Certain physical and mental illnesses and the pharmaceutical substances used to treat them can increase risk of obesity. Medical illnesses that increase obesity risk include several rare genetic syndromes (listed above) as well as some congenital or acquired

conditions: hypothyroidism, Cushing's syndrome, growth hormone deficiency, and the eating disorders: binge eating disorder and night eating syndrome.

2.8 Social Determinants

While genetic influences are important to understanding obesity, they cannot explain the current dramatic increase seen within specific countries or globally. Though it is accepted that energy consumption in excess of energy expenditure leads to obesity on an individual basis, the cause of the shifts in these (2) factors on the societal scale is much debated. There are a number of theories as to the cause but most believe it is a combination of various factors. [42]

The correlation between social class and BMI varies globally. No significant differences were seen among men of different social classes. In the developing world, women, men, and children from high social classes had greater rates of obesity. The decrease in strength of correlation was felt to be due to the effects of globalization.Among developed countries, levels of adult obesity, and percentage of teenage children who are overweight, are correlated with income inequality. A similar relationship is seen among US states: more adults, even in higher social classes, are obese in more unequal states. [43]

Many explanations have been put forth for associations between BMI and social class. It is thought that in developed countries, the wealthy are able to afford more nutritious food, they are under greater social pressure to remain slim, and have more opportunities along with greater expectations for physical fitness. Attitudes toward body weight held by people in one's life may also play a role in obesity. A correlation in BMI changes over time has been found among friends, siblings, and spouses. Stress and perceived low social status appear to increase risk of obesity. Smoking has a significant effect on an individual's weight. Those who quit smoking gain an average of 4.4 kilograms (9.7 lb) for men and 5.0 kilograms (11.0 lb) for women over (10) years. However, changing rates of smoking have had little effect on the overall rates of obesity. [44]

In the US the number of children a person has is related to their risk of obesity. A woman's risk increases by (7%) per child, while a man's risk increases by (4%) per child. This could be partly explained by the fact that having dependent children decreases physical activity in Western parents.

In the developing world urbanization is playing a role in increasing rate of obesity. In China overall rates of obesity are below (5%); however, in some cities rates of obesity are greater than (20%).

Malnutrition in early life is believed to play a role in the rising rates of obesity in the developing world. Endocrine changes that occur during periods of malnutrition may promote the storage of fat once more food energy becomes available.

Whether obesity causes cognitive deficits, or vice versa is unclear at present. [44][45]

2.9 Factors Associated with Obesity

A Ethnicity. Ethnicity factors may influence the age of onset and the rapidity of weight gain. African-American women and Hispanic women tend to experience weight gain earlier in life than Caucasians and Asians, and age-adjusted obesity rates are higher in these groups. Non-Hispanic black men and Hispanic men have a higher obesity rate then non-Hispanic white men, but the difference in prevalence is significantly less than in women.

B Childhood weight. A person's weight during childhood, the teenage years, and early adulthood may also influence the development of adult obesity. [42] For example,

- Being mildly overweight in the early 20th was linked to a substantial incidence of obesity by age (35);
- Being overweight during older childhood is highly predictive of adult obesity, especially if a parent is also obese;
- Being overweight during the teenage years is even a greater predictor of adult obesity.

C Hormones. Women tend to gain weight specially during certain events such as pregnancy, menopause, and in some cases, with the use of oral contraceptives. However, with the availability of the lower-dose estrogen pills, weight gain has not been as great a risk.

2.10 Body Fat Measurement

A. BMI is a calculated value and approximates the body's fat %. Actually, measuring a person's body fat % is not easy and is often inaccurate if the methods are not monitored

carefully. The following methods require special equipment, trained personnel, can be costly, and some are only available in certain research facilities.

B. Under water weighing (hydrostatic weighing): This method weighs a person underwater and then calculates lean body mass (muscle) and body fat. This method is one of the most accurate ones; however, it is generally done in special research facilities, and the equipment is costly. [43][44]

C. BOD POD: The BOD POD is a computerized, egg-shaped chamber. Using the same whole-body measurement principle as hydrostatic weighing, the BOD POD measures a subject's mass and volume, from which their whole-body density is determined. Using this data, body fat and lean muscle mass can then be calculated.

D. DEXA: Dual-energy X-ray absorptiometry (DEXA) is used to measure bone density. It uses X-rays to determine not only the % of body fat but also where and how much fat is located in the body. [44]

The following (2) methods are simple and straightforward:

A Skin calipers: This method measures the skinfold thickness of the layer of fat just under the skin in several parts of the body with calipers (a metal tool similar to forceps); the results are then used to calculate the percentage of body fat.

B Bioelectric impedance analysis (BIA): There are (2) methods of the BIA. One involves standing on a special scale with foot pads. A harmless amount of electrical current is sent through the body, and then percentage of body fat is calculated. The other type of BIA involves electrodes that are typically placed on a wrist and an ankle and on the back of the right hand and on the top of the foot. The change in voltage between the electrodes is measured. The person's body fat percentage is then calculated from the results of the BIA. Early on, this method showed variable results. Newer equipment and methods of analysis seem to have improved this method. [43]

Health clubs and weight-loss centers often use the skin caliper or bioelectric impedance analysis method; however, these can yield inaccurate results if an inexperienced person performs them or they are used on someone with significant obesity.

2.11 Body Fat Distribution

Deposits of adipose tissue throughout the body. The pattern of fat deposits in the body regions is an indicator of health status. Excess abdominal FAT increases health risks more than excess fat around the hips or thighs, therefore, waist-hip ratio is often used to determine health risks.

Fat distribution, female/male.

A. Gynecoid pattern (Female pattern):Fat is deposited in the lower body (abdomen, buttocks, hip s, thighs) by mesenchymal differentiation.

B. Android pattern (Male pattern):Fat is deposited in the upper body, especially around the abdo men. Adipocytes are moresensitive to insulin and catecholamines; fat accumulates by hypertroph y, possibly a function ofmembrane receptor density.The android pattern has greater lipolytic and lipogenic potential, and thus carries a greater risk for hypertension,CVD, DM and hyperinsulinae mia.

(45)

2.11.1 Gynoid Fat

Refers to the body fat that forms around the hips, breasts and thighs. Gynoid fat in women is used to provide nourishment for offspring and is often referred to as 'reproductive fat'. This is because it contains long-chain polyunsaturated fatty acids (PUFAs), which are important in the development of fetuses. However; it is also regarded as a physically attractive feature and serves additionally as an indication towards a woman's reproductive potential for mates. (46)

2.11.1.1Composition

Gynoid fat is mainly composed of long-chain polyunsaturated fatty acids. It is proposed that babies who are breast-fed are more likely to have increased cognitive capabilities due to these fatty acids being present in the breast milk, as they have been suggested to aid early brain development in fetuses and newborns. The most notable fatty acids found in human breast milk are Docosahexaenoic acid and Arachidonic acid, which have been shown to play crucial roles in the healthy formation and functions of neurons. (47)

2.11.1.2 Location

Gynoid fat contributes toward the female body shape that girls begin to develop at puberty; it is stored in the breasts and the hips, thighs and bottom. This process is modulated by estrogen, the female sex hormone, causing the female form to store higher levels of fat than the male form, which is affected primarily by testosterone. [48]

2.11.1.3Reproductive Function of Gynoid Fat:-

Gynoid fat is primarily a store of energy to be utilised in the nurturing of offspring, both to provide adequate energy resources during pregnancy and for the infant during the stage in which they are breastfeeding. When there are insufficient energy resources in the environment or health issues which require energy to combat a woman's storage of gynoid fat is likely to be reduced. Therefore, ancestrally, a female with high levels of gynoid fat would be signalling to males that they are in an optimal state for reproduction and nurturing of offspring. This can be seen in the fact that a female's Waist–hip ratio is at its optimal minimum during times of peak fertility - late adolescence and early adulthood, before increasing later in life. [48]

As a female's capacity for reproduction comes to an end, the fat distribution within the female body begins a transition from the gynoid type to more of an android type distribution. This is evidenced by the % of android fat being far higher in post-menopausal than pre-menopausal women. [49]

2.11.1.4 Sexual Dimorphism: -

The differences in gynoid fat between men and women can be seen in the typical "hourglass" figure of a woman, compared to the inverted triangle which is typical of the male figure. Women commonly have a higher body fat percentage than men and the deposition of fat in particular areas is thought to be controlled by sex hormones and GH. [50]

The hormone estrogen inhibits fat placement in the abdominal region of the body, and stimulates fat placement in the glutofemoral areas (the buttocks and hips). Certain hormonal imbalances can affect the fat distributions of both men and women. Women suffering from polycystic ovary syndrome, characterised by low estrogen, display more male type fat distributions such as a higher

waist-to-hip ratio. Conversely, men who are treated with estrogen to offset testosterone related diseases such as prostate cancer may find a reduction in their waist-to-hip ratio. [51]

Sexual dimorphism in distribution of gynoid fat was thought to emerge around puberty but has now been found to exist earlier than this.

2.11.2 Android Fat Distribution

Android fat distribution describes the distribution of human adipose tissue mainly around the trunk and upper body, in areas such as the abdomen, chest, shoulder and nape of the neck. This pattern may lead to an "apple-shaped" body or central obesity, and is more common in males than in females. Thus, the android fat distribution of men is about (48.6%), which is (10.3%) higher than that of premenstrual women. In other cases, an ovoid shape forms which does not differentiate between men and women. Generally during early adulthood, females tend to have a more peripheral fat distribution such that their fat is evenly distributed over their body. [53] This is to help centre a woman's gravity making her more stable when carrying offspring.

Android fat distribution is contrasted with gynoid fat distribution; fat around the hips, thighs and bottom, causing a "pear-shape". This is more female-patterned fat distribution, has been linked to risk factors for CVD, in both males and females. Android fat tends to be associated with cellulite and lumpy appearance of the skin - usually undesired by women. [54]

Jean Vague, a physician from Marseilles, was one of the first individuals to bring to attention the increased risk of developing certain diseases in individuals with an android distribution compared to a gynoid distribution. For example, DM and gout. There are other health consequences beyond these, including psychological consequences. [54]

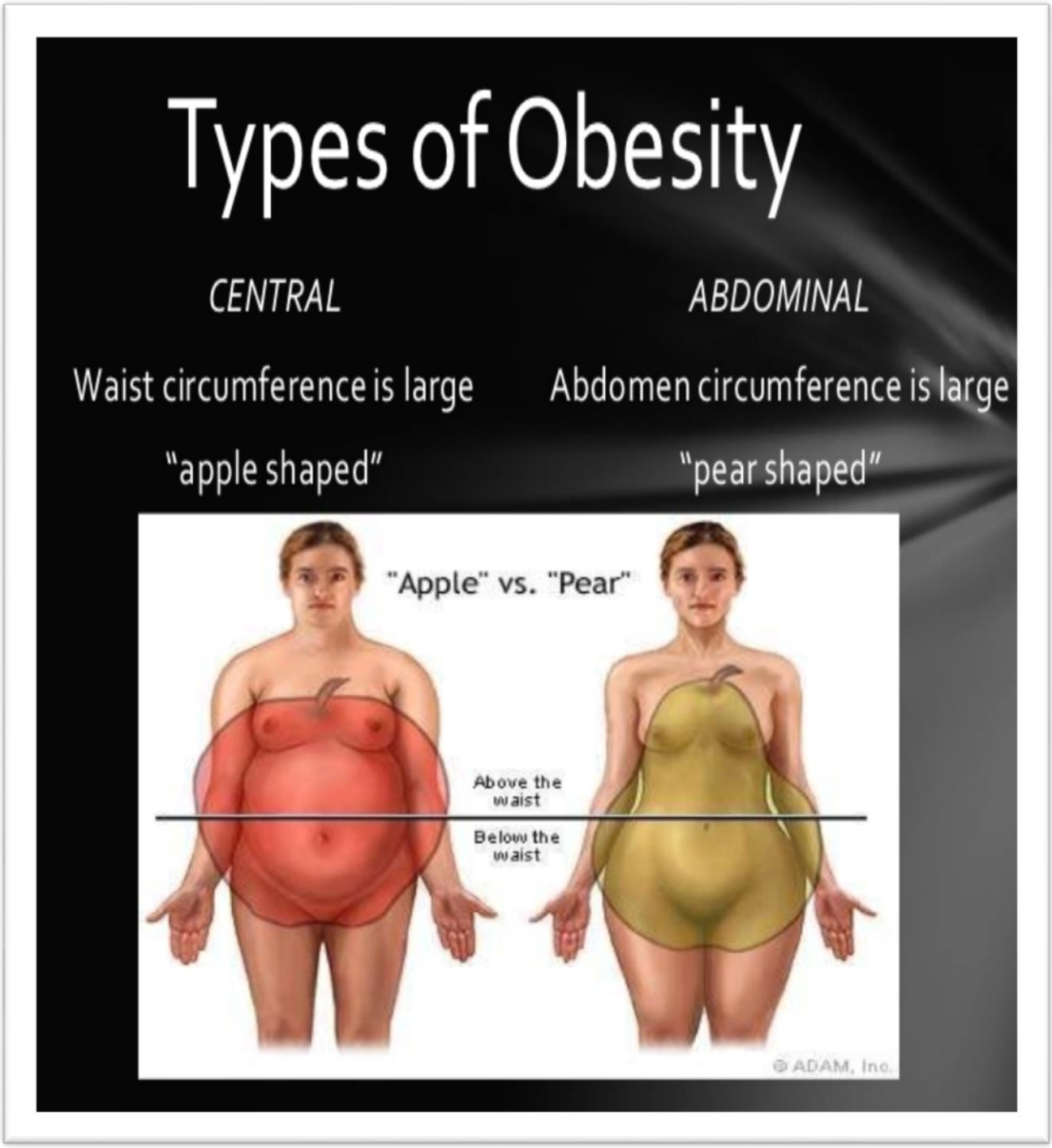


Figure (2-1): -Types of the obesity. [54]

2.11.2.1 Physiology

Android fat is readily mobilized by deficits in energy balance. It is stored in different depots to gynoid fat

Android fat cells are mostly visceral - they are large, deposited deep under the skin and are highly metabolically active. The hormones they secrete have direct access to liver. The presence of fat in the trunk and upper body in males is facilitated by testosterone. Testosterone circulation causes fat cells to deposit around the abdominal and gluteofemoral region, whereas in women oestrogen circulation leads to fat deposits around areas such as thighs, breasts and buttocks. Therefore, measuring a person's oestrogen to testosterone ratio can reveal their predicted gynoid to android fat distribution. Android fat develops as a back-up source of energy when the male body is experiencing an imbalance, whereas gynoid fat develops after puberty, in order to better prepare the body for supporting a potential infant (50%) of the variance in abdominal fat mass observed in humans is due to genetic factors. [55]

The cellular characteristics of adipose tissue in android and gynoid obese women are different. Android types have larger fat (Hypertrophy) cells whereas gynoid types have increased number of fat cells (Hyperplasia). This allows for hypertrophic obesity and hyperplastic obesity.(2) different receptors, **α** and β fat cell receptors vary in their ability to facilitate or inhibit fat mobilization. **α**-receptors are predominately in the lower body thus more abundant in gynoid patterns and β-receptors are predominantly in the upper body and so more abundant in android patterns. [56]

2.11.2.2 Causes

Hormonal disorders or fluctuations can lead to the formation of a lot of visceral fat and a protruding abdomen. Android fat can be controlled with proper diet and exercise. A poor diet with lack of exercise is likely to increase android fat level. [57]

2.11.2.3 Health Consequences Android Fat

Differences in body fat distribution are found to be associated with high blood pressure, high TG, lower high-density lipoprotein (HDL) cholesterol levels and high fasting and post-oral glucose insulin levels.

The android, or male pattern, fat distribution has been associated with a higher incidence of CAD, in addition to an increase in resistance to insulin in both obese children and adolescents.Studies have also related central abdominal obesity (indicated via increased waist-hip ratio) with increases in peripheral fasting insulin levels.[57]

Android fat is also associated with a change in pressor response in circulation. Specifically, in response to stress in a subject with central obesity the cardiac output dependent pressor response is shifted toward a generalised rise in peripheral resistance with an associated decrease in cardiac output.

There are differences in android and gynoid fat distribution among individuals, which relates to various health issues among individuals. Android body fat distribution is related to high cardiovascular disease and mortality rate. People with android obesity have higher hematocrit and red blood cell count and higher blood viscosity than people with gynoid obesity. Blood pressure is also higher in those with android obesity which leads to CVD. [58]

Women who are infertile and have polycystic ovary syndrome showed high amounts of android fat tissue. In contrast, patients with anorexia nervosa have increased gynoid fat %.[59] Women normally have small amounts of androgen, however when the amount is too high they develop male psychological characteristics and male physical characteristics of muscle mass. Women who have high amounts of androgen and thus an increase tendency for android fat distribution are in the lowest quintiles of levels of sex-hormone-binding globulin and more are at high risks of ill health associated with android fat .[60]

High levels of android fat have been associated with obesity and diseases caused by insulin insensitivity, such as DM. Insulin responsiveness is dependent on adipose cell size. The larger the adipose cell size the less sensitive the insulin. DM is more likely to occur in obese women with android fat distribution and hypertrophic fat cells. It is not just general obesity that is a consequence of android fat distribution but also other health consequences. There are connections between high android fat distributions and the severity of diseases such as acute pancreatitis - where the higher the levels of android fat are, the more severe the pancreatitis can be. An increase in android fat distribution is positively correlated with foot pain and disability associated with foot pain.Foot pain is reported to be the second most common musculoskeletal symptom in children

who are obese. Even adults who are overweight and obese report foot pain to be a common problem. [60][61]

2.2 Abdominal Obesity

Abdominal obesity, also known as central obesity, is when excessive abdominal fat around the stomach and abdomen has built up to the extent that it is likely to have a negative impact on health. There is a strong correlation between central obesity and CVD. [62]Abdominal obesity is not confined only to the elderly and obese subjects. Abdominal obesity has been linked to Alzheimer's disease as well as other metabolic and vascular diseases. [63]Visceral and central abdominal fat and waist circumference show a strong association with T2DM.

Researchers first started to focus on abdominal obesity in the 1980s when they realized that it had an important connection to cardiovascular disease, diabetes, and dyslipidemia. Abdominal obesity was more closely related with metabolic dysfunctions connected with cardiovascular disease than was general obesity. In the late 1980s and early 1990s insightful and powerful imaging techniques were discovered that would further help advance the understanding of the health risks associated with body fat accumulation. Techniques such as computed tomography and magnetic resonance imaging made it possible to categorize mass of adipose tissue located at the abdominal level into intra-abdominal fat and subcutaneous fat.

2.2.1 Health Risks

Central obesity is measured as increase by waist circumference or waist-hip ratio. Increase in waist circumference > 102 cm (40 in.) in males and > 88 cm (35 in.) in females. However increase in abdominal circumference may be due to increase in subcutaneous or visceral fat, and it is the visceral fat which increases the risk of coronary diseases. The visceral fat can be estimated with the help of MRI and CT scan. [107]

In females, measures of Waist to Hip ratio have been observed as an evolutionary sign of attractiveness and reproductive success. A female’s waist being smaller than her hips by a ratio of (0.7) is considered most attractiveas it indicated readiness to give birth to offspring, and overall health to ensure survival of offspring. Waist to hip ratio is determined by an individual's proportions of android fat and gynoid fat. A small waist to hip ratio indicates less android fat, high waist to hip ratio's indicate high levels of android fat.[108]

As WHR is associated with a woman's pregnancy rate, it has been found that a high waist-to-hip ratio can impair pregnancy, thus a health consequence of high android fat levels is its interference with the success of pregnancy and in-vitro fertilization. Body fat distribution is also related to the sex ratio of offspring. Women with large waists (a high WHR) tend to have an android fat distribution caused by a specific hormone profile, that is, having higher levels of androgens. This leads to such women having more sons.[109]

2.2.1.1 Diabetes

There are numerous theories as to the exact cause and mechanism in T2DM. Central obesity is known to predispose individuals for insulin resistance. Abdominal fat is especially active hormonally, secreting a group of hormones called adipokines that may possibly impair glucose tolerance. But adiponectin which is found in lower concentration in obese and diabetic individuals has shown to be beneficial and protective in T2DM. [110]

Insulin resistance is a major feature of T2DM, and central obesity is correlated with both insulin resistance and T2DM itself.Increased adiposity (obesity) raises serum resistin levels, which in turn directly correlate to insulin resistance. Studies have also confirmed a direct correlation between resistin levels and T2DM.And it is waistline adipose tissue (central obesity) which seems to be the foremost type of fat deposits contributing to rising levels of serum resistin. Conversely, serum resistin levels have been found to decline with decreased adiposity following medical treatment. [111]

2.2.1.2 Asthma

Developing asthma due to abdominal obesity is also a main concern. As a result of breathing at low lung volume, the muscles are tighter and the airway is narrower. It is commonly seen that people who are obese breathe quickly and often, while inhaling small volumes of air. People with obesity are also more likely to be hospitalized for asthma. A study has stated that (75%) of patients treated for asthma in the emergency room were either overweight or obese. [112]

2.2.1.3 Alzheimer’s Disease

Alzheimer's disease and abdominal obesity has a strong correlation and with metabolic factors added in, the risk of developing Alzheimer's disease was even higher. Based on logistic regression

analyses, it was found that obesity was associated with an almost (10-fold) increase risk of Alzheimer's disease. [113]

2.2.2 Causes

The currently prevalent belief is that the immediate cause of obesity is net energy imbalance—the organism consumes more usable calories than it expends wastes, or discards through elimination. Greater meat consumption has also been positively associated with greater weight gain, and specifically abdominal obesity, even when accounting for calories. Other environmental factors, such as maternal smoking, estrogenic compounds in the diet, and endocrine-disrupting chemicals may be important also. Obesity plays an important role in the impairment of lipid and carbohydrate metabolism shown in high-carbohydrate diets. It has also been shown that quality protein intake during a 24-hour period and the number of times the essential amino acid threshold of approximately (10g) has been achieved is inversely related to the % of central abdominal fat. Quality protein uptake is defined as the ratio of essential amino acids to daily dietary protein. [114]

Visceral fat cells will release their metabolic by-products in the portal circulation, where the blood leads straight to the liver. Thus, the excess of TG and FA created by the visceral fat cells will go into the liver and accumulate there. In the liver, most of it will be stored as fat. This concept is known as 'lipotoxicity'. [115]

Hypercortisolism, such as in Cushing's syndrome, also leads to central obesity. Many prescription drugs, such as dexamethasone and other steroids, can also have side effects resulting in central obesity, [115] especially in the presence of elevated insulin levels.

The prevalence of abdominal obesity is increasing in western populations, possibly due to a combination of low physical activity and high-calorie diets, and also in developing countries, where it is associated with the urbanization of populations. [116]

Waist measurement is more prone to errors than measuring height and weight. It is recommended to use both standards. BMI will illustrate the best estimate of total body fatness, while waist measurement gives an estimate of visceral fat and risk of obesity-related disease. [116]

2.2.2.1 Alcohol Consumption

After controlling for energy under-reporting, it was observed that increasing alcohol consumption significantly increased the risk of exceeding recommended energy intakes in male participants – but not in the small number of female participants (2.13%) with elevated alcohol consumption, even after establishing a lower number of drinks per day to characterize women as consuming a high quantity of alcohol. [117]

2.3 Visceral Fat

Visceral fat is technically excess intra-abdominal adipose tissue accumulation. In other words, it is known as a "deep" fat that's stored further underneath the skin than "subcutaneous" belly fat. It is a form of gel-like fat that's wrapped around major organs, including the liver, pancreas and kidneys.

The protruding belly and large waist, that's a clear sign you're storing dangerous visceral fat. While it's most noticeable and pronounced in obese individuals, anyone can have visceral fit, many without even knowing it.

Visceral fat is especially dangerous because, they also change the way your body operates. [65]

Carrying around excess visceral fat is linked with an increased risk for:

- CVD
- Cancer
- Stroke
- Dementia
- DM
- Depression
- Arthritis
- Obesity
- Sexual dysfunction
- Sleep disorders

Visceral fat is considered toxic and spells double-trouble in the body because it is capable of provoking inflammatory pathways, plus signaling molecules that can interfere with the body's normal hormonal functions. In fact, it acts almost like its very own organ since it is capable of having such a large impact on the body. (66)

Fat cells do more than simply store extra calories — they have proved to be much more involved in human physiology than previously thought. Tissue itself acts like its own organ by pumping out hormones and inflammatory substances. Storing excess fat around the organs increases production of pro-inflammatory chemicals, also called cytokines, which leads to inflammation; at the same time, it interferes with hormones that regulate appetite, weight, mood and brain function. (67)

2.3.1 Visceral Fat Development

Having a lean belly is a key indicator of health, so body tries to preserve this by controlling appetite and energy expenditure. To prevent dangerous fat buildup, the body basically works like an orchestra of chemicals that tells us when to eat and when we are full. This chemical feedback system, which is built on communication between the brain and other major organs. the brain/body connection — is what's responsible for either keeping us at a healthy weight or making us more susceptible to weight gain and visceral fat storage. (68)

At the core of your weight, appetite and mood control are blood sugar levels, which are controlled largely by the hormone insulin. Insulin balances blood sugar levels by bringing them down after we've eaten a high-carbohydrate or sugary meal. When we digest food, our body breaks down sugar and starch molecules into simpler units called glucose or fructose.

These simple sugars enter blood stream and trigger the release of insulin from the pancreas, and then insulin has the important job of ushering blood sugar into cells throughout body. This process supplies us with energy for things like brain, tissue and muscular function when it is working properly. (69)

At the same time, insulin also corresponds to body fat stores, including the visceral fat stored deep within bodies. This is why people often call insulin our "fat-storage hormone."

When there's too much glucose in blood stream and our cells already have filled glycogen stores, glucose is stored as fat. This happens a lot more quickly and easily when consuming refined

processed carbohydrates and sugary foods. Processed starches, like white bread or white rice, along with high-sugar foods, are rapidly converted into simple sugars that enter the blood stream and trigger a larger release of insulin from the pancreas. The result is usually weight gain, plus even more hunger, which leads to continued overeating and a vicious cycle that makes it hard to stop eating sweets. [70]

The more often and longer that blood insulin levels remain high, the more likely a person is to accumulate excess body fat and to battle weight problems. Insulin also communicates with many other hormones needed for various functions, including those made in the adrenal glands, such as the stress hormone cortisol, so abnormally high levels and hormonal imbalances result in powerful urges to eat, mood changes, lack of energy and various other factors that contribute to disease formation.[71]

Why is more fat stored as visceral fat in some people but not in others? Specific mechanisms responsible for proportionally increasing visceral fat storage include eating too many calories ("positive energy balance"), sex hormones, cortisol production, growth hormones and dietary fructose (sugar).

2.3.2 Risks of High Levels of Visceral Fat

2.3.2.1 Increased Inflammation

A major concern is that visceral fat produces hormonal and inflammatory molecules that get dumped directly into the liver, leading to even more inflammation and hormone-disrupting reactions. If more fat stored than you need, especially around visceral organs like the liver, heart, kidneys, pancreas and intestines, your body becomes inflamed and your metabolism suffers, making it a hard cycle to break out of. [72]

Visceral fat does more than just lead to inflammation down the road — it becomes inflamed itself by producing something known as interleukin-6, a type of inflammatory molecule. This kind of fat stores inflammatory white blood cells and kicks off a series of autoimmune reactions. Inflammation is at the root of most diseases, and this is why inflammatory belly fat is linked with cognitive decline, arthritis, diabetes and so on. [72][73]

2.3.2.2 Higher Risk of Diabetes

More than other types of fats, visceral fat is thought to play a large role in insulin resistance, which means a heightened risk for developing DM. For example, abdominal fat is viewed as a bigger health risk than hip or thigh fat, not only for DM but for many other chronic diseases too. Some evidence suggests that pear-shaped women are better protected from metabolic diseases like DM compared to big-bellied people. While men are more likely to store noticeable levels of visceral fat, women are definitely at risk, too. Reducing visceral fat through a healthy diet and other means is one of the most important natural DM treatments there is that's within your control. (74)

2.3.2.3 Makes It Harder to Lose Weight

People tend to get heavier and heavier as time goes on — and one of the main reasons is that stored body fat affects hunger levels, especially visceral fat. It might seem hard to imagine, but metabolism is largely governed by level of existing stored fat. Fat messes with appetites and makes it easier to overeat due to hormonal changes that take place. (75)

Higher levels of insulin also promote more efficient conversion of calories into body fat, so this vicious cycle continues. Eating refined carbohydrates, as opposed to complex carbohydrates in their natural state like vegetables and fruit, can cause the body's "set point" for body weight to increase. (76)

When you eat refined carbohydrates such as white flour and sugar, the fat-storing hormones are produced in excess, raising the set point and making it hard to follow a moderate-calorie, healthy diet. This is why it's important to kick your addiction and address weight gain and visceral fat formation early on, as opposed to letting the situation escalate. (77)

2.3.2.4 Higher Risk for Heart Disease and Strokes

Fat-generated inflammatory cytokines are the main contributors to heart disease and other inflammatory disorders. When your body is inflamed, liver becomes overwhelmed with cholesterol and toxins, which leads to plaque buildup in your arteries.(78)Visceral fat is associated with an increased risk for CVD markers like high TG, high blood pressure and high cholesterol. According to a 2013 report done by the University Center Hospital of Quebec, visceral fat is closely related to clustering cardiometabolic risk factors. Hypertriglyceridemia; increased free fatty acid availability; adipose tissue release of pro-inflammatory cytokines; liver insulin resistance and

inflammation; increased liver VLDL synthesis and secretion; reduced clearance of triglyceride-rich lipoproteins; presence of small, dense LDL particles; and reduced HDL cholesterol levels are among the many metabolic alterations closely related to this condition.[79]

2.3.2.5 More Likely to Battle Dementia

A growing body of evidence points to the fact that there's a strong link between obesity, vascular disease, inflammation and cognitive decline, including dementia. In fact, it seems that excess pounds on the body equates with less brain volume and, therefore, poorer function into older age. [80]

This is even true even for people with excess belly fat but who are overall at a normal weight! The bigger the belly (or a person's waist-to-hip ratio), the more negative impact felt on the brain's memory center called the hippocampus. In fact, many experts now feel that visceral adipose tissue (VAT) levels rather than BMI should be considered as an important risk factor in the development of dementia. [80]

Results from a 2010 study done by the Department of Cardiology at Oita Red Cross Hospital in Japan found that elevated levels of visceral fat in non-dementia patients with T2DM is characterized by abnormal changes in hippocampus volume and insulin resistance.[81] Other studies have also found that the higher someone's waist-to-hip ratio, the higher the risk for small strokes, which are associated with declining brain function.

We still don't know exactly how visceral fat and dementia are linked, but it's believed it has to do with the hormone leptin, which is released by stored fat and has adverse effects on the brain, appetite regulation, learning and memory. Leptin and ghrelin are (2) of the most hormones to pay attention to in reference to losing weight naturally. [82]

2.3.2.6 Higher Likihood to Have Depression and Mood Problems

Since excess body f*l*at is linked to hormonal changes, including those of serotonin, galanin and other brain neurotransmitters, excess body fat can negatively impact mood. [83]

A 2014 study conducted by Boston University School of Medicine found that depressive symptoms are associated with visceral adiposity in middle-aged adults. To examine the relationship between measures of adiposity (fat) and depression, researchers examined visceral

adipose tissue (VAT) and depressive symptoms in (1,581) women (mean age 52.2 years) and (1,718) men (mean age 49.8 years).[84]

After adjusting for age, body mass index, smoking, alcohol and other factors, results showed that higher levels of stored VAT translated to higher likelihood of experiencing depression.[85]

Depression is especially associated with greater fat storage in women, so it might be even more crucial for women to follow a depression-freediet. In a study of middle-aged women over (50 years) old, visceral fat, but not subcutaneous belly fat or waist circumference, was related to depressive symptoms. [86]

2.3.3 Visceral Fat and Metabolism

2.3.3.1 Small, Dense Low-Density Lipoprotein

By its peculiar location, the expanded visceral fat depot has easy access to the liver via the portal circulation, where it could influence metabolism and promote insulin resistance. It has been hyperlipolytic state of the expanded visceral adipose depot leads to the delivery of large amounts of nonesterified FFA to the liver. According to the FFA drainage hypothesis, the delivery of FFA to the liver would contribute to the synthesis of VLDLs enriched with TGs. [87] Then, after the activity of cholesteryl ester transfer protein, which promotes the exchange of TGs from VLDLs to LDLs and the reverse transport of cholesteryl esters to VLDLs, TG-enriched LDL particles are produced in large amounts. By the action of hepatic lipase, TG-enriched LDLs become smaller and denser. However, it should be pointed out that, although experimental animal models support the portal FFA hypothesis, this issue is unsettled in humans. For instance, although a correlation between portal FFA levels and visceral fat has been reported, evidence indicates that most FFA delivered to the liver comes from the systemic circulation.[88]Thus, other factors than FFA could explain the relation of visceral obesity to altered liver TG metabolism. In this regard, the release of proinflammatory substances by large intra-abdominal adipocytes combined to reduced secretion of an important cytokine, adiponectin, could also contribute to impair hepatic lipoprotein metabolism. [89] Small, dense LDL is (1) key feature of visceral obesity and is closely associated with the hypertriglyceridemic state of insulin resistance. Studies have shown that small, dense LDL particles have a greater ability to penetrate within the vascular wall and have a high susceptibility

to oxidation and are thereby potentially highly atherogenic.[90] In line with this, population prospective studies have generally reported that a high proportion of small, dense LDLs is predictive of an increase risk of developing CAD.[91] In fact, in the Quebec Cardiovascular Study, men having some of the key metabolic features of visceral obesity, eg, fasting hyperinsulinemia, small, dense LDLs, and high apolipoprotein B (ApoB) concentrations, were at a very high risk of having a first coronary event.[92]

2.3.3.2 High-Density Lipoprotein

It is noteworthy that, in the same study, a low HDL concentration was a better predictor of IHD than LDL levels. From a clinical standpoint, it should be stressed that a decreased HDL concentration is rarely an isolated finding in patients. Rather, low HDL levels are often found in association with high TG, high Apo B, and insulin resistance, which are, incidentally, also associated with a high proportion of small, dense LDL. At this point, it should also be emphasized that, in a majority of studies, the independent effect of TG as a predictor of CAD is at best weak when HDL variation is taken into account. In this regard, the physiological interactions between TG and HDL should not be overlooked and may explain the confusion among clinicians with regard to proper interpretation of hypertriglyceridemia. In fact, plasma TG concentration shows a strong negative correlation with HDL level and is a marker of visceral obesity and insulin resistance. Rather, hypertriglyceridemia should be considered as a simple and convenient marker of a cluster of metabolic abnormalities conferring a high CVD risk, particularly when accompanied by intra-abdominal obesity. Incidentally, in the Prospective Cardiovascular Munster and Helsinki Heart studies, the groups of patients having both a high plasma TG and low HDL levels were at the highest risk of developing CAD.[94][95]

2.3.4 Visceral Fat and Inflammation

Whether there is a cause-and-effect relationship between CRP and coronary heart disease event remains unclear, but studies have shown that CRP levels are markedly increased in individuals with abdominal obesity, particularly among subjects with a selective excess of visceral adipose tissue. Therefore, the expanded intra-abdominal fat depot may contribute to a proinflammatory state, which, in turn, is linked to clinical events. Although it is still unclear whether CRP is a

marker or a component of the atheroinflammatory process, it is believed that it may help identify individuals at higher risk. [98]

Adiponectin, an adipocyte-specific peptide, has been reported to be involved in an array of functions in different target organs, including the brain, the liver, the skeletal muscle, and the vascular wall. Moreover, adiponectin has been identified as having a potent anti-inflammatory role. In peripheral tissues, adiponectin promotes the oxidation of FFA and favorably affects insulin sensitivity. Thus, it appears that adiponectin could be at the center stage in having multiple metabolic effects along with anti-inflammatory and antiatherosclerotic properties. [98]

2.3.5 Visceral Obesity and Hypertension

2.3.5.1 Renin-Angiotensin System

Hypertension, a major cardiovascular risk factor, is closely associated with obesity. Indeed, it is estimated that between (65%) and (78%) of cases of hypertension could be attributed to obesity. [100] Activation of the RAS in hypertension is well known and is suspected of having a role in insulin resistance. Importantly, the discovery that adipose tissue is, in addition to the liver, an extra source of angiotensinogen (AGN), has contributed to the heralding of intense research efforts aiming at uncovering specific mechanisms involved in the obesity-associated hypertension. In fact, renin produced by the kidney allows the transformation of AGN to angiotensin (Ang) I and then, through the action of angiotensin-converting enzyme (ACE), Ang I is transformed to Ang II, a powerful vasoconstrictor. Angiotensin II type 1 receptor (AR-1) is expressed by adipocytes, where it exerts potential important functions. In fact, differentiation of preadipocyte to adipocyte is hampered by Ang II. [101] Thus, it is likely that, by interfering with preadipocyte differentiation, Ang II contributes to the formation of large and dysfunctional adipocytes. In turn, expression of AGN is increased in large adipocytes and, therefore, suggests that a vicious circle between the RAS and the dysfunctional adipose tissue is involved in obesity-associated hypertension. Increasing evidence indicates that large adipocytes are producing elevated levels of leptin, reactive oxygen species, and proinflammatory cytokines.[102] Furthermore, accumulation of ectopic fat and development of insulin resistance are abetted by the insufficient capacity of oversized adipocytes to appropriately handle excess energy intake.[93][103] Importantly, adipose depots with large

adipocytes are infiltrated by macrophages, which have reciprocal communications with fat cells. Accordingly, FFAs released by adipocytes promote the production of tumor necrosis factor-α by macrophages, which, in turn, induce the production of interleukin-6 by fat cells. [104]

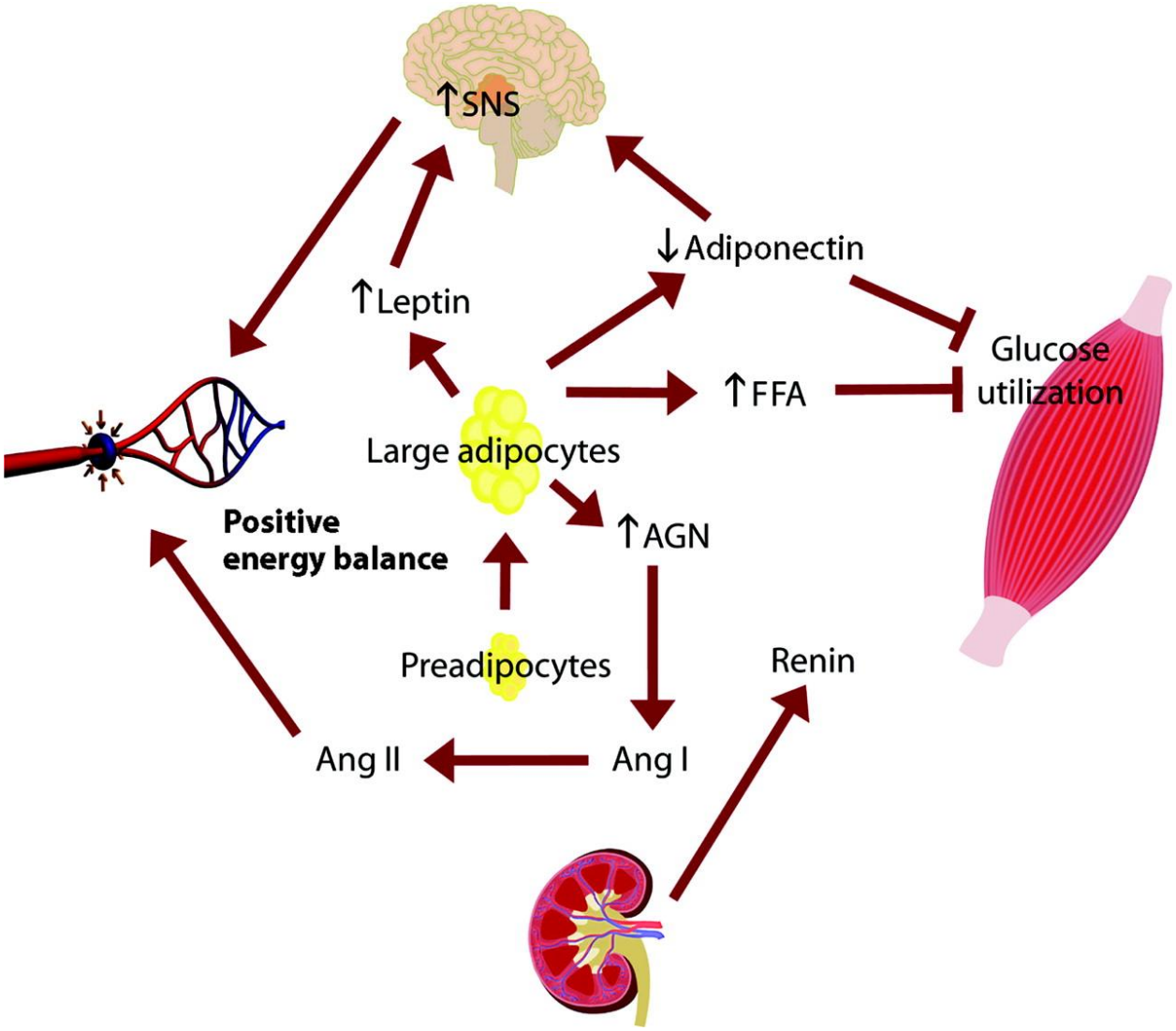


Figure (2-2): Physiopathologic relationships between obesity and hypertension. Chronic positive energy balance promotes the accumulation of excessive ectopic/visceral fat, which, in turn synthesizes AGN and thereby contributes to the activation of the RAS. In addition, Ang II hampers the development of preadipocytes and, therefore, promotes the accumulation of large dysfunctional adipocytes, which produce an increased amount of leptin and nonesterified FFAs, as well as reduced quantity of adiponectin. In turn, a decreased level of adiponectin and increased load of FFA impede glucose use by the skeletal muscle. Furthermore, higher levels of leptin and lower amounts of circulating adiponectin activate the SNS, a key component of the hypertensive response. [106]

2.4 Diagnosis of Visceral Fat and Abdominal Obesity

There are various ways of measuring abdominal obesity including:

- Absolute waist circumference (>102 cm (40 in) in men and >88 cm (35 in) in women)
- WHR (the circumference of the waist divided by that of the hips of (>0.9) for men and (>0.85) for women).
- WHtR.
- SAD.

In those with a BMI under (35), intra-abdominal body fat is related to negative health outcomes independent of total body fat.Intra-abdominal or visceral fat has a particularly strong correlation with CVD.

BMI and waist measurements are well recognized ways to characterize obesity. However, waist measurements are not as accurate as BMI measurements. For this reason, it is recommended to use both methods of measurements. [118]

While central obesity can be obvious just by looking at the naked body, the severity of central obesity is determined by taking waist and hip measurements. The absolute waist circumference (102) centimetres (40 in) in men and (88) centimetres (35 in) in women and the WHR (>0.9 for men and >0.85 for women)[119]are both used as measures of central obesity. A differential diagnosis includes distinguishing central obesity from ascites and intestinal bloating. In the cohort of (15,000) people participating in the National Health and Nutrition Examination Survey (NHANES III), waist circumference explained obesity-related health risk better than the BMI when metabolic syndrome was taken as an outcome measure and this difference was statistically significant. In other words, excessive waist circumference appears to be more of a risk factor for metabolic syndrome than BMI.[120] Another measure of central obesity which has shown superiority to BMI in predicting CVD risk is the Index of Central Obesity (waist-to-height ratio - WHtR), where a ratio of (>=0.5) (i.e. a waist circumference at least half of the individual's height) is predictive of increased risk. Another diagnosis of obesity is the analysis of intra-abdominal fat having the most risk to one's personal health.[121] An increasing acceptance of the importance of central obesity within the medical profession as an indicator of health risk has led to new developments in obesity diagnosis such as the Body Volume Index, which measures central

obesity by measuring a person's body shape and their weight distribution. The effect of abdominal adiposity occurs not just in those who are obese, but also affects people who are non-obese and it also contributes to insulin sensitivity.

2.4.1 Waist–hip Ratio

Waist–hip ratio or waist-to-hip ratio (WHR) is the ratio of the circumference of the waist to that of the hips. This is calculated as waist measurement divided by hip measurement *(*W ÷ H). For example, a person with a 25″ (64 cm) waist and 38″ (97 cm) hips has a waist–hip ratio of about 0.66 (Figure 2-3).

The WHR has been used as an indicator or measure of health, and the risk of developing serious health conditions. WHR correlates with fertility (with different optimal values for males and females).[122]

2.4.1.1 Measurement

2.4.1.1.1 World Health Organisation protocol

According to the WHO's data gathering protocol, the waist circumference should be measured at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest, using a stretch-resistant tape that provides a constant (100 g) tension. Hip circumference should be measured around the widest portion of the buttocks, with the tape parallel to the floor. Other organizations use slightly different standards.[123]

For both measurements, the individual should stand with feet close together, arms at the side and body weight evenly distributed, and should wear little clothing. The subject should be relaxed, and the measurements should be taken at the end of a normal respiration. Each measurement should be repeated twice; if the measurements are within (1 cm) of one another, the average should be calculated. If the difference between the (2) measurements exceeds (1 cm), the (2) measurements should be repeated.

2.4.1.1.2 Practical Measurements

Practically, however, the waist is more conveniently measured simply at the smallest circumference of the natural waist, usually just above the belly button, and the hip circumference may likewise be measured at its widest part of the buttocks or hip. Also, in case the waist is convex

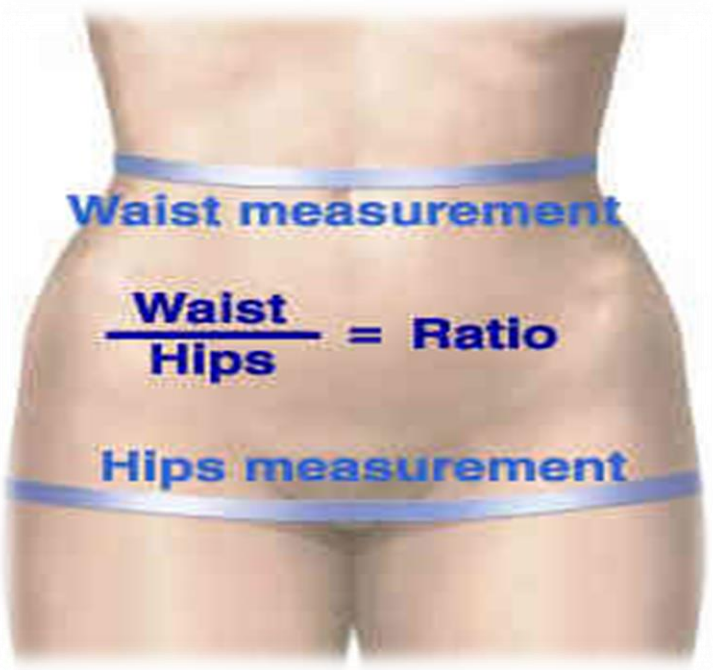
rather than concave, such as the case in pregnancy, different body types, and obesity, the waist may be measured at a horizontal level (1 inch) above the navel.[123]

2.4.1.2 Methods of waist-to-hip ratio (WHR) estimation

It is the ratio of the circumference of the waist to that of the hips. This is calculated as waist measurement divided by hip measurement (W ÷ H). The WHR has been used as an indicator or measure of health, and the risk of developing serious health conditions. [123]

Table (2-2):-Waist-to-hip ratio (WHR) Categories: [123]

Male	Female	Health risk based on WHR
0.95 or < less	0.80 or < less	Low risk
0.96 to 1.0	0.81 to 0.85	Moderate risk
1.0 or > greater	0.86 or > greater	High risk



(Figure 2-3): Waist measurement. [123]

2.4.1.3 Indicator of Health

The WHR has been used as an indicator or measure of health, and the risk of developing serious health conditions. Research shows that people with "apple-shaped" bodies (with more weight around the waist) face more health risks than those with "pear-shaped" bodies who carry more weight around the hips.

WHR is used as a measurement of obesity, which in turn is a possible indicator of other more serious health conditions. The WHO states that abdominal obesity is defined as a waist–hip ratio above (0.90) for males and above (0.85) for females, or BMI above (30). The National Institute of Diabetes, NIDDK states that women with whr of more than (0.8), and men with more than (1.0), are at increased health risk because of their fat distribution.[124]

WHR has been found to be a more efficient predictor of mortality in older people (>75 years of age) than waist circumference or BMI. [125] If obesity is redefined using WHR instead of BMI, the proportion of people categorized as at risk of heart attack worldwide increases (3-fold).The body

fat % is considered to be an even more accurate measure of relative weight. Of these three measurements, only the waist–hip ratio takes account of the differences in body structure. Hence, it is possible for two women to have vastly different body mass indices but the same waist–hip ratio, or to have the same body mass index but vastly different waist–hip ratios. [126]

2.4.1.3 Stress

The stress hormone cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and has been associated with higher levels of abdominal fat and therefore a higher WHR.[128] Abdominal fat is a marker of visceral fat (stored around important internal organs such as the liver, pancreas and intestines) and has greater blood flow and more receptors for cortisol than peripheral fat. The greater the number of cortisol receptors, the more sensitive the visceral fat tissue to cortisol. This heightened sensitivity to cortisol stimulates fat cells to further increase in size. Women who have a combination of normal BMI and high WHR experience elevated cortisol reactivity to acute stressors and failure to habituate to repeated stressors, compared to women with normal WHR.[129] This suggests that high WHR might also indicate HPA-axis dysregulation and over-exposure to cortisol.

Evidence for the relationship between cortisol and central fat distribution has primarily been studied in individuals with Cushing's syndrome. This is characterized by over-exposure to cortisol due to elevated activity of the HPA axis. A primary component of Cushing's syndrome is the accumulation of fat in the abdominal region, and it is hypothesized that elevated cortisol levels contribute to this accumulation. However, this hypothesis remains contested as cortisol levels only modestly explain variation in central fat distribution. It is more likely that a complex set of biological and neuroendocrine pathways related to cortisol secretion contribute to central adiposity, such as leptin, neuropeptide y, corticotropin releasing factor and the sympathetic nervous system. [130]

2.4.1.5 Growth and Development

In general, adults with growth hormone deficiencies also have increased WHRs. Adults with untreated congenital isolated growth hormone deficiency have increased WHRs, possibly from increased cortisone: cortisol ratios and insulin sensitivities. However, because of the growth hormone deficiency, this insulin resistance point cannot be reached and these individuals are more

sensitive to insulin. Increased adipose deposits are therefore more likely to be formed in these individuals, causing the high WHR. Growth hormone deficiencies have also been correlated with WHRs in prepubertal children; the specific baseline body statistics, such as WHRs, of pre-pubertal children with growth hormone deficiencies can predict growth response effectiveness to artificial growth hormone therapies, such as RhGH treatments.

2.4.1.6 Sex Characteristics

Males with congenital adrenal hyperplasia, determined by CYP21A2 mutations, have increased WHRs.

2.4.1.7 Fertility

A WHR of (0.9) for men and (0.7) for women has been shown to correlate strongly with general health and fertility. Women within the (0.7) range have optimal levels of estrogen and are less susceptible to major diseases such as diabetes, cardiovascular disorders and ovarian cancers.Women with high WHR (0.80 or higher) have significantly lower pregnancy rates than women with lower WHRs (0.70–0.79), independent of their BMIs. Men with WHRs around (0.9), similarly, have been shown to be more healthy and fertile with less prostate cancer and testicular cancer. [132]

Evidence suggests that WHR is an accurate somatic indicator of reproductive endocrinological status and long-term health risk. Among girls with identical body weights, those with lower WHRs show earlier pubertal endocrine activity, as measured by high levels of lutenizing hormone and follicle-stimulating hormone, as well as sex steroid (estradiol) activity. [133]

Menopause, the natural or surgical cessation of the menstrual cycle, is due to an overall decrease in ovarian production of the hormones estradiol and progesterone. These hormonal changes are also associated with an increase in WHR independent of increases in body mass. Significantly; studies find that large premenopausal WHRs are associated with lower estradiol levels and variation in age of menopause onset. Circulating estrogen preferentially stores lipid deposits in the gluteofemoral region, including the buttocks and thighs, and evidence suggests that menopause-associated estrogen deficiency results in an accumulation of adipose deposits around the abdomen.These menopause-induced changes in body fat distribution can be counteracted with hormone

replacement therapy.In contrast, aging males gradually accumulate abdominal fat, and hence increased WHR, in parallel with declining androgen levels. [134]

2.4.2 Sagittal Abdominal Diameter

Sagittal abdominal diameter (SAD) is a measure of visceral obesity, the amount of fat in the gut region. SAD is the distance from the small of the back to the upper abdomen. SAD may be measured when standing or supine. SAD may be measured at any point from the narrowest point between the last rib and the iliac crests to the midpoint of the iliac crests. [136][137]

SAD is a strong predictor of coronary disease, with higher values indicating increased risk independent of BMI.

For persons of normal BMI, SAD should be less than (25) centimetres (9.8 in). If the amounts of measure exceeds (30) centimetres (12 in) correlates to increased cardiovascular risk and insulin resistance. SAD measure of men in their (40s), greater than (25 cm), also predicts significantly higher risk of Alzheimer's disease (30 years) later. An article in Annals of Neurology links visceral fat to lower brain volume. [137][138]

A related measurement is SAH, the abdominal height as measured in the supine position.The SAH method is easier for self-monitoring, but gives slightly lower results due to gravity; the values are not directly comparable. [138]

Finding the anthropometric measure of visceral obesity is essential to clinical practice, because it predicts cardiovascular and metabolic risks. SAD has been proposed as an estimate of VAT.

Sagittal diameter is the distance between the back and the highest point of the abdomen. Increased sagittal diameter is linked to intra-abdominal fatness and metabolic risk. It is unclear whether sagittal diameter has any clinical use beyond that of waist circumference alone. [139]

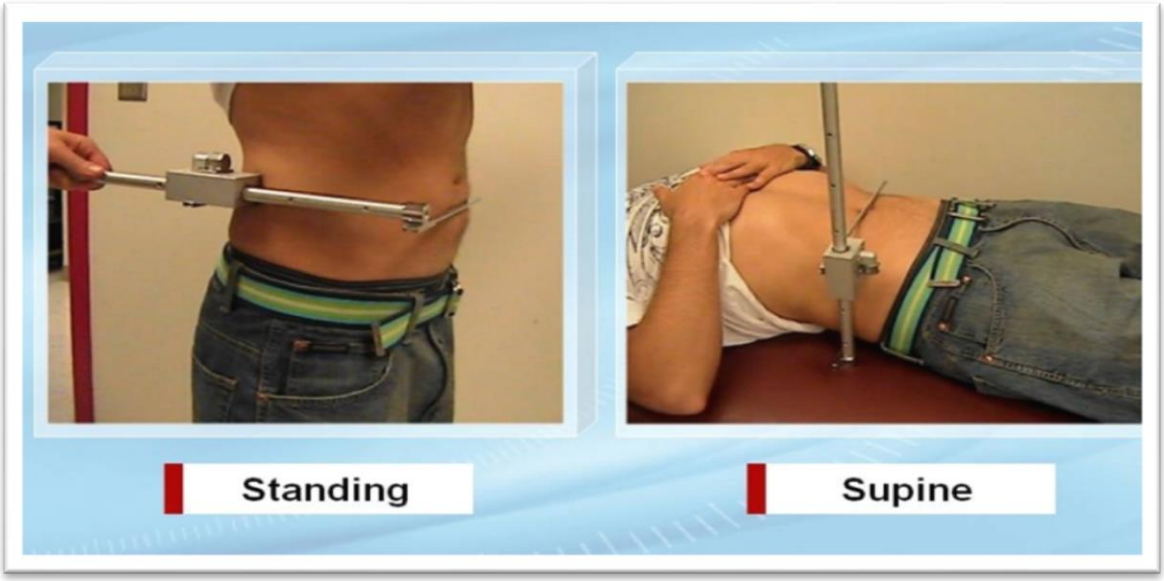
The SAD is associated with CVD and mortality. There is some evidence to suggest that SAD is more useful than waist circumference in assessing health risk. In general, most studies show that sagittal diameter and waist circumference are similarly linked to health risk. To date, there are no established sagittal diameter values to denote health risk and/or abdominal obesity. Currently, waist circumference is a more common measure of abdominal obesity in clinical settings, perhaps due to simplicity of measurement. Moreover, it is unclear whether measures of abdominal sagittal

diameter provide any clear advantage over waist circumference alone. Nevertheless, SAD is a simple, inexpensive tool that can be used to assess abdominal obesity and related health risk. [140]

SAD is commonly assessed using a sagittometer (i.e., a sliding beam caliper with a ruler). As with waist circumference, sagittal diameter is typically measured between the top of the iliac crest and the minimal waist. There is no consensus as to optimal landmarking or methodology. Sagittal diameter should be measured at the end of a normal expiration while the individual is relaxed and in a standing or supine position. To measure sagittal diameter, place one of the calipers on the individual's back at the level of the landmark and close the other caliper until it touches the individual's abdomen. The calipers should be parallel to the ground if the individual is standing and perpendicular to the ground if the individual is supine. The calipers should be snug without indenting the individual's skin. [140] [141]

There is no firm evidence to prove whether measuring sagittal diameter in the standing or supine position provides a better indicator of intra-abdominal fat or health risk. Sagittal diameter measures in the standing position will generally be larger than measures in the supine position. The measurement difference will depend on the individual's degree of obesity and abdominal fat distribution. However, as measures of standing and supine sagittal diameters are related, both may provide comparable estimates of health and intra-abdominal fat.

Sagittal diameter is a good measure of intra-abdominal fat and health risk. Measures of sagittal diameter are not as well studied as waist circumference, and there is no clear evidence to suggest that sagittal diameter provides any advantage over waist circumference in assessing abdominal adiposity or cardiometabolic risk. [142]



(Figure 2-4): Sagittal abdominal diameter [142]

2.5 Cardiovascular Disease

Cardiovascular disease (CVD) is a class of diseases that involve the heart or blood vessels. CVD includes coronary artery diseases (CAD) such as angina and myocardial infarction (commonly known as a heart attack). Other CVDs include ; stroke, heart failure, hypertensive heart disease, rheumatic heart disease, cardiomyopathy, heartarrhythmia, congenital heart disease, valvular heart disease, carditis, aortic aneurysms, peripheral artery disease, thromboembolic disease, and venous thrombosis. [143]

The underlying mechanisms vary depending on the disease in question. CAD, stroke, and peripheral artery disease involve atherosclerosis. This may be caused by high blood pressure, smoking, diabetes, lack of exercise, obesity, high blood cholesterol, poor diet, and excessive alcohol consumption, among others. High blood pressure results in (13%) of CVD deaths, while tobacco results in (9%), diabetes (6%), lack of exercise (6%) and obesity (5%). Rheumatic heart disease may follow untreated strep throat. [143] [144]

It is estimated that (90%) of CVD is preventable. Prevention of atherosclerosis involves improving risk factors through: healthy eating, exercise, avoidance of tobacco smoke and limiting alcohol intake.Treating risk factors, such as high blood pressure, blood lipids and DM is also beneficial

.Treating people who have strep throat with antibiotics can decrease the risk of RHD. The effect of the use of aspirin in people who are otherwise healthy is of unclear benefit.

CVD are the leading cause of death globally. This is true in all areas of the world except Africa.Together they resulted in (17.9) million deaths (32.1%) in 2015 up from (12.3) million (25.8%) in 1990.Deaths, at a given age, from CVD are more common and have been increasing in much of the developing world, while rates have declined in most of the developed world since the 1970s.Coronary artery disease and stroke account for (80%) of CVD deaths in males and (75%) of CVD deaths in females. [145] Most CVD affects older adults. In the United States (11%) of people between (20) and (40) have CVD, while (37%) between (40) and (60, 71%)of people between (60) and (80), and (85%) of people over (80) have CVD.The average age of death from coronary artery disease in the developed world is around (80) while it is around (68) in the developing world. Disease onset is typically seven to (10) years earlier in men as compared to women. [146]

2.5.1 Types of Cardiovascular Disease

There are many CVDs involving the blood vessels. They are known as vascular diseases.

- Coronary artery disease (also known as CHD and IHD).
- PAD – disease of blood vessels that supply blood to the arms and legs.
- CVD – disease of blood vessels that supply blood to the brain (includes stroke).
- Renal artery stenosis.
- Aortic aneurysm.

There are also many CVDs that involve the heart.

- Cardiomyopathy – diseases of cardiac muscle.[146]
- Hypertensive heart disease – diseases of the heart secondary to high blood pressure or hypertension.
- Heart failure - a clinical syndrome caused by the inability of the heart to supply sufficient blood to the tissues to meet their metabolic requirements.
- Pulmonary heart disease – a failure at the right side of the heart with respiratory system involvement.
- Cardiac dysrhythmias – abnormalities of heart rhythm.

- Inflammatory heart disease.
  - Endocarditis – inflammation of the inner layer of the heart, the endocardium. The structures most commonly involved are the heart valves.
  - Inflammatory cardiomegaly.
  - Myocarditis – inflammation of the myocardium, the muscular part of the heart.
- Valvular heart disease.
- Congenital heart disease – heart structure malformations existing at birth.
- Rheumatic heart disease – heart muscles and valves damage due to rheumatic fever caused by Streptococcus pyogenes a group A streptococcal infection.

2.5.2 Risk Factors

There are many risk factors for heart diseases: age, gender, tobacco use, physical inactivity, excessive alcohol consumption, unhealthy diet, obesity, genetic predisposition and family history of CVD, raised blood pressure (hypertension), raised blood sugar (diabetes mellitus), raised blood cholesterol (hyperlipidemia), psychosocial factors, poverty and low educational status, and air pollution. While the individual contribution of each risk factor varies between different communities or ethnic groups the overall contribution of these risk factors is very consistent. Some of these risk factors, such as age, gender or family history/genetic predisposition, are immutable; however, many important cardiovascular risk factors are modifiable by lifestyle change, social change, drug treatment (for example prevention of hypertension, hyperlipidemia, and DM). [147]

2.5.2.1 Genetics

Cardiovascular disease in a person's parents increases their risk by 3 fold. Multiple single nucleotide polymorphisms (SNP) have been found to be associated with CVD in genetic association studies, but usually their individual influence is small, and genetic contributions to CVD are poorly understood. [148]

2.5.2.2 Age

Age is by far the most important risk factor in developing cardiovascular or heart diseases, with approximately a tripling of risk with each decade of life. Coronary fatty streaks can begin to form in adolescence. It is estimated that (82%) of people who die of coronary heart disease are (65) and older. At the same time, the risk of stroke doubles every decade after age (55). [149]

Multiple explanations have been proposed to explain why age increases the risk of cardiovascular/heart diseases. One of them is related to serum cholesterol level. In most populations, the serum total cholesterol level increases as age increases. In men, this increase levels off around age (45 to 50) years. In women, the increase continues sharply until age (60 to 65) years.

Aging is also associated with changes in the mechanical and structural properties of the vascular wall, which leads to the loss of arterial elasticity and reduced arterial compliance and may subsequently lead to CAD. [150]

2.5.2.3 Sex

Men are at greater risk of heart disease than pre-menopausal women. Once past menopause, it has been argued that a woman's risk is similar to a man's although more recent data from the WHO and UN disputes this. If a female has DM, she is more likely to develop heart disease than a male with DM.

CHD are (2 to 5) times more common among middle-aged men than women. [151] In a study done by the WHO, sex contributes to approximately (40%) of the variation in sex ratios of coronary heart disease mortality.Another study reports similar results finding that gender differences explains nearly half the risk associated with cardiovascular diseases. One of the proposed explanations for gender differences in cardiovascular diseases is hormonal difference. Among women, estrogen is the predominant sex hormone. Estrogen may have protective effects through glucose metabolism and hemostatic system, and may have direct effect in improving endothelial cell function.[152]The production of estrogen decreases after menopause, and this may change the female lipid metabolism toward a more atherogenic form by decreasing the HDL cholesterol level while increasing LDL and total cholesterol levels.

Among men and women, there are notable differences in body weight, height, body fat distribution, heart rate, stroke volume, and arterial compliance. In the very elderly, age-related large artery pulsatility and stiffness is more pronounced among women than men. This may be caused by the women's smaller body size and arterial dimensions which are independent of menopause. [153]

2.5.2.4 Tobacco

Cigarettes are the major form of smoked tobacco. Risks to health from tobacco use result not only from direct consumption of tobacco, but also from exposure to second-hand smoke. Approximately (10%) of cardiovascular disease is attributed to smoking; however, people who quit smoking by age (30) have almost as low a risk of death as never smokers. [154]

2.5.2.5 Physical Inactivity

Insufficient physical activity (defined as less than (5 x 30 minutes) of moderate activity per week, as or less than (3 x 20 minutes) of vigorous activity per week) is currently the (4) leading risk factor for mortality worldwide. In 2008, (31.3%) of adults aged (15) or older (28.2% men and (34.4%) women) were insufficiently physically active. The risk of IHD and DM is reduced by almost a ⅓ in adults who participate in (150 minutes) of moderate physical activity each week (or equivalent). In addition, physical activity assists weight loss and improves blood glucose control, blood pressure, and lipid profile and insulin sensitivity. These effects may, at least in part, explain its cardiovascular benefits. [155]

2.5.2.6 Diet

High dietary intakes of saturated fat, trans-fats and salt and low intake of fruits, vegetables and fish are linked to cardiovascular risk, although whether all these associations are a cause is disputed. The WHO attributes approximately (1.7) million deaths worldwide to low fruit and vegetable consumption. The amount of dietary salt consumed is also an important determinant of blood pressure levels and overall cardiovascular risk. Frequent consumption of high-energy foods, such as processed foods that are high in fats and sugars, promotes obesity and may increase cardiovascular risk. Cutting down on saturated fat reduced risk of CVD by (17%) including heart disease and stroke. High trans-fat intake has adverse effects on blood lipids and circulating inflammatory markers, and elimination of trans-fat from diets has been widely advocated. There is evidence that higher consumption of sugar is associated with higher blood pressure and unfavorable blood lipids, and sugar intake also increases the risk of DM. High consumption of processed meats is associated with an increased risk of CVD, possibly in part due to increased dietary salt intake. [156]

The relationship between alcohol consumption and CVD is complex, and may depend on the amount of alcohol consumed. There is a direct relationship between high levels of alcohol consumption and risk of CVD. Drinking at low levels without episodes of heavy drinking may be associated with a reduced risk of CVD. Overall alcohol consumption at the population level is associated with multiple health risks that exceed any potential benefits. [157]

2.5.2.7 Socioeconomic Disadvantage

CVD affects low- and middle-income countries even more than high-income countries.There is relatively little information regarding social patterns of CVD within low- and middle-income countries, but within high-income countries low income and low educational status are consistently associated with greater risk of CVD. Policies that have resulted in increased socio-economic inequalities have been associated with greater subsequent socio-economic differences in CVD implying a cause and effect relationship. Psychosocial factors, environmental exposures, health behaviours, and health-care access and quality contribute to socio-economic differentials in cardiovascular disease. The Commission on Social Determinants of Health recommended that more equal distributions of power, wealth, education, housing, environmental factors, nutrition, and health care were needed to address inequalities in CVD and non-communicable diseases. [158]

2.5.2.8 Air Pollution

Particulate matter has been studied for its short- and long-term exposure effects on CVD. Currently, $PM_{2.5}$ is the major focus, in which gradients are used to determine CVD risk. For every (10 $\mu g/m^3$) of $PM_{2.5}$ long-term exposure, there was an estimated (8–18%) CVD mortality risk. Women had a higher relative risk (RR) (1.42) for $PM_{2.5}$ induced CAD than men (0.90) did. Overall, long-term PM exposure increased rate of atherosclerosis and inflammation. In regard to short-term exposure (2 hours), every (25 $\mu g/m^3$) of $PM_{2.5}$ resulted in a (48%) increase of CVD mortality risk. In addition, after only (5 days) of exposure, a rise in systolic (2.8 mmHg) and diastolic (2.7 mmHg) blood pressure occurred for every (10.5 $\mu g/m^{3)}$ of $PM_{2.5}$. Other research has implicated $PM_{2.5}$ in irregular heart rhythm, reduced heart rate variability (decreased vagal tone), and most notably heart failure. $PM_{2.5}$ is also linked to carotid artery thickening and increased risk of acute myocardial infarction. [159]

2.5.3 Cardiovascular Risk Assessment

Existing CVD or a previous cardiovascular event, such as a heart attack or stroke, is the strongest predictor of a future cardiovascular event. Age, sex, smoking, blood pressure, blood lipids and DM are important predictors of future CVD in people who are not known to have cardiovascular disease.These measures, and sometimes others, may be combined into composite risk scores to estimate an individual's future risk of CVD. Numerous risk scores exist although their respective merits are debated. Other diagnostic tests and biomarkers remain under evaluation but currently these lack clear-cut evidence to support their routine use. They include family history, coronary artery calcification score, high sensitivity C-reactive protein (hs-CRP), ankle–brachial pressure index, lipoprotein subclasses and particle concentration, lipoprotein(a), apolipoproteins A-I and B, fibrinogen, white blood cell count, homocysteine, N-terminal pro B-type natriuretic peptide (NT-proBNP), and markers of kidney function. High blood phosphorus is also linked to an increased risk. [160]

Occupational CVD is disease of the heart or blood vessels that are caused by working conditions, making them a form of occupational illness. Little is known about occupational risks for heart disease, but links have been established between CVD and certain toxins (including carbon disulfide, nitroglycerin, and carbon monoxide), extreme heat and cold, exposure to tobacco smoke, depression, and occupational stress. Other occupational hazards potentially related to CVD include noise exposure at work, shift work, and physical activity at work.

"Best buys" or very cost-effective interventions that are feasible to be implemented even in low-resource settings have been identified by WHO for prevention and control of CVD. They include (2) types of interventions: population-wide and individual, which are recommended to be used in combination to reduce the greatest CVD burden. [160] [161]

Examples of population-wide interventions that can be implemented to reduce CVDs include:

- Comprehensive tobacco control policies.
- Taxation to reduce the intake of foods that are high in fat, sugar and salt.
- Building walking and cycle paths to increase physical activity.
- Strategies to reduce harmful use of alcohol.
- Providing healthy school meals to children.

At the individual level, for prevention of first heart attacks and strokes, individual health-care interventions need to be targeted to those at high total cardiovascular risk or those with single risk factor levels above traditional thresholds, such as hypertension and hypercholesterolemia. The former approach is more cost-effective than the latter and has the potential to substantially reduce cardiovascular events. This approach is feasible in primary care in low-resource settings, including by non-physician health workers.

For secondary prevention of CVD in those with established disease, including diabetes, treatment with the following medications is necessary:

- Aspirin.
- β -blockers.
- Angiotensin-converting enzyme inhibitors.
- Statins.

The benefits of these interventions are largely independent, but when used together with smoking cessation, nearly (75%) of recurrent vascular events may be prevented. Currently there are major gaps in the implementation of these interventions particularly at the primary health care level.

In addition costly surgical operations are sometimes required to treat CVDs. They include:

- Coronary artery bypass.
- Balloon angioplasty (where a small balloon-like device is threaded through an artery to open the blockage).
- Valve repair and replacement.
- Heart transplantation.
- Artificial heart operations.

Medical devices are required to treat some CVDs. Such devices include pacemakers, prosthetic valves, and patches for closing holes in the heart.

2.6 Coronary Artery Disease

CAD, also known as ischemic heart disease (IHD), is a group of diseases that includes: stable angina, unstable angina, MI, and sudden cardiac death. It is within the group of cardiovascular diseases of which it is the most common type. A common symptom is chest pain or discomfort which may travel into the shoulder, arm, back, neck, or jaw. Occasionally it may feel like heartburn. Usually symptoms occur with exercise or emotional stress, last less than a few minutes, and get better with rest. Shortness of breath may also occur and sometimes no symptoms are present.The first sign is occasionally a heart attack. Other complications include HF or an irregular heartbeat. [161]

Risk factors include: high blood pressure, smoking, diabetes, lack of exercise, obesity, high blood cholesterol, poor diet, and excessive alcohol, among others.Other risks include depression.The underlying mechanism involves atherosclerosis of the arteries of the heart. A number of tests may help with diagnoses including: ECG, cardiac stress testing, coronary computed tomographic angiography, and coronary angiogram, among others. [162]

Prevention is by eating a healthy diet, regular exercise, maintaining a healthy weight and not smoking.Sometimes medication for diabetes, high cholesterol, or high blood pressure are also used.There is limited evidence for screening people who are at low risk and do not have symptoms.Treatment involves the same measures as prevention.Additional medications such as antiplatelets including aspirin, beta blockers, or nitroglycerin may be recommended. PCI or CABG may be used in severe disease. In those with stable CAD it is unclear if PCI or CABG in addition to the other treatments improves life expectancy or decreases heart attack risk. [163]

In 2015 CAD affected (110) million people and resulted in (8.9) million deaths.It makes up (15.9%) of all deaths makes it the most common cause of death globally. The risk of death from CAD for a given age has decreased between 1980 and 2010 especially in developed countries. The number of cases of CAD for a given age has also decreased between 1990 and 2010. In the United States in 2010 about (20%) of those over (65) had CAD, while it was present in (7%) of those (45) to (64), and (1.3%) of those (18) to (45). Rates are higher among men than women of a given age. [164]

2.7 Peripheral Artery Disease

PAD is a narrowing of the arteries other than those that supply the heart or the brain.When narrowing occurs in the heart it is called CAD while in the brain it is called cerebrovascular disease. PAD most commonly affects the legs, but other arteries may also be involved. The classic symptom is leg pain when walking which resolves with rest, known as intermittent claudication. Other symptoms including skin ulcers, bluish skin, cold skin, or poor nail and hair growth may occur in the affected leg. Complications may include an infection or tissue death which may require amputation; coronary artery disease, or stroke. Up to (50%) of cases of PAD are without symptoms. [165]

The main risk factor is cigarette smoking.Other risk factors include DM, high blood pressure, and high blood cholesterol. The underlying mechanism is usually atherosclerosis.Other causes include artery spasm. PAD is typically diagnosed by finding an ankle-brachial index (ABI) less than (0.90), which is the systolic blood pressure at the ankle divided by the systolic blood pressure of the arm. Duplex ultrasonography and angiography may also be used. Angiography is more accurate and allows for treatment at the same time; however, it is associated with greater risks. [166]

It is unclear if screening for disease is useful as it has not been properly studied.In those with intermittent claudication from PAD, stopping smoking and supervised exercise therapy improves outcomes. Medications, including statins, ACE inhibitors, and cilostazol also may help. Aspirin does not appear to help those with mild disease but is usually recommended in those with more significant disease. Anticoagulants such as warfarin are not typically of benefit. Procedures used to treat the disease include bypass grafting, angioplasty, and atherectomy. [167]

In 2010 about (202) million people had PAD worldwide.In the developed world it affects about (5.3%) of (45) to (50) years olds and (18.6%) of (85-) to (90-) year-olds. In the developing world it affects (4.6%) of people between the ages of (45) to (50) and (15%) of people between the ages of (85) to (90). In the developed world PAD is equally common among men and women while in the developing world women are more commonly affected. In 2013 PAD resulted in about (41,000) deaths up from 16,000 deaths in 1990. [168]

2.8 Heart Failure

HF, often referred to as CHF, occurs when the heart is unable to pump sufficiently to maintain blood flow to meet the body's needs Signs and symptoms commonly include shortness of breath, excessive tiredness, and leg swelling. The shortness of breath is usually worse with exercise, while lying down, and may wake the person at night. A limited ability to exercise is also a common feature. Chest pain, including angina, does not typically occur due to HF. [169]

Common causes of HF include CAD including a previous myocardial infarction (heart attack), high blood pressure, atrial fibrillation, valvular heart disease, excess alcohol use, infection, and cardiomyopathy of an unknown cause. These cause HF by changing either the structure or the functioning of the heart.There are two main types of heart failure: heart failure due to left ventricular dysfunction and HF with normal ejection fraction depending on whether the ability of the left ventricle to contract is affected, or the heart's ability to relax. The severity of disease is usually graded by the degree of problems with exercise. HF is not the same as MI (in which part of the heart muscle dies) or cardiac arrest (in which blood flow stops altogether). Other diseases that may have symptoms similar to HF include obesity, kidney failure, liver problems, anemia, and thyroid disease. [170]

The condition is diagnosed based on the history of the symptoms and a physical examination with confirmation by echocardiography. Blood tests, electrocardiography, and chest radiography may be useful to determine the underlying cause. Treatment depends on the severity and cause of the disease. In people with chronic stable mild heart failure, treatment commonly consists of lifestyle modifications such as stopping smoking, physical exercise, and dietary changes, as well as medications. In those with heart failure due to left ventricular dysfunction, angiotensin converting enzyme inhibitors or angiotensin receptor blockers along with β blockers are recommended.For those with severe disease, aldosterone antagonists, or hydralazine with a nitrate may be used. Diuretics are useful for preventing fluid retention. Sometimes, depending on the cause, an implanted device such as a pacemaker or an implantable cardiac defibrillator may be recommended. In some moderate or severe cases cardiac resynchronization therapy (CRT) may be suggested or cardiac contractility modulation may be of benefit.A ventricular assist device or occasionally a heart transplant may be recommended in those with severe disease despite all other measures. [171]

HF is a common, costly, and potentially fatal condition. In 2015 it affected about (40) million people globally. In developed countries, around (2%) of adults have heart failure and in those over the age of (65), this increases to (6–10%). In the year after diagnosis the risk of death is about (35%) after which it decreases to below (10%) each year. This is similar to the risks with a number of types of cancer. In the United Kingdom the disease is the reason for (5%) of emergency hospital admissions.Heart failure has been known since ancient times with the Ebers papyrus commenting on it around 1550 BCE. [172]

2.9 Obesity and Associated Comorbidities

Obesity is associated with numerous comorbidities such as CVD, T2DM, and hypertension, certain cancers, and sleep apnea. In fact, obesity is an independent risk factor for CVD, and CVD risks have been documented in obese children. Indeed, a relationship exists between BMI in adolescence and all-cause mortality. After a follow-up of (31.5 years), with those with a BMI between the 25th and 75th percentiles used as control subjects, it was reported that a BMI above the 95th percentile in adolescence predicted adult mortality rates in both male (80% increment) and female (≈100% increment) patients. A (30%) increase in all-cause mortality was also seen in female and male subjects when baseline BMI was between the 85th and 95th percentiles. Another study, after (55 years) of follow-up, reported an excess mortality rate among male but not female subjects who were overweight (BMI >75th percentile in the US reference population) in adolescence as compared with those who were lean (BMI 25th to 49th percentiles). The observed increased risk of death was independent of adult BMI. Thus, obesity is associated with an increased risk of morbidity and mortality and is associated with reduced life expectancy. [180] [181] [182]

Besides an altered metabolic profile, a variety of adaptations/alterations in cardiac structure and function occur in the individual as adipose tissue accumulates in excess amounts, even in the absence of comorbidities. Hence, obesity may affect the heart through its influence on known risk factors such as dyslipidemia, hypertension, glucose intolerance, inflammatory markers, obstructive sleep apnea/hypoventilation, and the prothrombotic state, as well as through yet-unrecognized mechanisms. Overweight/obesity predisposes or is associated with numerous cardiac complications such as CHD, HF, and sudden death through its impact on the cardiovascular

system. The pathophysiology of these entities linked to obesity will be discussed in the following sections. [183]

2.10 Cardiovascular Impact of Increased Adipose Tissue Mass

2.10.1 Adipose Tissue Circulation

It has long been recognized that an extensive capillary network surrounds adipose tissue. Adipocytes are located close to vessels with the highest permeability, the lowest hydrostatic pressure, and the shortest distance for transport of molecules to and from the adipocytes. Resting blood flow is usually (2 to 3 mL/min per 100 g) of adipose tissueand can increase (≈10-fold). This increment is still lower (≈20 mL/min per 100 g) than that seen in skeletal muscle (50 to 75 mL/min per 100 g). Adipose tissue blood flow increases after meal intake, but this modulation varies and may be decreased in patients with the features of the obesity-related MetS. [184]

Also, adipose tissue comprises a substantial proportion of total body weight. Therefore, a large quantity of fluid is present in the interstitial space of adipose tissue, as the interstitial space is (≈10%) of the tissue wet weight. Excess fluid in this compartment may have important repercussions in obese individuals with HF if this extra volume is redistributed into the circulation; however, modulation of blood flow through adipose tissue typically prevents this from occurring. This is because blood flow in adipose tissue is regulated by β_1-receptors that mediate vasodilation, in contrast to those of skeletal muscle, which are mainly β_2. As a consequence of this decrease in blood flow in adipose tissue, the fluid present in the interstitial compartment is not readily accessible. Although cardiac output increases with total fat mass, the perfusion per unit of adipose tissue actually decreases with increasing obesity, that is, from (2.36 mL/min per 100 g) to (1.53 mL/min per 100 g) of adipose tissue (≈35%) in patients who have (15% to 26%) body fat compared with those with (>36%) body fat. Accordingly, the increase in systemic blood flow encountered in obesity cannot be explained solely by increased requirements caused by adipose tissue perfusion because the enlarged vascular bed of adipose tissue is less vascularized than other tissue. Probably, the concomitant increase in lean body mass in these individuals accounts for some of the increased cardiac output. Indeed, it has been reported that stroke volume, cardiac output, and left ventricular mass may be more related to fat-free mass than to fat mass. [185] [186]

The adipose tissue is not simply a passive storehouse for fat but an endocrine organ that is capable of synthesizing and releasing into the bloodstream an important variety of peptides and nonpeptide compounds that may play a role in cardiovascular homeostasis. This is of importance because IL-6 modulates CRP production in the liver, and CRP may be a marker of a chronic inflammatory state that can trigger acute coronary syndrome. [188]

2.11 Hemodynamic Repercussion of Obesity

Obesity produces an increment in total blood volume and cardiac output that is caused in part by the increased metabolic demand induced by excess body weight. Thus, at any given level of activity, the cardiac workload is greater for obese subjects. Obese subjects have higher cardiac output and a lower total peripheral resistance than do lean individuals. The increased cardiac output is attributable mostly to increased stroke volume because heart rate increases little if at all. Also, in obesity, the Frank-Starling curve is shifted to the left because of incremental increases in left ventricular filling pressure and volume, which over time may produce chamber dilation. Ventricular chamber dilation may then lead to increased wall stress, which predisposes to an increase in myocardial mass and ultimately to left ventricular hypertrophy,[189][190] [191]characteristically of the eccentric type. Left atrial enlargement may also occur in normotensive obese individuals but typically in the setting of increased left ventricular mass. Left atrial enlargement may not be mediated solely through left ventricular diastolic dysfunction impairment but may simply reflect a physiological adaptation to the expanded blood volume. As a consequence, left atrial dilation may mediate the excess risk of atrial fibrillation associated with obesity. However, left ventricular hypertrophy (LVH) in long-standing obesity and/or the effects of concomitant hypertension may also be contributing factors to left atrial enlargement. [192]

Weight loss through diet and exercise is recommended in the management of obesity, but it is important to recognize that obesity is associated with persistence of elevated cardiac filling pressures during exercise. Increased cardiac output during exercise is typically accompanied by an increase in left ventricular filling pressure, often exceeding (20 mm Hg). Therefore, the average left ventricular filling pressure is often within the upper limits of normal at rest but increases disproportionately with increased venous return during exercise. [193]This is consistent with a high-pressure system, and, accordingly, obese patients may demonstrate higher right heart filling

pressures, systolic pressure, cardiac output, and pulmonary vascular resistance index. The latter may reflect intrinsic pulmonary disease, abnormal left ventricular function, or undiagnosed causes of pulmonary hypertension such as sleep apnea/hypoventilation or recurrent pulmonary thromboembolism. With increased venous return, small increments of central blood volume are associated with a significant increase in left ventricular end-diastolic pressure. A decrease in central blood volume accompanies weight reduction, and, when present, relief of edema and dyspnea may accompany this improvement. [194]

2.12 Effects on Ventricular Function

Eccentric LVH, which is commonly present in morbidly obese patients (BMI≥40 kg/m^2), is often associated with left ventricular diastolic dysfunction. Moreover, as with left ventricular mass, longer durations of obesity are associated with poorer left ventricular systolic function and greater impairment of left ventricular diastolic function. Because of the presence of nonspecific symptoms, the evaluation of the presence of left ventricular diastolic dysfunction is clinically important in obese subjects. Age and cardiac hypertrophy of the concentric or, more commonly, the eccentric type predispose to left ventricular systolic dysfunction. In humans and most animal models, the development of obesity leads not only to increased fat depots in classic adipose tissue locations but also to significant lipid deposits in other organs. With fat gain, lipid deposition can impair tissue and organ function in (2) possible ways: (1) The size of fat pads around key organs may increase substantially, modifying organ function either by simple physical compression or because periorgan fat cells may secrete various locally acting molecules, and (2) lipid accumulation can occur in nonadipose cells and may lead to cell dysfunction or cell death, a phenomenon known as lipotoxicity. Abnormal cellular adaptations may unfavorably affect the cardiac muscle, which is one of the several mechanisms leading to cardiomyopathy. [197]

2.13 Cardiomyopathy of Obesity (Adipositas Cordis)

Obesity cardiomyopathy was recognized as early as 1818. The case described by Cheyneis of historic interest, not only because it is a carefully recorded documentation of a fatty heart but because it was the first reported case of Cheyne-Stokes respiration.[198] Metaplasia is a reversible change in which one adult cell type (epithelial or mesenchymal) is replaced by another adult cell

type. It may represent an adaptative substitution of cells that are sensitive to stress by cell types better able to withstand the adverse environment. Cords of cells can gradually accumulate fat between muscle fibers or cause myocyte degeneration, resulting in cardiac conduction defects. These cords of fat cells may also emanate from epicardial fat. When the right ventricle is involved, the sinus node musculature, the atrioventricular node, the right bundle branch [(199)] and, ultimately, the entire myocardium of the atrioventricular region might be replaced by fat. Occasionally, a pattern of restrictive cardiomyopathy develops.In this situation, small irregular aggregates and bands of adipose tissue separate myocardial cells, a potential result of pressure-induced atrophy from the intervening fat. An alternative explanation could be, as discussed previously, the lipotoxicity of the myocardium induced by free fatty acids, which can cause apoptosis of lipid-laden cells such as cardiomyocytes.

Thus, through different mechanisms (increased total blood volume, increased cardiac output, LVH, left ventricular diastolic dysfunction, adipositas cordis), obesity may predispose to HF. Because dyspnea with exertion and lower-extremity edema are often nonspecific signs of heart disease in obesity, it may be difficult to clinically assess an obese individual because of several limitations inherent to the subject's morphology. [(200)]

2.14 Clinical and Laboratory Assessment of Obese Individuals

2.14.1 History and Physical Examination

The physical examination and ECG often underestimate the presence and extent of cardiac dysfunction in obese patients. Cardiovascular manifestations likely occur on a continuum from the overweight to the morbidly obese individuals because symptoms and signs of obesity cardiomyopathy occur mainly in patients with a relative weight (≥175%) or a BMI (≥40 kg/m^2). On physical examination, jugular venous distention and hepatojugular reflux may not be seen, and heart sounds are usually distant. However, dorsal hand veins, if visible, can estimate central venous pressure. The hand is lowered beneath the sternal angle until the dorsal veins are distended. [(201)] The arm is then gradually and passively raised while the dorsal veins are observed. Normally, the dorsal hand veins empty at the level of the sternal angle when the patient's trunk is (30° to 45°) above the horizontal. Although this bedside technique remains a crude evaluation with several limitations, persistent distention is recorded as the vertical distance above the angle of

Louis.In the very obese patient, symptoms of heart disease may remain nonspecific, but the clinician should carefully search for the presence of cor pulmonale. In the majority of individuals, the splitting of the S_2 is most often heard at the 2^{nd} or 3^{rd} left interspace parasternally, but in obese patients, the split S_2 is either inaudible or very poorly defined in the 2^{nd} interspace and is often best heard at the 1^{st} left interspace. An electronic stethoscope may be helpful. This is of importance because pulmonary artery systolic pressure has been reported to be above the suggested normal limit (≤30 mm Hg) in (51%) of obese patients, and for each increase in BMI, the pulmonary artery systolic pressure is increased by (≈0.1 to 0.4 mm Hg). [(202)]

2.14.2 Electrocardiogram

Like physical evaluation, the ECG is influenced by morphological changes induced by obesity, such as (1) displacement of the heart by an elevated diaphragm in the supine position, (2) increased cardiac workload with associated cardiac hypertrophy, (3) increased distance between the heart and the recording electrodes induced by the accumulation of adipose tissue in the subcutaneous tissue of the chest wall (and possibly increased epicardial fat), and (4) the potential associated chronic lung disease secondary to the sleep apnea/hypoventilation syndrome. [(203)]

2.14.3 Echocardiography

In times past, the cardiac status of obese individuals was difficult to assess, and obesity-induced cardiac abnormalities were found only after death.Even since the development of echocardiography, transthoracic echocardiography can be technically difficult in obese patients.Differentiation between subepicardial adipose tissue and pericardial effusion is often difficult in obese patients. [(209)]Epicardial adipose tissue is known to be a common cause of false-positive effusion (pseudopericardial effusion), and this adipose tissue depot may cause an underestimation of the amount of pericardial fluid.

2.15 Metabolic Syndrome (Mets)

Mets is the name for a group of risk factors that raises your risk for heart disease and other health problems, such as DM and stroke. The term "metabolic" refers to the biochemical processes

involved in the body's normal functioning. Risk factors are traits, conditions, or habits that increase chance of developing a disease. [224]

Plaque hardens and narrows the arteries, reducing blood flow tobheart muscle. This can lead to chest pain, a heart attack, heart damage, or even death.

2.15.1 Metabolic Risk Factors

The (5) conditions described below are metabolic risk factors. [225]

- A large waistline. This also is called abdominal obesity or "having an apple shape." Excess fat in the stomach area is a greater risk factor for heart disease than excess fat in other parts of the body, such as on the hips.
- A high TG level. TG is a type of fat found in the blood.
- A low HDL cholesterol level. HDL sometimes is called "good" cholesterol. This is because it helps remove cholesterol from your arteries. A low HDL cholesterol level raises your risk for heart disease.
- High blood pressure . Blood pressure is the force of blood pushing against the walls of your arteries as your heart pumps blood. If this pressure rises and stays high over time, it can damage your heart and lead to plaque buildup.
- High fasting blood sugar. Mildly high blood sugar may be an early sign of DM.[225] [226]

2.15.2 Risk for Metabolic Syndrome

People at greatest risk for Mets have these underlying causes:

- Abdominal obesity (a large waistline).
- An inactive lifestyle.
- Insulin resistance.

Some people are at risk for Mets because they take medicines that cause weight gain or changes in blood pressure, blood cholesterol, and blood sugar levels. These medicines most often are used to treat inflammation, allergies, HIV, and depression and other types of mental illness.[228] [229]

2.15.3.1 A Large Waistline

Having a large waistline means that carry excess weight around your waist (abdominal obesity). This is also called having an "apple-shaped" figure. The doctor will measure waist to find out whether have a large waistline.

A waist measurement of (35 inches) or more for women or (40 inches) or more for men is a metabolic risk factor. A large waistline means you're at increased risk for heart disease and other health problems.

2.15.3.2 A High Triglyceride Level

Triglycerides are a type of fat found in the blood. TG level of (150 mg/dL) or higher (or being on medicine to treat high TG) is a metabolic risk factor. (The mg/dL is milligrams per deciliter—the units used to measure TG, cholesterol, and blood sugar.)

2.15.3.3 A Low HDL Cholesterol Level

HDL cholesterol sometimes is called "good" cholesterol. This is because it helps remove cholesterol from arteries.

An HDL cholesterol level of less than (50 mg/dL) for women and less than (40 mg/dL) for men (or being on medicine to treat low HDL cholesterol) is a metabolic risk factor.

2.15.3.4 High Blood Pressure

A blood pressure of (130/85 mmHg) or higher (or being on medicine to treat high blood pressure) is a metabolic risk factor. (The mmHg is millimeters of mercury—the units used to measure blood pressure.)

If only (1) of (2) blood pressure numbers are high, still at risk for Mets.

2.15.3.5 High Fasting Blood Sugar

A normal fasting blood sugar level is less than (100 mg/dL). A fasting blood sugar level between (100–125 mg/dL) is considered prediabetes. A fasting blood sugar level of (126 mg/dL) or higher is considered diabetes.

A fasting blood sugar level of (100 mg/dL) or higher (or being on medicine to treat high blood sugar) is a metabolic risk factor.

About (85%) of people who have T2DM—the most common type of DM—also have metabolic syndrome. These people have a much higher risk for heart disease than the (15%) of people who have T2DM without metabolic. [234]

2.16 Wnt Signaling Pathway

The Wnt signaling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. (3)Wnt signaling pathways have been characterized: the canonical Wnt pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway.[235]The noncanonical planar cell polarity pathway regulates the cytoskeleton that is responsible for the shape of the cell. The noncanonical Wnt/calcium pathway regulates calcium inside the cell. Wnt signaling pathways use either nearby cell-cell communication (paracrine) or same-cell communication (autocrine). They are highly evolutionarily conserved in animals, which mean they are similar across animal species from fruit flies to humans. [236]

Wnt signaling was first identified for its role in carcinogenesis, then for its function in embryonic development. The embryonic processes it controls include body axis patterning, cell fate specification, cell proliferation and cell migration. These processes are necessary for proper formation of important tissues including bone, heart and muscle. Its role in embryonic development was discovered when genetic mutations in Wnt pathway proteins produced abnormal fruit fly embryos. Wnt signaling also controls tissue regeneration in adult bone marrow, skin and intestine.Later research found that the genes responsible for these abnormalities also influenced breast cancer development in mice. This pathway's clinical importance was

demonstrated by mutations that lead to various diseases, including breast and prostate cancer, glioblastoma, type II diabetes and others. [237]

2.16.1 Proteins

Wnt comprises a diverse family of secreted lipid-modified signaling glycoproteins that are (350–400) amino acids in length.The type of lipid modification that occurs on these proteins is palmitoylation of cysteines in a conserved pattern of (23–24) cysteine residues. Palmitoylation is necessary because it initiates targeting of the Wnt protein to the plasma membrane for secretion and it allows the Wnt protein to bind its receptor due to the covalent attachment of fatty acids. Wnt proteins also undergo glycosylation, which attaches a carbohydrate in order to ensure proper secretion. In Wnt signaling, these proteins act as ligands to activate the different Wnt pathways via paracrine and autocrine routes.These proteins are highly conserved across species.They can be found in mice, humans, Xenopus, zebrafish, Drosophila and many others.[239] [240]

2.16.2 The Basics: Wnt Genes and Predicted Protein Products

All metazoan species express Wnt genes, with the genome of Hydra vulgaris carrying (13) and mice and humans carrying (19) independent genes (additional information can be found on the Wnt homepage. Based on their primary amino acid sequence, all Wnt genes are predicted to encode secreted proteins. The defining property of Wnt proteins is a nearly invariant positioning of (22) cysteine residues, most of which are postulated to form disulfide bridges that maintain a globular secondary structure

Intermolecular disulfide linkages; however, this has not been observed to be the case for purified and biologically active Wnt proteins. In addition, the recent high-resolution structure of a Wnt protein suggests that all conserved cysteine residues are occupied in intramolecular rather than intermolecular disulfide bridges. Wnt proteins carry several stretches of highly charged amino acids and have a predicted isoelectric point of nearly (9), which, in combination with multiple glycosylation events, would lead one to expect that Wnt proteins are readily soluble in an aqueous environment. [240] [241]

The primary amino acid sequence of Wnt shows several hallmarks of secreted proteins, most notably a signal sequence for secretion, stretch of approximately (20) hydrophobic amino acids. Cleavage of this signal peptide can be predicted using several computer algorithms; however, the

true first amino acid of a Wnt protein was identified by amino-terminal sequencing of a purified Wntprotein. Interestingly, in the case of Wnt5a, the amino-terminal residue was found to be located (62) amino acids from the predicted translational start site. This observation serves as a cautionary note for those who wish to append amino-terminal tags (His, HA, or GFP) onto Wnts, because such tags may be cleaved from the mature protein upon signal sequence cleavage. [242]

Aside from targeting Wnt proteins for secretion, the amino terminus may harbor additional critical biological functions. A survey of isoform and alternative splicing databases reveals that multiple Wnt genes carry distinct 5′ untranslated regions (UTRs) and are predicted to encode distinct amino termini. In the case of Wnt16, two isoforms with distinct 5′ UTRs are expressed from alternative promoters. Although little is known regarding the biological significance of these two isoforms, it is intriguing that one of the two isoforms has a broad expression pattern, whereas the other is restricted to the pancreas. Changes in the amino terminus of Wnt proteins may represent a common mechanism by which signaling activity can be affected a gene encoding a transmembrane protein that antagonizes Wnt signaling .Tiki protein acts cell-autonomously as a protease to cleave eight amino terminal residues from a Wnt protein, thereby reducing receptor-binding and signaling activities. Such observations suggest that the Wnt family of proteins is significantly more complex and diverse than expected for 19 genes. [242] [244]

It should be noted that in contrast to Wnt signaling, which has been conveniently—and perhaps inappropriately—categorized as either canonical or noncanonical, no sequence or structural basis for this distinction has been identified in Wnt proteins. Although many studies make reference to either canonical or noncanonical Wnts, this difference is most likely conferred by cellular context as determined by the expressed repertoire of receptors and signal transducers rather than by an intrinsic property of the Wnt proteins. The hypothesis that Wnt signaling activity is conferred by cellular context rather than by the Wnt protein sequence is supported by the observations that a so-called noncanonical Wnt5a can act “canonically” by activating β-catenin signaling in certain contexts. Furthermore, maternal Wnt11, which has been largely studied for its roles in noncanonical Wnt signaling, specifies the dorsal axis in *Xenopus* by localizing β-catenin to dorsal nuclei, thus promoting a canonical signaling pathway. [245]

2.16.3 Structure of Wnt Proteins

While it required (20 years) to obtain a pure and biologically active Wnt protein, it took another (10) years to achieve its crystallization and provide a high-resolution structure of a Wnt protein. The feat was accomplished in Chris Garcia's laboratory and has revealed several interesting properties of Wnt proteins. The (3.25) Å crystal structure was determined for Xenopus Wnt8 (XWnt8) in complex with the CRD of mouse Frizzled8 and reveals a highly unusual two-domain structure with amino-terminal and carboxy-terminal domains (NTD and CTD) forming a protein fold previously not identified in any other protein structure **(Fig 2-5)**. The NTD is composed of a cluster of α-helices with (10) of the conserved cysteine residues forming (5) disulfide bridges, whereas the CTD is dominated by (2) β-sheets and maintained by six disulfide bridges. [246]

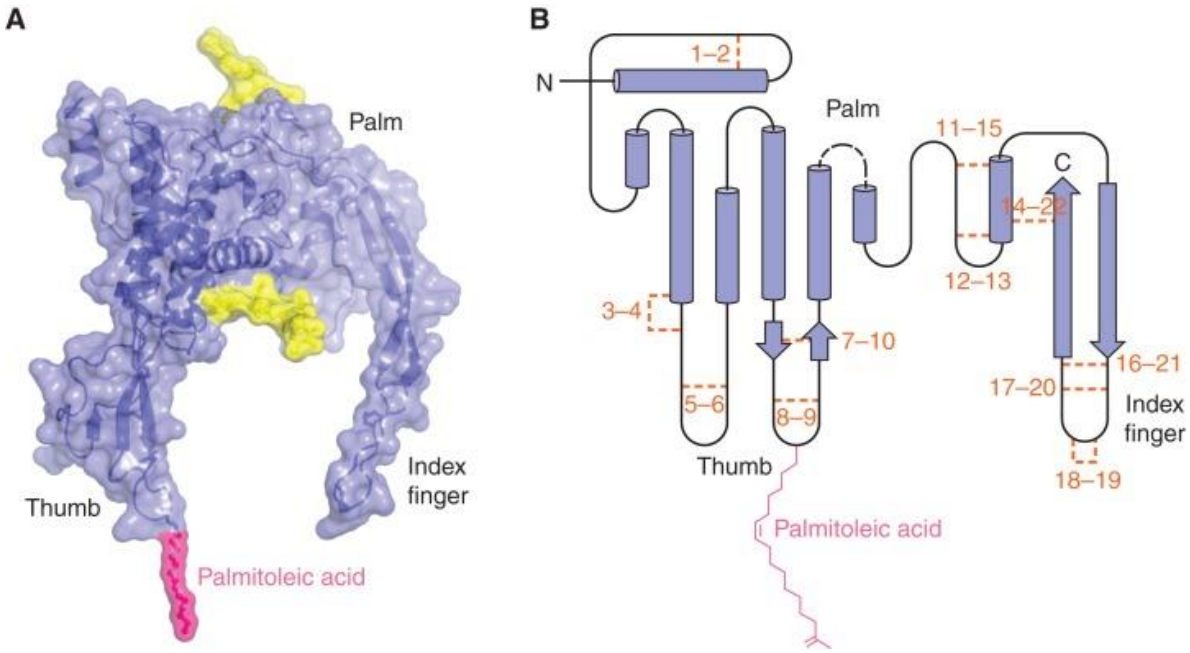


Figure (2-5): Structure of Wnt. (*A*) Space-filling model of XWnt8. The Frizzled CRD structure has been removed. (Yellow clusters) The *N*-linked glycosylations in XWnt8. (*B*) Secondary structure for Wnt. (Orange) the conserved 22 cysteine residues are numbered to indicate the pairs that form disulfide bridges. *N*-linked glycosylations are not shown because the numbers and positions of *N*-linked glycosylations are highly variable among Wnts. (Dashed line) the approximate position of the linker region where Wg carries an insert of about

80 amino acids. (Figures were generated with the kind assistance of C. Janda and C. Garcia, Stanford University.) [246]

In this structure, Wnt extends a thumb from the NTD and an index finger from the CTD to grasp the globular Frizzled CRD. Interestingly, the thumb extends a lipid at serine 187, the highly conserved residue previously identified to carry a covalently attached palmitoleic acid. This protein–lipid thumb structure is nestled in a deep hydrophobic groove of the CRD, where it makes multiple contacts to completely traverse the CRD surface. As is the case with the thumb, the points of contact of the index finger in the CTD with the CRD are also dominated by hydrophobic and highly conserved residues. Some of the contact points on the Frizzled8 CRD are substituted in other Frizzled CRDs, thus providing a possible mechanism to influence Wnt–Frizzled binding specificities. To further extend the hand analogy for the Wnt structure, the region between the thumb and the index finger is akin to the palm, where the NTD and CTD are intimately associated. Interestingly, the solvent-exposed linker region between NTD and CTD corresponds to the region with the greatest flexibility among Wnt proteins; in particular, Wg carries a large insert of 80 amino acids not present in other Wnts. This "Wg insert" has been used to generate arguably the best antisera to any Wnt protein, further supporting the model that this nonconserved linker region is solvent exposed and likely not involved in binding to the Frizzled CRD. [247] [248]

This Wnt structure will enable a more rational approach to interrogate Wnt–Frizzled interactions and design Wnt agonists and antagonists. A remaining unanswered question concerns the structure of an uncomplexedWnt protein. The covalently attached lipid necessitates some type of interaction, either with a carrier protein, such as Swim, or with membranous domains, to shield this hydrophobic moiety in a largely aqueous environment. The highly accessible presentation of the lipid moiety on Wnt makes the interaction with carrier molecules or the plasma membrane a likely scenario. An alternative possibility is that the hydrophobic portions of the thumb and index finger fold in to form a fist; however, the current crystal structure does not provide evidence for such intramolecular folding. [249]

2.16.4 Secretion of Wnt

S ER and Golgi-processed Wnt protein is targeted for secretion, a sorting receptor encoded by the *Wntless* gene (Wls, also known as Eveness interrupted/Evi, Sprinter, MIG-14, and Gpr177), a multispan transmembrane protein, binds and accompanies Wnt to the cell surface. Wls binding of

Wnt3a requires acylation on Ser-209, indicating that it acts downstream from Porcn. A comprehensive mutational analysis indicated that acylation of the Ser equivalent of Wg^{S239} is required for the interaction of Wls with all Wnts, except WntD. Furthermore, by using a membrane-tethered Wg protein, WgNRT, Herr and Basler provided evidence that mere membrane association is not sufficient for Wg association with Wls, thus suggesting that acylation by Porcn enables Wnt's functional interaction with Wls. In addition, in the case of Wg, glycosylation on conserved residues does not affect the dependence of Wnt on Wls. [250]

Vacuolar acidification is required for release of Wnt protein and small drug inhibition of the V-ATPase, a proton pump required for vacuolar acidification, prevents Wls from releasing Wnt so that Wnt–Wls complexes accumulate both in cells and at the plasma membrane. However, although essential, a decrease in pH is not sufficient to dissociate the Wnt–Wls complex. [251]

RNA interference screens in two separate laboratories identified members of the p24 protein family as cargo receptors for Wnt in anterograde transport and secretion. Although the (2) groups differ slightly on which of the (9) fly p24 family members are involved in Wg secretion, they agree in the basic finding that these cargo proteins specifically regulate Wg secretion and that secretion of other signaling molecules (e.g., Decapentaplegic, Hedgehog, and Unpaired) is unaffected. Therefore, Wnt protein does not exit a cell through passive transport or bulk flow but requires specific cargo proteins, such as the p24 family of highly conserved transmembrane receptors, to exit from the ER. [252]

Once Wnt is released from the cell, Wls is recycled via endosomes and the retromer complex to the Golgi, where it acts again to escort a newly processed Wnt protein to the cell surface .As a result, interfering with either Wls expression or it is recycling via the retromer inhibits Wnt secretion. In contrast to the classical retromer complex, which involves the sorting nexins SNX1–SNX2 and SNX5–SNX6 (referred to as SNX–BAR sorting nexins) and cargo-selective VPS26, VPS29, and VPS35, Wls recycling requires the distantly related sorting nexin SNX3.Therefore, retrograde transport of Wls is distinct from other recycled cargo, raising the possibility of specifically interfering with Wnt secretion through targeted disruption of Wls recycling via SNX3. [253]

If retromer components Vps35 and Vps26 are mutant, Wls is targeted for degradation rather than for endosomal recycling to the Golgi so that it is no longer available to facilitate Wnt secretion.

Such defects in the retromer complex can be rescued by overexpression of Wls. In Caenorhabditis elegans, it has been shown that the recycling of the Wls homolog MIG-14 from the cell surface to the Golgi requires the MTM-6/MTM-9 myotubularin complex, which dephosphorylates PIP_3, a central regulator of endosomal trafficking. A conserved endocytosis motif required for Wls recycling has been identified in the third intracellular loop of Wls; its mutation results in Wls accumulation on the cell surface and impair Wg secretion and signaling. (254)

Although it is clear that theWls/retromer system is essential for Wnt secretion. Long-range, but not short-range action of Egl-20 (a worm Wnt) was impaired in retromer mutants, suggesting that the retromer complex is important for packaging Wnt for long-range signaling. In contrast, flies carrying mutations in Vps35 are defective in short-range signaling as evidenced by strong reduction in expression of the Wg target senseless. (255)

In motor neurons in Drosophila, Wls not only transports Wnt from the Golgi to the plasma membrane, but also functions to shuttle Wnt across the synaptic cleft of the neuromuscular junction in exosome-like vesicles. Furthermore, postsynaptically in the muscle, Wls also guides Wg-activated Frizzled-2 trafficking before Frizzled-2 is proteolytically cleaved and its carboxyl terminus is imported into the nucleus. (256)

Structural modeling suggests that the ER luminal portion of Wls contains a lipocalin-family fold, which has been shown to interact with lipids. Interestingly, a lipocalin fold has also been predicted by NMR analysis of the Wnt inhibitory factor (WIF) domain, which is found in the secreted Wnt antagonist WIF1 and the Wnt receptor Ryk. Furthermore, a fly lipocalin, named Swim (secreted Wnt interacting molecule) has been proposed to act as Wnt chaperone and shield the hydrophobic moieties on Wnt, thereby enabling efficient Wnt diffusion or transport in a largely aqueous environment. Expression of certain lipid-binding proteins, including Lipocalin2 and FABP5, is up-regulated in cells overexpressing Wnt1, Additionally, Wls/Gpr177 is also a target of Wnt signaling in mouse cells, suggesting the presence of feed-forward regulation that ensures sufficient chaperones are available to usher Wnt through the secretory pathway. However, in flies, Wls appears not to be a target of canonical Wntsignaling. Alternatively, it is tempting to speculate that Wnt expression triggers a signaling system similar to that of the unfolded protein response, thereby activating expression of genes that encode fatty acid-binding proteins, such as lipocalins, which facilitate the export of hydrophobic Wnt proteins from the ER and to the cell surface. During the

transit from the Golgi to the plasma membrane, Wnt protein is passed from Wls to a lipocalin, which then accompanies it in the extracellular space and may regulate its distribution. However, it has been shown that the secretion of the swim protein is independent of Wg. [257]

Following release from the secretory machinery, it has been suggested that Wnts become tethered to the plasma membrane via their lipid moieties, a feasible model given the lipid's accessibility as revealed by the protein structure.Although this is a formal possibility, it is clear that a significant proportion of Wnt (e.g., Wnt3a and Wnt5a) can be purified in a biologically active form from conditioned media. Alternatively, Wnt proteins may become incorporated into lipoprotein complexes. In Drosophila, Wg (and Hh) was found to colocalize and associate with the lipoprotein Lipophorin, a particle with similar characteristics to ApoB-based lipoproteins. Knockdown of Lipophorin expression by RNA interference significantly reduced the signaling range of both Wgand Hh, suggesting that lipoprotein complexes regulate long-range Wnt signaling. In mammalian cells, Wnt3a was shown to cofractionate by KBr isopycnic density centrifugation with the lipoprotein marker hApoB100 and was associated with both high- and low-density lipoproteins (HDL and LDL).Furthermore, addition of HDL, but not LDL, supported Wnt3a solubility in medium containing delipidated fetal calf serum. Taken together with its hydrophobic properties, Wnt solubility in the extracellular environment necessitates molecules or complexes that interact with hydrophobic moieties [258]

2.16.5.1 Canonical Pathway

The canonical Wnt pathway (or Wnt/β-catenin pathway) is the Wnt pathway that causes an accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus to act as a transcriptional coactivator of transcription factors that belong to the TCF/LEF family. Without Wnt, β-catenin would not accumulate in the cytoplasm since a destruction complex would normally degrade it. This destruction complex includes the following proteins: Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). It degrades β-catenin by targeting it for ubiquitination, which subsequently sends it to the proteasome to be digested. However, as soon as Wnt binds Fz and LRP5/6, the destruction complex function becomes disrupted. This is due to Wnt causing the translocation of the negative Wnt regulator, Axin, and the destruction complex to the plasma membrane. Phosphorylation by other proteins in the destruction complex subsequently binds Axin

to the cytoplasmic tail of LRP5/6. Axin becomes de-phosphorylated and its stability and levels decrease. This allows β-catenin to accumulate and localize to the nucleus and subsequently induce a cellular response via gene transduction alongside the TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors. [265] [266]

2.16.5.2 Noncanonical Pathways

The noncanonical planar cell polarity (PCP) pathway does not involve β-catenin. It does not use LRP-5/6 as its co-receptor and is thought to use NRH1, Ryk, PTK7 or ROR2. The PCP pathway is activated via the binding of Wnt to Fz and its co-receptor. The receptor then recruits Dsh, which uses its PDZ and DIX domains to form a complex with Dishevelled-associated activator of morphogenesis 1 (DAAM1). Daam1 then activates the small G-protein Rho through a guanine exchange factor. Rho activates Rho-associated kinase (ROCK), which is one of the major regulators of the cytoskeleton. Dsh also forms a complex with rac1 and mediates profilin binding to actin. Rac1 activates JNK and can also lead to actin polymerization. Profilin binding to actin can result in restructuring of the cytoskeleton and gastrulation. [267]

The noncanonical Wnt/calcium pathway also does not involve β-catenin. Its role is to help regulate calcium release from the endoplasmic reticulum (ER) in order to control intracellular calcium levels. Like other Wnt pathways, upon ligand binding, the activated Fz receptor directly interacts with Dsh and activates specific Dsh-protein domains. The domains involved in Wnt/calcium signaling are the PDZ and DEP domains. However, unlike other Wnt pathways, the Fz receptor directly interfaces with a trimeric G-protein. This co-stimulation of Dsh and the G-protein can lead to the activation of either PLC or cGMP-specific PDE. If PLC is activated, the plasma membrane component PIP2 is cleaved into DAG and IP3. When IP3 binds its receptor on the ER, calcium is released. Increased concentrations of calcium and DAG can activate Cdc42 through PKC. Cdc42 is an important regulator of ventral patterning. Increased calcium also activates calcineurin and CaMKII. CaMKII induces activation of the transcription factor NFAT, which regulates cell adhesion, migration and tissue separation.Calcineurin activates TAK1 and NLK kinase, which can interfere with TCF/ß-Catenin signaling in the canonical Wntpathway.However, if PDE is activated, calcium release from the ER is inhibited. PDE

mediates this through the inhibition of PKG, which subsequently causes the inhibition of calcium release. [268] [269]

2.17 The R-spondin Protein Family

The four vertebrate R-spondin proteins are secreted agonists of the canonical Wnt/β-catenin signaling pathway. These proteins are approximately (35 kDa) and are characterized by two amino-terminal furin-like repeats, which are necessary and sufficient for Wnt signal potentiation, and a thrombospondin domain situated more towards the carboxyl terminus that can bind matrix glycosaminoglycans and/or proteoglycans. Although R-spondins are unable to initiate Wnt signaling, they can potently enhance responses to low-dose Wnt proteins. In humans, rare disruptions of the gene encoding R-spondin1 cause a syndrome of XX sex reversal (phenotypic male), palmoplantar keratosis (a thickening of the palms and soles caused by excess keratin formation) and predisposition to squamous cell carcinoma of the skin. Mutations in the gene encoding R-spondin4 cause anonychia (absence or hypoplasia of nails on fingers and toes). Recently, leucine-rich repeat-containing G-protein-coupled receptor (Lgr)4, Lgr5 and Lgr6, three closely related orphans of the leucine-rich repeat family of G-protein-coupled receptors, have been identified as receptors for R-spondins. Lgr5 and Lgr6 are markers for adult stem cells. Because R-spondins are potent stimulators of adult stem cell proliferation in vivo and in vitro, these findings might guide the therapeutic use of R-spondins in regenerative medicine. [277]

2.17.1 Gene Organization and Evolutionary History

The R-spondins are members of a superfamily of thrombospondin type 1 repeat (TSR-1)-containing proteins. The prototype member (discovered in 1971) was isolated from platelets that had been stimulated with thrombin, and was therefore designated 'thrombin-sensitive protein' . The TSR-1 repeat (also known as properdin repeat) was then characterized in the thrombospondin proteins (TSPs), in which it is repeated three times. TSP1 and TSP2 are secreted multimeric matricellular proteins that, in addition to the TSP repeat, share homology in an amino-terminal globular region, von Willebrand factor domain, type II repeats (epidermal growth factor (EGF)-like), type III repeats (calcium binding) and the carboxy-terminal region. These modular proteins act by bringing together cytokines, growth factors, membrane receptors and extracellular proteases. Several proteins involved in the complement pathway (properdin, C6, C7, C8A, C8B,

C9) and extracellular matrix proteins, such as mindin, F-spondin and SCO-spondin, contain one or more TSR-1 repeats. [277] [278]

The prefix R in the R-spondin subfamily of TSR-1-containing proteins derives from the expression of the gene encoding murine R-spondin1. This gene is transiently expressed in the neural tube at 10 and 12 days post-conception, in the boundary region between the roof plate and neuroepithelium, hence its name R(oof plate specific)-spondin . In addition to the presence of the TSR-1 domain, all four R-spondin members are characterized by the presence of a carboxy-terminal region with positively charged amino acids and, importantly, two furin-like cysteine-rich repeats near the amino terminus of the mature protein. Furin repeats (first seen in the endoproteasefurin) are also present in receptors for growth factors such as EGF, insulin, hepatocyte growth factor (HGF) and neurotrophic factors. The R-spondin family was discovered over a (4 year) period. R-spondin3 was discovered in 2002, whereas descriptions of R-spondin1 and R-spondin2 followed in 2004. Finally, R-spondin4 was characterized in 2006. [279]

R-spondin homologs (defined by 2 Fu domains followed by a TSP1 domain) are present in all vertebrates, in primitive chordates such as the lancelet Branchiostomafloridae, in the hemichordate acorn worm Saccoglossuskowalevskii and in the echinodermate sea urchin Strongylocentrotus purpuratus. No homologs with an R-spondin domain composition are found in invertebrate model organisms such as Drosophila or Caenorhabditis, or any other primitive animal. Given this phylogenetic distribution, an R-spondin-like gene was likely to have been present in the deuterostome ancestor and, given its absence outside the deuterostome clade, also originated there. [280]

The mammalian R-spondins have a similar five-exon gene organization and protein domain structure. The human family members share a pair-wise amino acid similarity of (40%) to (60%).The amino-terminal hydrophobic signal peptide ensures that secretion is encoded by the first exon, whereas the two cysteine-rich furin-repeat domains are encoded by exons (2) and (3), and a single TSP1 domain is encoded by exon (4). Exon (5) encodes a region in the protein that is solely characterized by its high density of basic amino acids. [281]

2.17.2 Characteristics Structural Features

The four R-spondin proteins share common domain architecture. An amino-terminal endoplasmic reticulum signal peptide ensures entry into the secretory pathway. The processed mature protein has two cysteine-rich furin-like repeats at the amino terminus. The central TSR-1 domain is followed by a region with a high number of basic amino acids at the carboxyl terminus(Figure 2-6). The two furin-like repeats near the amino terminus are related to a domain seen in the subtilisin-like proprotein convertase family member furin. Although the function of this domain in furin is unknown, its prevalence in several important receptors for growth factors, such as EGF, insulin, HGF and neurotrophic factors, suggests it makes a significant functional contribution. [282]

In a purified peptide containing both furin-like repeats of R-spo2, they determined the free and interconnected cysteine residues. In total, five free cysteine residues were found: three in furin repeat (1) and (2) in furin repeat (2). All interconnected cysteine residues appeared to be separated by only two or three intervening amino acids. No crystallographic study of furin-like repeats in R-spondins is yet available. However, such analyses have been performed for the EGF receptor and insulin growth factor receptor (1). These revealed the existence of three pairs of linked cysteine residues in furin-like repeat 1 that successively bridge (5), (8) and (18) intervening residues. No unbound cysteine residues remained. It is unclear whether these divergent outcomes reflect consequences of the techniques used or structural differences underlying the specific roles of these domains in the proteins studied. [283]

The second domain that is common to all four R-spondins is a TSR-1 domain. The human genome harbors 41 proteins that contain TSR-1 domains. The number of the TSR-1 domains in these proteins varies from 1 to 18. All of the TSRs occur either in secreted proteins or in the extracellular portion of transmembrane proteins. The TSR-1 domain in R-spondin may have a role related to glycosaminoglycan (GAG)/proteoglycan binding. Several observations supporting such a role have been made in other TSR-1-domain-containing proteins. Multiple amino acid sequence alignments of TSRs show that a typical TSR domain consists of 60 amino acids, of which 12 are highly conserved. X-ray crystallography of the TSR-1s of human TSP1 led to the discovery of the CWR layer, an architecture composed of three antiparallel strands. Strand A assumes a rippled conformation, whereas strands B and C assume regular β-sheets. The side chains of the tryptophan residues in the A strand make up two W-layers. Two arginine residues in the B strand comprise the

R-layers. The alternate stacking of the cationic guanidinium groups of the arginine residues with the aromatic side chains of the tryptophan residues provide vital stabilization in the structure of this small domain. Additional solidity derives from the C-layers, disulfide bonds capping the amino-terminal and carboxy-terminal ends of the strands. The exposed tryptophan residues and arginine residues define the front face of the domain and are likely to contact the negatively charged repeating disaccharide units of GAGs and proteoglycans. Moreover, the disaccharide units in GAGs span approximately 9 Å, enabling two units to fit into the recognition groove of the TSR-1. The three-dimensional structure of R-spondins is not yet available, but molecular modeling techniques have also predicted a GAG-binding site for the TSR of R-spondin 4. A recently reported binding of R-spondin3 to the transmembrane proteoglycan syndecan-4 is consistent with these findings. It will be of interest to determine the GAG-binding specificity of the four R-spondin TSR-1s and to translate this knowledge into functional models. [284] [285]

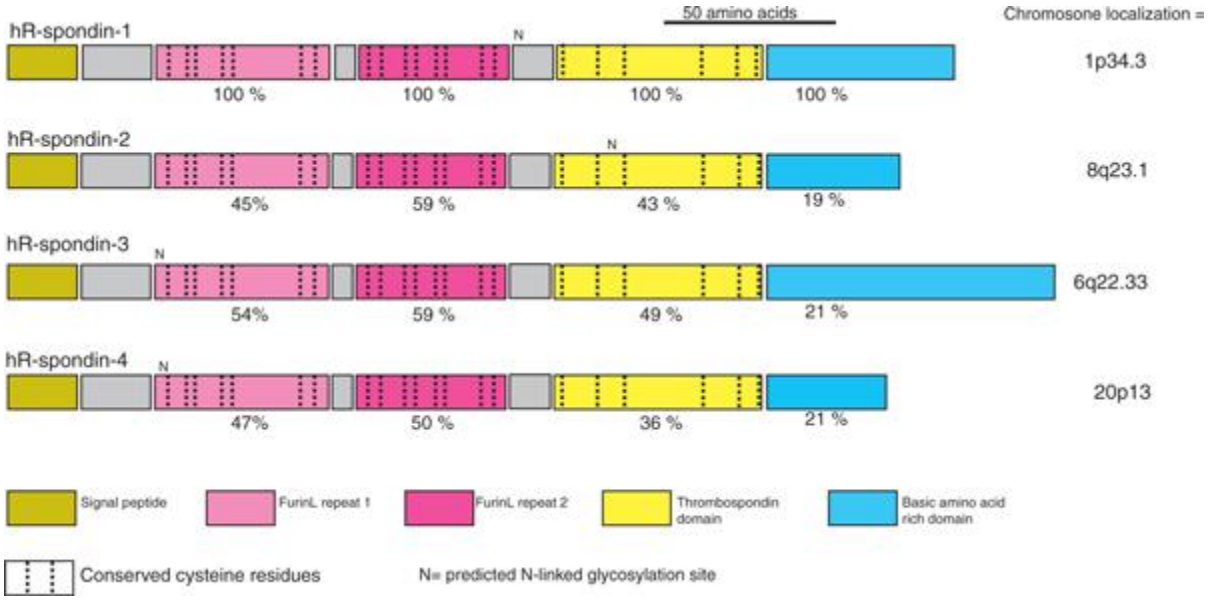


Figure (2-6): Protein domain architecture and chromosome location of human R-spondins. Schematic representations are shown for all four human R-spondin proteins. The total lengths of R-spondin1, 2, 3 and 4 are 263, 243, 292 and 234 amino acids, respectively. Three types of domains are detected: two cysteine-rich furin-like repeats, a single thrombospondin domain, and a basic amino-acid-rich domain. The relative protein sequence conservation, as a percentage of identical amino acids, within these domains is indicated. The two furin repeats jointly contain 15 conserved cysteines, conforming to the consensus sequence for this domain in each repeat. Twelve out of 60 amino acid residues are highly conserved in thrombospondin protein 1 (TSP1) domains, six of which are cysteines. Secretion is mediated by an amino-terminal endoplasmic reticulum signal peptide. Putative *N*-linked glycosylation sites are indicated (N). [285]

2.17.3 Localization and Function

Extensive functional analysis of the R-spondin proteins, using Wnt reporter assays in 293T cells, uncovered a link with the canonical Wnt/β-catenin pathway. The latter plays a central role in cellular proliferation, differentiation and stem cell maintenance. Activity is initiated when secreted proteins of the Wnt family bind to Frizzled (Fzd) receptors and the low-density lipoprotein receptor related protein 5 or 6 (LRP5/6) co-receptors. At this level, the pathway is controlled by a series of extracellular antagonists. R-spondins uniquely synergize with Wnt proteins. Accordingly, R-spondin activation showed sensitivity to the presence of the extracellular Wnt inhibitor Dickkopf-1 (DKK1) and no synergy could be induced by overexpression of any of the known intracellular components of the pathway. Protein domain analysis showed that furin repeats are essential and sufficient to mediate the Wnt-potentiating effect of the R-spondins. The first *in* vivo experiments documenting this Wnt potentiating phenomenon were performed in early frog embryos. Depletion of R-spondin2 in one blastomere at the eight-cell stage resulted in disorganized somites and a reduction in myotomes at the injected site. Depletion at the gastrula stage resulted in a failure to transcriptionally activate the myoD and myf5 genes, later leading to impaired muscle development. Manipulation of Wnt activity at this developmental stage, in chick and mammals, strikingly phenocopies these effects. Canonical Wnt pathway potentiation by R-spondins has also been seen in experimentally induced tumors. A sustained high level of Wnt activity in the tumor was explained by the finding that mammary tumor virus integration sites were seen in both genes for Wnt family members and the gene for R-spondin2. [285] [286]

2.17.4 R-spondin3

R-Spondin 3 (RSPO3), also called cysteine-rich and single thrombospondin domain containing-1 (CRISTIN1), Protein with TSP type-1 repeat (PWTSR), is a member of the R-Spondin protein family. R-spondins (RSPO) are a recently discovered secretory protein family with four members in human. Although all four RSPO proteins activate the canonical Wnt pathway, RSPO2 and RSPO3 are more potent than RSPO1, whereas RSPO4 is relatively inactive. RSPO-3 is expressed ubiquitously and expressed at higher level in placenta, small intestine, fetal thymus and lymph node. RSPO3 is the activator of the beta-catenin signaling cascade, leading to TCF-dependent gene activation. RSPO3 acts both in the canonical Wnt/beta-catenin-dependent pathway and in non-

canonical Wnt signaling pathway, probably by acting as an inhibitor of ZNRF3, an important regulator of the Wnt signaling pathway. RSPO3 also acts as a ligand for frizzled FZD8 and LRP6 and may negatively regulate the TGF-beta pathway. This gene belongs to the R-spondin family. The encoded protein plays a role in the regulation of Wnt (wingless-type MMTV integration site family)/beta-catenin and Wnt/planar cell polarity (PCP) signaling pathways, which are involved in development, cell growth and disease pathogenesis R-spondin-3: Activator of the beta-catenin signaling cascade, leading to TCF-dependent gene activation. Acts both in the canonical Wnt/beta-catenin-dependent pathway and in non-canonical Wnt signaling pathway, probably by acting as an inhibitor of ZNRF3, an important regulator of the Wnt signaling pathway. Acts as a ligand for frizzled FZD8 and LRP6. May negatively regulate the TGF-beta pathway. Belongs to the R-spondin family. 2 isoforms of the human protein are produced by alternative splicing. [(286)]

Coronary arteries are essential to support the heart with oxygen and coronary diseases are the leading cause of death worldwide. Identifying the signaling pathways involved in the formation and specification of coronaries is therefore essential, as it could inspire novel regenerative treatments for cardiac diseases. The coronary arteries are derived from the vascular plexus of the heart that arises at E11.5 and is remodeled until the postnatal period. An integral part in this remodeling process is arterial venous differentiation. Arterial specification in the embryonic vasculature and postnatal vessels of the retina appears to require specific activation of the transcription factor SOX17. How coronary specification is acheived and – more importantly – which signaling molecules drive this process remains elusive. Here we identify R-spondin3 (Rspo3), a secreted activator of β – catenin signaling, as a crucial regulator of coronary artery differentiation. Rspo3 deficient embryos die early in development due to vascular defects in the placenta and conditional deletion of Rspo3 in the heart with the Islet1-Cre causes impaired development of the secondary heart field. However, Rspo3 expression persists in angiogenic regions with high Wnt/β-catenin signaling in the heart. Temporal deletion of Rspo3 11.5 days post coitum with the ubiquitously expressed cCAGCreERT2 line leads to a complete absence of the coronary arteries and a drastic reduction in proliferation of the compact myocardium. Closer inspection of Rspo3 expression reveals it is specifically expressed in the cardiomyoblasts surrounding the first order branch vessels of the left and right coronary arteries, and that Sox17 is highly expressed in the endothelial cells of these vessels. Ablation of Rspo3 leads to decreased Wnt/Bcatenin signaling and, consequently, a significant reduction of Sox17 in these vessels. These

results identify Rspo3 as a key regulator of arterial/venous differentiation in the first order branch vessels of the heart by controlling the expression of Sox17 in a Wnt/B-catenin-dependentfashion. Body fat distribution is a heritable trait that independently predicts type 2 diabetes and cardiovascular risk. Genome-wide association studies (GWAS) meta-analyses have identified sexually dimorphic associations, with greater effect in women, between loci within RSPO3 (e.g. rs9491696) and BMI-adjusted waist-to-hip ratio (WHR). RSPO3 is a LGR4 receptor ligand and a Wnt/β-catenin signalling agonist. Consistent with a role in modulating regional adiposity, RSPO3 expression was higher in abdominal vs gluteal The WHR-increasing allele (G) at rs9491696 was associated with ~2-fold higher RSPO3 expression in both abdominal AT, despite being associated with increased android and reduced leg fat mass. Accordingly, RSPO3 had distinct effects on abdominal and gluteal AP biology due, in part, to differential modulation of Wnt/β-catenin signalling. Specifically, ectopic RSPO3 expression led to increased proliferation selectively in abdominal APs and adipogenesis was impaired in both abdominal and gluteal RSPO3 over-expressing cells. RSPO3 signals through LGR4 to differentially regulate abdominal and gluteal adipose progenitor cell proliferation and adipogenesis, thereby modulating body fat distribution. (287)

Chapter Three

Materials and Method

3. Materials and Methods

3.1 Study Design

This is a prospective study to apply of genetic polymorphism & gene experience of R-SPONDIN3 as biomarker of cardiometabolic traits associated with or without obesity in sample ofsudaneese pateins in Khartoum state.

3.2 Study Area

The study was conducted in Khartoum State in Ahmed Gasim hospital Cardiac Surgery and Renal Transplant Center, AlshaabTeaching Hospital and Obesity Centers; Ajmal Medical, Hiba Mutamed Diet Center, sport city Center, Aldar Diet Center, Almaleka Diet Center.

Khartoum State is located in the middle of the populated areas in Sudan almost northeast center of the country between (16°) latitude north and (15°) latitude south and longitude (21°) west and (24°) longitude east, and expands an area amounting to (22,142 km^2 12884 Mile).Most of the population works in government service, the private sector, and banking. There are also many merchants, and migrants and displaced people working in marginal activities.

3.3 Study Duration

The study was carried out during the period from August 2016 to March 2019.

3.4 Ethical Considerations

This study was approved by the research committee – College of Medical Laboratory Sciences - Shendi University. Informed consent was obtained from each participant before taking the samples.

3.5 Sample Size and Study Population

The study was including (300) participants (males and females) classified into (3) groups. The first group was including one (100) participants with abdominal obesity (obese), the second group was including (100) participants already diagnosed with CVD entangled with obesity (positive control group), while the third group was include (100) healthy lean volunteers (negative control group).

For sample size we were used this equation: [30]

$$n = \frac{X^2 \times N \times P \times (1-P).}{(ME^2 \times (N-1)) + (X^2 \times P \times (1-P))}$$

Where:

n = sample size

X^2 = Chi-square for required confidence level at 1 degree of freedom

N = population size

P = population proportion (percentage picking a choice)

ME = required margin of error (confidence interval)

3.6 Sampling Technique

Simple random samples were taken, (7) milliliters venous blood samples were withdrawn from fasting participants and were divided into (2) tubes under aseptic conditions using sterile evacuated tubes from each subject as follows:

- (3) Milliliters venous blood was put into serum separator gel (SSG) tube for performing lipid profile.
- (4) Milliliters venous blood was put into a sterile ethylene di-amine tetra-acetic acid (EDTA) tube.

3.7 Sample Separation

All the serum samples were separated after (10 minutes) after collection. Then samples were centrifuged for (5 minutes) at (3000 rpm), the serum were immediately transported to labeled Eppendorf safe–lock tubes with cap.

3.8 Sample Storage

- All the EDTA samples were stored at (–20ºC) to be used for the genotyping technique. All the samples were analysis in Mouwasat Hospital-Eldamam Branch Kingdom of Saudi Arabia.
- Serum Samples were kept in a laboratory refrigerator (4 - 8 °C) till time of analysis.

3.9 Data Collection

Personal data were obtained either by reviewing medical form and/or by standard questionnaire.

3.10 Laboratory Tests

3.10.1 Methods of Estimation

Identification of genetic polymorphisms of RSPO 3 gene (rs9491696):

The test was done in 2 main steps:

I. Extraction of genomic DNA from blood leucocytes of EDTA anti-coagulated blood.

II. Amplification of the extracted DNA and genotypic analysis by convensional PCR & Real time PCR. [31]

I- Extraction of Genomic DNA from Peripheral Blood Leucocytes:

TINAamp Genomic DNA extraction kit was used (Cat. no. # DP304).[TANGEN BIOTECH (BEIJING) CO., LTD. Building C7-3, Zhongguancun Dongsheng Science Park, No.66 Xixiaokou Road].

Principle:

Samples were digested with proteinase K and lysis solution. The lysate is then mixed with ethanol and loaded onto the purification column, where the DNA binds to the silica membrane. Impurities are effectively removed by washing the column with the prepared wash buffers. Genomic DNA was then eluted under low ionic strength conditions with the elution buffer.

Reagents:

- Wash Buffer GD concentrate: prepared by adding (17 ml) of absolute ethanol to reach a final volume of (30 ml) and stored closed at room temperature.
- Wash Buffer PW concentrate: prepared by adding (60 ml) of absolute ethanol to reach a final volume of 75 ml and stored closed at room temperature.
- Buffer TE: ready for use and stored at room temperature.
- Proteinase K Solution: ready for use and stored at room temperature till time of opening & then divided into aliquots and stored at (– 20°C).

- Buffer GB: ready for use and stored at room temperature.
- Ethanol (96%).

Blood Spin Protocol:

All samples and reagents were equilibrated to room temperature.

1. 20µl of Proteinase K Solution was added to 200µl of anti- coagulated whole blood in a 1.5 ml micro-centrifuge tube & mix by vortex.
2. 200µl of Buffer GB was added to the sample and all wasmixed by vortex for 20 seconds.
3. The micro-centrifuge tube wasincubated at 70ºC for 10 minutes until the cells were completely lysed.
4. (200µl) of absolute ethanol was added to the sample and mixed again by pipetting. The mixture from step (4) was carefully applied to the spin column (in a 2 ml collection tube). The cap was closed and then the spin column was centrifuged at (12000 rpm) for (45 seconds). The spin column was placed in a clean (2 ml) collection tube and the tube containing the filtrate was discarded.
5. The spin column was carefully open and (500µl) wash buffer GD (with ethanol added) was added. The cap was closed, and the spin column was centrifuged at (12000 rpm) for (45 seconds).
6. The filtrate from the (2 ml) collection tube was discarded. The spin column was carefully opened and (600µl) buffer PW (with ethanol added) was added. The cap was closed and centrifuged at full speed (12000 rpm) for (45 seconds).
7. Step (6) was repeated.
8. The spin column was centrifuged at (12000 rpm) for (2 minutes) to dry the membrane completely.
9. The spin column was placed in a clean (1.5 ml) micro-centrifuge tube and the collection tube containing the filtrate was discarded. The spin column was carefully be opened and (200 µl) buffer TE was added and incubated for (2 minutes) at room temperature (to increase DNA yield) and then centrifuged at (12000 rpm) for (2 minutes).

10. DNA elute in the elution buffer was stored at (– 20ºC) till the assay time.

3.10.2 Primer Design

Degenerate oligonucleotide primers (Table 3-1) from conserved regions of the RSPO 3 gene was designed by primer3plus (www.bioinformatics.nl/primer3plus) from the RSPO 3 gene sequences in the GenBank database (NCBI) and synthesized by Macrogen (South Korea).

No	Oligo Name	Sequence (5' -> 3')	Yield {OD}	Yield {ug}	Yield {umol/ul}	Vol for 100 pmol/ul	Tm {C}	MW {G/MOL}	CG – Content	Synthesis scale	Purifications
1	Rspo3 F	CGGGATCCGCCGCCGCCATGCACTTGCGACTGATTTC (37)	7.27	220	19.5	195	>75	11278	64.9%	0.01 umol	HPSF
2	Rspo3 R	CCTCTAGAGTGTACAGTGCTGACTGATACCG (31)	3.59	102	10.7	107	69.5	9511	51.6%	0.01 umol	HPSF

Table (3-1) Primers used for detection of RSPO 3 Gene

3.10.3 Amplification of RSPO 3 Gene

The amplification was done using (CLASSIC K960 China thermal cycler). DNA amplifies was done using Maxime PCR Premix kit (*I*-Taq) (iNtRON, Korea). The PCR assay was carried out in a total volume of (20 µL) of mixture containing (0.5 µL) of the gene-specific primers (1 µL), (2 µL) of template DNA and (17 µL) of water for injection (WFI). The amplification conditions included three steps: heating at (94°C) for (5 minutes); (35 cycles) of denaturation at (94°C) for (30 seconds), annealing at (55°C) for (30 seconds), and extension at (72°C) for (30 seconds); and the final extension at (72°C) for (3 minutes).

3.10.3.1 Visualization of the DNA

The gel casting tray was placed into the electrophoresis system, tank flooded with 1x TBE buffer just to cover the gel surface, (5 µl) of PCR products from each sample was added to wells of electrophoreses, (5 µl) of DNA ladder (100-bp DNA ladder, iNtRON, Korea) , was added to the well in each run. The gel electrophoresis apparatus was connected to power supply (Primer, 100 V, 500 mA, UK). The electrophoresis was carried out at (75Volts) for (30 minutes) and the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized for DNA bands. By U.V transilluminator and photographed (Uvitec– UK).

3.10.4 Amplification and Real-time Polymerase Chain Reaction (PCR) Assays:

Real-Time PCR with sequence-specific primers were used to define the RSPO 3 gene SNP (rs9491696). Real-time PCR allelic discrimination assay was designed using Taq-Man SNP Genotyping Assays (Applied Biosystems, MA, nand USA).

Principle:

During PCR the following steps were occurring:

1. Each TaqMan Minor Groove Binder (MGB) probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
2. When the oligonucleotide probe is intact, the proximity of the reporter dye to the quencher dye results in quenching of the reporter fluorescence primarily by forster-type energy transfer.
3. AmpliTaq Gold DNA polymerase extends the primers bound to the template DNA.
4. AmpliTaq Gold DNA polymerase cleaves only probes that are hybridized to the target.
5. Cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter.
6. The increase in fluorescence signal occurs when probes that have hybridized to the complementary sequence are cleaved. Thus, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.

Reagents:

TaqMan SNP Genotyping Assays:

The required are only (3) components:

- Purified DNA sample per well, its concentration was adjusted according to DNA concentration of the sample.
- 20x SNP Genotyping Assay.
- TaqMan Universal PCR Master Mix.

SNP Genotyping Assay Contents:

Each of the 20xTaqMan SNP Genotyping assay consists of a single tube containing:

1. Sequence-specific forward and reverse primers to amplify the required region of R-SPONDIN3 gene.

2. Two TaqMan MGB probes for distinguishing between the two alleles:

- One probe labeled with VIC dye detects the allele 1 (C variant) sequence.
- One probe labeled with FAM dye detects the allele 2 (T variant) sequences.

About the Probes; Each TaqMan MGB probe contains:

A reporter dye at the 5′ end of each probe:

- VIC dye is linked to the 5′ end of the Allele 1 probe.
- FAM dye (6-carboxyfluorescein) is linked to the 5′ end of the Allele 2 probe.
    - A Minor Groove Binder (MGB) at the 3′ end of each probe. This modification increases the melting temperature (Tm) for a given probe length which allows the design of shorter probes.

A non-fluorescent quencher (NFQ) at the 3′ end of each probe.

Technique:

Preparing the Reaction Mix:

- The reaction mixture was prepared for each assay before transferring it to the optical reaction plate for thermal cycling.
- After adding the reagents to the DNA samples, they will be mixed thoroughly to avoid air bubbles in the well.
- The number of reactions to be performed for Assay was calculating. One Negative Control (NTC) on the plate will be added.

The total volume of each component needed for the assay was calculated as follow: (10µl) Taqman Universal PCR Master Mix was added to (1µl) R-SPONDIN3 Taqman SNP Genotyping Assay (20x) and (4 µl) DNAase free water (adjusted according to DNA concentration of each sample) + DNA template, providing (20 µl) total volume per well.

- The bottle of TaqMan Universal PCR Master Mix (2x) will be gently swirled to ensure that

it is well mixed before use.

- The (20 x) SNP Genotyping Assay will be mixed my vortex and centrifuged briefly.
- The required total volumes of universal master mix and (20 xs) SNP Genotyping Assay was pipetted into a sterile micro-centrifuge tube.
- The tube was capped and inverted several times to mix.
- The tube was centrifuged briefly to spin down the contents and to eliminate any air bubbles from the reaction mix.

Adding the DNA to the prepared reaction mix in the reaction plate:

Into each well of the reaction plate, the DNA was pipetted as below:

- Quantification of the amount of genomic DNA in samples before using TaqMan SNP genotyping assays. DNA concentration should be measured using Qubit (2.0) Fluorometer (Invitrogen, CA, USA) then the required volume was me of DNA of each sample was calculated to see the nano-grams (ng) of DNA in each reaction.
- It is important to be sure that no cross-contamination occurred from well to well during pipetting.
- The plate was sealed with the appropriate cover.

PCR Amplification:

During the first step of a TaqMan SNP genotyping assay experiment, AmpliTaq Gold DNA polymerase from the taqman universal PCR master mix amplified the target DNA using sequence-specific primers. TaqMan MGB probes from the SNP genotyping assay provided a fluorescence signal for the amplification of each allele.

Steps of Performing PCR and the Thermal Cycling Conditions:

The thermal cycling conditions were programmed as follows:

1. AmpliTaq Gold Enzyme activation at (95°C) for (10 minutes).
2. (50) Cycles of amplification consisting of: -Denaturation at (92°C) for (15) seconds. -Annealing/Extension at (60°C) for (1 minutes).
3. Specifying the reaction volume (20µl/Well) in a (48) well plate.
4. Loading the reaction plate into the thermal cycler, and then starting the run.

Each Cycle of PCR Consisted of 3 steps:

1. **A denaturation step**, in which target DNA should incubate at high temperature, so that the target strands are melted apart and make accessible to hybridization with specific oligonucleotide primers.
2. **An annealing step**, in which the reaction mixture was cool to allow the primers to anneal to their complementary target sequences.
3. **An extension reaction** usually does at an intermediate temperature, in which the primers will extend on the DNA template by a DNA polymerase. Thus, repeating the thermal cycle resulted in a geometric accumulation of amplified target sequences.
4. **Allelic Discrimination Plate Read and Analysis:** After PCR amplification, an end-point plate read was performed using Applied Bio-systems Real-Time PCR System.

The Sequence Detection System (SDS) Software will use the fluorescence measurements make during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicated which allele in each sample.

3.10.5 Method of Cholesterol Estimation:

3.10.5.1 Cholesterol Oxidase Method:

Principle:

Free and esterified cholesterol in the sample originates, cholestrase will break cholesterol ester into cholesterol and free fatty acid, cholesterol-oxidase will break cholesterol into cholesten one and hydrogen peroxide, peroxidase will break hydrogen peroxide with present of phenol and 4-aminoantipyrine to produce color it intensity depend on amount of cholesterol in sample. [32]

3.10.6 Method of HDL-cholesterol Estimation:

3.10.6.1 Phosphotungstic acid end point method (HDL-C):

When serum is treated with phosphotungustate in the presence of magnesium ion, the LDL, VLDL and chylomicron are precipitated from serum. The HDL cholesterol remains dissolved in the supernatant. The supernatant then acts as a sample and assayed for cholesterol by an enzymatic method. [32]

3.10.7 Method of Triglycerides estimation:

3.10.7.1 Enzymatic-colorimetric method (GPO-PAP):

Serum triglycerides are hydrolyzed to glycerol and free fatty acids by lipoprotein lipase. In the presence of ATP and glycerol kinase (GK), the glycerol is converted to glycerol-3-phosphate, which then is oxidized by glycerol phosphate oxidase (GPO) to yield hydrogen peroxide. The oxidative condensation of 4-Chlorophenol and (4-AAP) 4-aminophenazone in the presence of Peroxidase (POD) and hydrogen peroxide produces a rose-colored dye which is measured at 550 nm. The intensity of the color formed is directly proportional to the triglycerides concentration in the sample. [32]

3.10.8 Method of LDL estimation:

3.10.8.1 Enzymatic-colorimetric method:

This direct method for quantifying cholesterol in low-density lipoproteins (LDL) is a homogeneous enzymatic test in which the differential precipitation and further sedimentation of the rest of lipoproteins and quilomicrons is avoided. The procedure comprises two steps. In the first step cholesterol in lipoproteins other than LDL in the test sample are decomposed by the simoultaneaus action of cholesterol esterase (CE) and cholesterol oxidase (CO) at pH (7.0), giving as end products cholestenone and hydrogen peroxide, the latter being decomposed to water and oxygen by catalase. In the second step a surfactant which specifically acts on LDL is added to the reaction product of the first step being the remaining cholesterol quantified by a Trinder´s type reaction in which the aniline derivate, HDAOS, and 4-aminoantipyrine (4-AA) as acoupling reagent are condensed by the H_2O_2 in presence of peroxidase (POD) to form a red quinoneimine dye proportional to the concentration of LDL-cholesterol present in the sample. [33]

3.10.9 Methods of waist-to-hip ratio (WHR) estimation:

It is the ratio of the circumference of the waist to that of the hips. This is calculated as waist measurement divided by hip measurement (W ÷ H). The WHR has been used as an indicator or measure of health, and the risk of developing serious health conditions.[34]

Table (3-2): -Waist-to-hip ratio (WHR) Categories: [34]

Male	Female	Health risk based on WHR
0.95 or < less	0.80 or < less	Low risk
0.96 to 1.0	0.81 to 0.85	Moderate risk
1.0 or > greater	0.86 or > greater	High risk

Methods of BMI estimation:

It calculates a value indicative of the fat content of the body by dividing the weight by the square of height. [35]

$$\mathrm{BMI} = \frac{\mathrm{mass(kg)}}{(\mathrm{height(m)})^2}$$

Table (3-3): -BMI Categories: [34]

Categories	BMI
Underweight	Less than 18.5
Normal weight	18.5 – 24.9
Overweight	25 – 29.9
Obese	30 or higher

3.11 Quality controls and Managements

Blood was collected with care and adequate safety precautions to ensure test results were reliable. Quality Assurance (QA) and standard Operating System should be followed for all molecular biological and clinical chemistry tests to achieve validity and reliability of test results.

3.12 Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. **(**Armonk, NY: IB was M Corp**)** Qualitative data were described using number and percent.The Kolmogorov-Smirnov test was used to verify the normality of distribution .Quantitative data were described using range (min imum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

The used tests were

1 - Chi-square test

For categorical variables, to compare between different groups

2 - Student t-test

For normally distributed quantitative variables, to compare between two studied groups

3 - F-test (ANOVA)

For normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) (LSD) for pairwise comparisons

4 - Pearson coefficient

To correlate between two normally distributed quantitative variables

5 - Mann Whitney test

For abnormally distributed quantitative variables, to compare between two studied groups

6 –Kruskal Wallis test

For abnormally distributed qu antitative variables, to compare between more than two studied groups and Post Hoc (Dunn's multiple comparisons test) for pairwise comparisons

3.13 Implications of study Results on Public Health:

Detection of mutation in RSPO 3 gene and confirmation of its effect on fat distribution and increased risk of cardiovascular disease can develop medical practices that can alleviate the suffering associated with human disease and provide strong support to basic research that it gives us a powerful tool for understanding and describing more human obesity in Sudanese population.

ChaptarFour

(Results)

4. Results

4.1 Demographic Data

(300) consenting participants were enrolled in this study. The study group consisted of 156 (52.0%) males and 144 (48.0%) females with an average age of 40.70 ± 4.81 and 43.73 ± 8.85 years respectively. From Ahmed Gasim hospital Cardiac Surgery and Renal Transplant Center (40), AlshaabTeaching Hospital (60), Ajmal Medical (40), Hiba Mutamed Diet Center (15), sport city Center (25), Aldar Diet Center (10), Almaleka Diet Center (10) Also other data were registered in the submitted questionnaire.

4.2 Molecular Findings

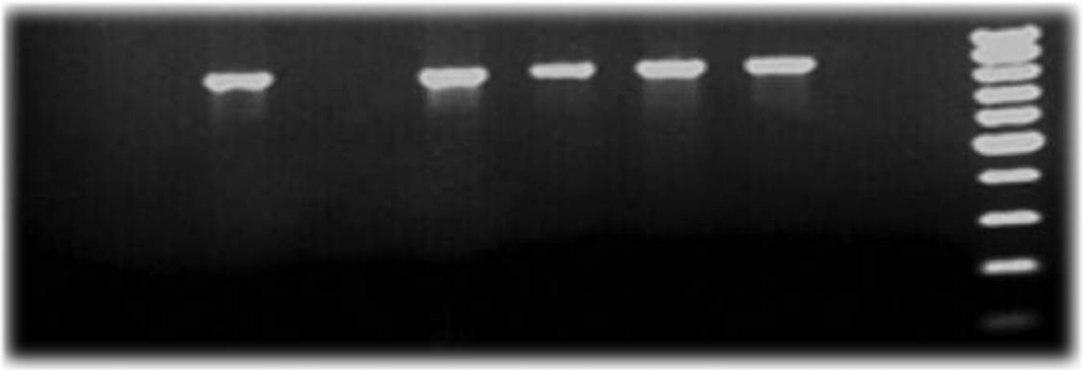
4.2.1 Purity of the Extracted DNA Chain Reaction and Amplification

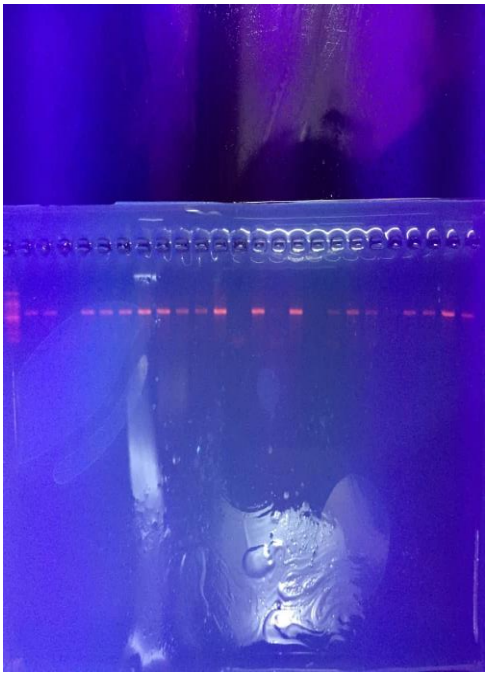
RSPO 3 gene extracted purity was detected by (2%) agarose gel, the extracted DNA was clearly seen in pure form and high amounts compared to the DNA marker which contain (40 ng) in (5µL) loading (all fragments except typical band DNA). The typical band of DNA fragments is (100 ng) Figure (4 - 1).

Blood samples were collected from volunteers via venipuncture in fasting state. PCR was done to all the (3) groups (control, obesity and heart group)**.** In this study, the results of PCR were significantly different (P <0.001) in Heart group subjects as compared to healthy controls and obese group (Table 4-1). Among heart disease group mutation was detected in some subjects (19%) and the rest without mutation (81 %) but for obese group no mutation was detected.

4.2.2 PCR for the amplification ofRspon 3 gene

Degenerate oligonucleotide primers from conserved regions of the RSPO 3 gene was designed from alignments of known DNA sequences in the GenBank database (NCBI). PCR amplified RSPO 3 gene product (80688 bp) which encoded (26896) amino acids on the agarose gel.





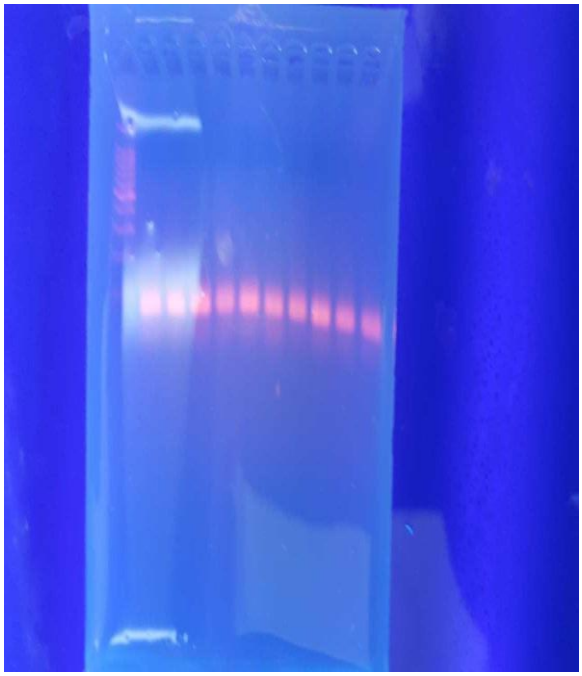


Figure (4 - 1). RSPO 3 gene extracted separated by 2 % Agrosol Gel

Table (4 - 1): Comparison between the different studied groups according to conventional PCR

Conventional PCR	Control group (n = 100)		Heart Group (n = 100)		Obesity group (n = 100)		χ^2	P
	No.	%	No.	%	No.	%		
Negative for mutation	100	100.0	81	81.0	100	100.0	40.569*	<0.001*
Positive for mutation	0	0.0	19	19.0	0	0.0		

χ^2: **Chi square test**

P: p value for comparing between the studied groups

*: Statistically significant at p ≤ 0.05

4.2.3 Relative Quantification of RSPO3 Gene

Performed gene expression studies of RSPO3 Gene for (3) hundred consenting participants (100) healthy and (100) obese with abdominal obesity and (100) participants with CVD disease due to abdominal obesity, To see the amount of gene expression in the (3) group for all fat phenotype (visceral, subcuteanous and gluteal fat) to determine if amount of gene expression and rs9491696 was associated with fat distribution and CVD.

RSPO3 expression was higher in heart group than obesity group compared to normal individuals (4.5 –fold and 2.4-fold respectively) (Table 4 - 2) Figure (4 - 1).Also the amount of gene expression in individuals with visceral fat was higher than individuals with subcuteanous fat (5.0-fold and 4.1-fold respectively) in heart group .Amount of gene expression in individuals with visceral ,subcuteanous fat and Gluteal Fat almost the same (2.4-fold, 2.5-fold and 2.4-fold respectively).

Comparison between the different studied groups according to gene expression showed significant differences (P <0.001) mean value of gene expression in healthy group subjects was (1.0 ± 0.0),

Obesity group was (2.44 ± 0.50) and heart disease group subjects was (4.54 ± 0.87) respectively (Table 4 - 2) Figure (4 - 1).

Correlation between gene expression and age in heart disease group and obesity group showed weak positive correlation with the r value of age in heart group was (0.034) and obesity group was (0.007) respectively (Table 4 - 3).

Correlation between gene expression and BMI in heart and obesity group showed weak Negative correlation with the r value of age in heart disease group was (-0.259) and obesity group was (0.078) respectively (Table 4 - 3).

Correlation between gene expression and WHR in heart and obesity group showed weak Negative correlation with the r value of age in heart disease group was -0.064 and obesity was (- 0.145) respectively (Table 4 - 3).

Comparison between gene expression and fat phenotype among the heart disease group showed significant association (P <0.001) mean value of gene expression among heart disease group subjects was (5.0 ± 1.05) visceral fat and (4.15 ± 0.36) subcutaneous fat respectively and Comparison between gene expression and fat phenotype among the obesity group showed insignificant association (P = 0.663) mean value of gene expression among obesity group subjects was (2.45 ± 0.51) visceral fat , (2.53 ± 0.51) gluteal fat and (4.15 ± 0.36) subcutaneous fat respectively (Table 4 - 4) Figure (4 -2) .

Comparison between gene expression and sex among the heart disease group showed insignificant association (P = 0.926) mean value of gene expression among heart disease group subjects was (4.53 ± 0.91) male and (4.55 ± 0.81) female respectively and respectively and Comparison between gene expression and sex among the obesity group showed insignificant association (P = 0.154) mean value of gene expression among obesity group subjects was (2.52 ± 0.51) male and (2.38 ± 0.49) female respectively and respectively (Table 4 - 5) Figure (4 -3).

Comparison between the heart disease group and obesity groups according to fat phenotypes showed significant differences (P <0.001) number (46%) and (46 %) visceral fat, number (0%) and (0 %) gluteal Fat and number (54%) and (54 %) subcutaneous fat respectively among the heart group and number (31%) and (31 %) visceral fat, number (17%) and (17 %) gluteal fat and number (52%) and (52 %) subcutaneous fat respectively among the obesity group respectively (Table 4 - 6).

Comparison between the different studied groups according to sex showed significant differences (P = 0.035) number (54%) and (54 %) male and number (46%) and (46 %) female among control group, number (60%) and (60 %) male and number (40%) and (40 %) female among heart disease group and number (42%) and (42 %) male and number (58%) and (58 %) female among obesity group respectively (Table 4-7).

Comparison between the different studied groups according to age showed significant differences (P <0.001) mean value of age in healthy group subjects (40.70 ± 4.81), Obesity group was (43.73 ± 8.85) and heart disease group subjects was (50.61 ± 8.65) respectively. Comparison between the control and heart groups according to age showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to age showed significant differences (P_2 = 0.015). Comparison between the heart disease group and obesity groups according to age showed significant differences (P_3 = <0.001*) (Table 4-7).

Comparison between the different studied groups according to BMI showed significant differences (P <0.001) mean value of BMI in healthy group subjects (40.70 ± 4.81), Obesity group was (43.73 ± 8.85) and heart disease group subjects was 50.61 ± 8.65 respectively. Comparison between the control and heart disease group according to BMI showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to BMI showed significant differences (P_2 = 0.015). Comparison between the heart and obesity groups according to BMI showed insignificant differences (P_3=0.131) (Table 4-7) .

Comparison between the different studied groups according to WHR showed significant differences (P <0.001) mean value of WHR in healthy group subjects (40.70 ± 4.81), Obesity group was (43.73 ± 8.85) and heart disease group subjects was (50.61 ± 8.65) respectively. Comparison between the control and heart disease group according to WHR showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to WHR showed significant differences (P_2 = 0.015). Comparison between the heart disease group and obesity groups according to WHR showed insignificant differences (P_3=0.316) (Table 4-7).

Comparison between the different studied groups according to cholesterol showed significant differences (P <0.001) mean value of cholesterol in healthy group subjects (134.4 ± 31.15), Obesity group was (210.8 ± 75.23) and heart disease group subjects was (235.4 ± 91.24) respectively. Comparison between the control and heart groups according to cholesterol showed

significant differences (P_1<0.001). Comparison between the control and obesity groups according to cholesterol showed significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to cholesterol showed insignificant differences (P_3 = 0.061) (Table 4-8) Figure (4 - 4).

Comparison between the different studied groups according to TG showed significant differences (P <0.001) mean value of TG in healthy group subjects (94.31 ± 43.16), Obesity group was (558.6 ± 311.5) and heart disease group subjects was (589.2 ± 303.4) respectively. Comparison between the control and heart groups according to TG showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to triglyceride showed significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to TG showed insignificant differences (P_3 = 0.061) (Table 4-8) Figure (4 - 4).

Comparison between the different studied groups according to HDL showed significant differences (P <0.001) mean value of HDL in healthy group subjects (42.42 ± 9.70), Obesity group was (28.45 ± 9.99) and heart disease group subjects was 30.26 ± 14.69 respectively. Comparison between the control and heart groups according to HDL showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to HDL showed significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to HDL showed insignificant differences (P_3 = 0.899) (Table 4-8) Figure (4 - 4).

Comparison between the different studied groups according to LDL showed significant differences (P <0.001) mean value of LDL in healthy group subjects (81.27 ± 25.38), Obesity group was (113.9 ± 31.18) and heart disease group subjects was 108.4 ± 36.19 respectively. Comparison between the control and heart groups according to LDL showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to LDL showed significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to LDL showed insignificant differences (P_3 = 0.346) (Table 4-8) Figure (4 - 4).

Comparison between heart and obesity group according to duration of obesity showed significant differences (P_2<0.001) mean value of duration of obesity among heart disease group subjects was (29.12 ± 8.36) and obesity group subjects was (24.28 ± 8.01) respectively Figure (4 - 5).

There was a significant correlation between WHR and TG among heart disease group (P<0.020) and obesity groups (P<0.006) (Table 4-9)

The findings of current study prevailed that there was an insignificant correlation WHR and cholesterol among heart disease group (P<0.941) and obesity groups (P<0.953) (Table 4-9). Also there was an insignificant correlation WHR and LDL-C among heart disease group (P<0.086) and obesity groups (P<0.381) (Table 4-9). Also there was an insignificant correlation WHR and HDL-C among heart disease group (P<0.323) and obesity groups (P<0.150) (Table 4-9).

There was an insignificant correlation between BMI and TG among heart disease group (P<0.274) and obesity groups (P<0.808) (Table 4-10).

The findings of current study prevailed that there was an insignificant correlation BMI and cholesterol among heart disease group (P<0.150) and obesity groups (P<0.061) (Table 4-10). Also there was an insignificant correlation BMI and LDL-C among heart disease group (P<0.635) and obesity groups (P<0.818) (Table 4-10). Also there was an insignificant correlation BMI and HDL-C among heart disease group (P<0.271) and obesity groups (P<0.869) (Table 4-10).

Table (4 - 2): Comparison between the different studied groups according to gene expression

Gene expression	Control group (n = 100)	Heart disease Group (n = 100)	Obesity group (n = 100)	F	P
Mean ± SD.	1.0 ± 0.0	4.54 ± 0.87	2.44 ± 0.50	946.172*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*,p_3<0.001*				

F: **F for ANOVA test**, pairwise comparison bet. Each 2 groups were done using **Post Hoc Test (Tukey)**

p: p value for comparing between the studied groups

p_1: p value for comparing between control group and heart disease group

p_2: p value for comparing between control group and obesity group

p_3: p value for comparing between heart disease group and obesity group

*: Statistically significant at $p \leq 0.05$

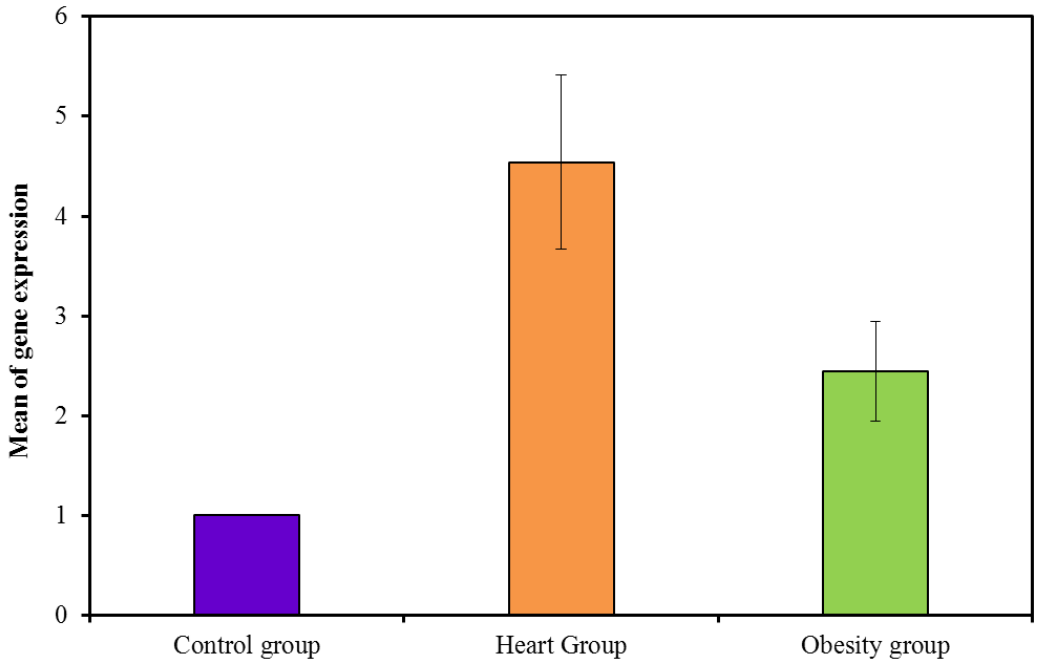


Figure (4 - 2): Comparison between the different studied groups according to gene expression

Table (4 - 3): Correlation between Gene expression and different parameters in each group

	Gene expression			
	Heart disease Group		Obesity group	
	r	p	r	p
Age (years)	0.034	0.739	0.007	0.948
BMI (kg/m2)	-0.259	0.009*	0.078	0.441
WHR	-0.064	0.525	-0.145	0.150

r: Pearson coefficient

*: Statistically significant at $p \leq 0.05$

Table (4 - 4): Relation between Fat phenotypes and gene expressionin each group

Gene expression	Fat phenotypes			Test of sig.	p
	Visceral fat	Gluteal Fat	Subcutaneous fat		
Heart disease Group	**(n= 46)**	**(n= 0)**	**(n= 54)**		
Mean ± SD.	5.0 ± 1.05	-	4.15 ± 0.36	t=5.229*	<0.001*
Obesity group	**(n= 31)**	**(n= 17)**	**(n= 52)**		
Mean ± SD.	2.45 ± 0.51	2.53 ± 0.51	2.40 ± 0.50	F=0.413	0.663

F: **F for ANOVA test**t: **Student t-test**

p: p value for association between Fat phenotypes and gene expression

*: Statistically significant at $p \leq 0.05$

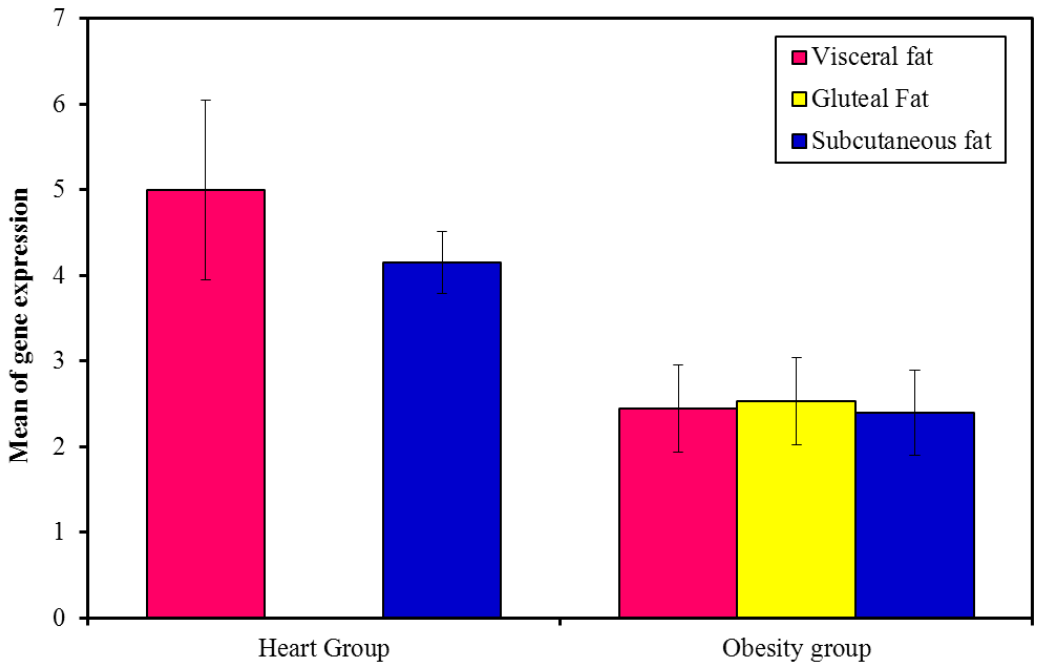


Figure (4 -3): Relation between Fat phenotypes and gene expression in each group

Table (4 - 5): Relation between sex and gene expression in each group

Gene expression	Sex		t	p
	Male	Female		
Heart disease Group	**(n = 60)**	**(n = 40)**		
Mean ± SD.	4.53 ± 0.91	4.55 ± 0.81	0.093	0.926
Obesity group	**(n = 42)**	**(n = 58)**		
Mean ± SD.	2.52 ± 0.51	2.38 ± 0.49	1.437	0.154

t: **Student t-test**

p: p value for association between sex and gene expression

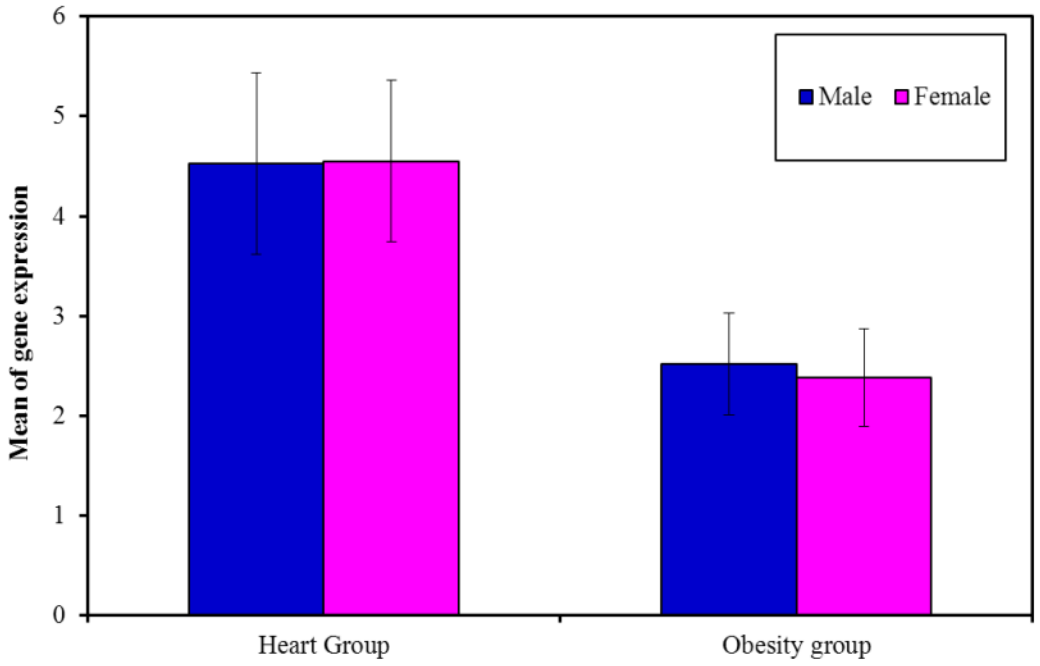


Figure (4 - 4): Relation between sex and gene expression in each group

Table (4 - 6): Comparison between the different studied groups according to fat phenotypes

Fat phenotypes	Heart disease Group (n = 100)		Obesity group (n = 100)		χ^2	P
	No.	%	No.	%		
Visceral fat	46	46.0	31	31.0		
Gluteal Fat	0	0.0	17	17.0	19.960*	<0.001*
Subcutaneous fat	54	54.0	52	52.0		

χ^2: **Chi square test**

p: p value for comparing between the studied groups

*: Statistically significant at $p \leq 0.05$

Table (4 - 7): Comparison between the different studied groups according to demographics data

	Control group (n = 100)		Heart disease Group (n = 100)		Obesity group (n = 100)		Test of Sig.	p
	No.	%	No.	%	No.	%		
Sex								
Male	54	54.0	60	60.0	42	42.0	χ^2= 6.731*	0.035*
Female	46	46.0	40	40.0	58	58.0		
Age (years)								
Mean ± SD.	40.70 ± 4.81		50.61 ± 8.65		43.73 ± 8.85		F= 43.884*	<0.001*
Sig. bet. Grps	p_1<0.001*,p_2=0.015*,p_3<0.001*							
BMI (kg/m^2)								
Mean ± SD.	21.06 ± 1.41		41.01 ± 7.17		39.44 ± 6.75		F= 373.477*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.131							
WHR								
Mean ± SD.	0.84 ± 0.06		1.05 ± 0.16		1.07 ± 0.14		F= 106.401*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.316							

χ^2: **Chi square test**

F: **F for ANOVA test**, pairwise comparison bet. Each 2 groups were done using **Post Hoc Test (Tukey)**

p: p value for comparing between the studied groups

p_1: p value for comparing between controlgroup and heart disease group

p_2: p value for comparing between controlgroup and obesity group

p_3: p value for comparing between heart disease group and obesity groups

*: Statistically significant at $p \leq 0.05$

Table (4 - 8): Comparison between the different studied groups according to lipid profile

Lipid profile	Control group (n = 100)	Heart disease Group (n = 100)	Obesity group (n = 100)	H	P
Cholesterol (mg/dl) Mean ± SD.	134.4 ± 31.15	235.4 ± 91.24	210.8 ± 75.23	178.233*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.061				
Triglycerides (mg/dl) Mean ± SD.	94.31 ± 43.16	589.2 ± 303.4	558.6 ± 311.5	178.233*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.061				
LDL (mg/dl) Mean ± SD.	81.27 ± 25.38	108.4 ± 36.19	113.9 ± 31.18	45.830*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.346				
HDL(mg/dl) Mean ± SD.	42.42 ± 9.70	30.26 ± 14.69	28.45 ± 9.99	92.947*	<0.001*
Sig. bet. Grps	p_1<0.001*, p_2<0.001*, p_3=0.899				

H: H for **Kruskal Wallis test,** Pairwise comparison bet. Each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test)**

p: p value for comparing between the studied groups

p_1: p value for comparing between control group and heart disease group

p_2: p value for comparing between control group and obesity group

p_3: p value for comparing between heart disease group and obesity groups

*: Statistically significant at $p \leq 0.05$

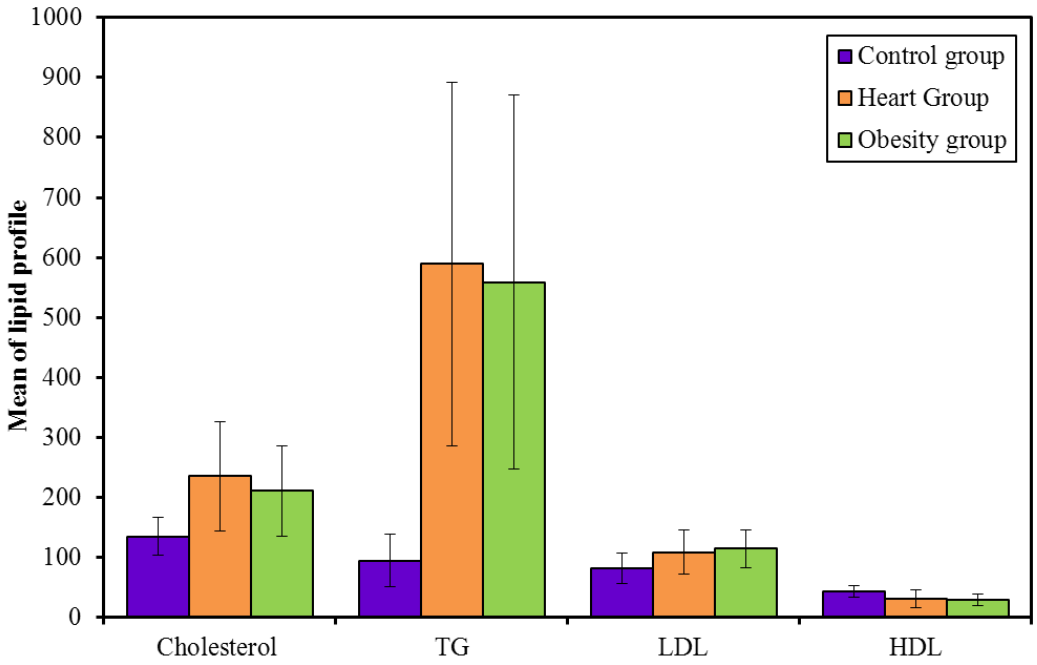


Figure (4 - 5): Comparison between the different studied groups according to lipid profile

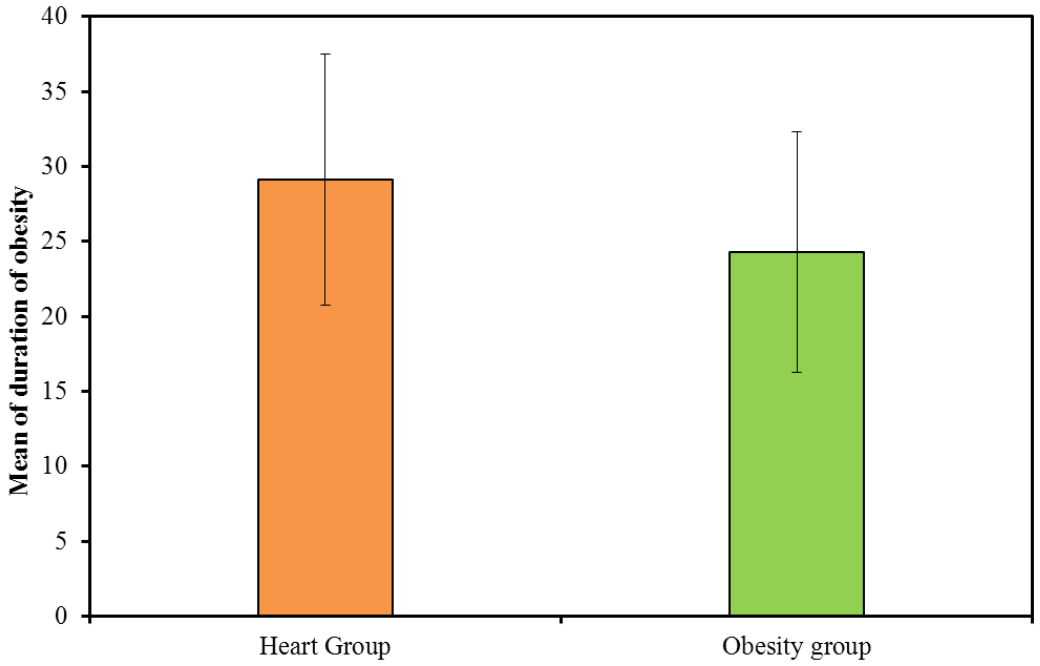


Figure (4 - 6): Comparison between the two studied groups according to duration of obesity

Table (4 - 9): Correlation between WHR and different parameters (n = 100)

	WHR			
	Heart disease group		Obesity group	
	r	p	r	p
Cholesterol (mg/dl)	0.008	0.941	0.006	0.953
Triglycerides (mg/dl)	-0.232*	0.020*	-0.272*	0.006*
LDL-C (mg/dl)	-0.173	0.086	-0.089	0.381
HDL-C (mg/dl)	0.100	0.323	-0.145	0.15

r: Pearson coefficient

*: Statistically significant at $p \leq 0.05$

Table (4 - 10): Correlation between BMI and different parameters (n = 100)

	BMI			
	Heart disease group		Obesity group	
	r	p	r	p
Cholesterol (mg/dl)	0.145	0.15	0.188	0.061
Triglycerides (mg/dl)	-0.111	0.274	0.025	0.808
LDL-C (mg/dl)	0.048	0.635	0.023	0.818
HDL-C (mg/dl)	0.111	0.271	-0.017	0.869
Duration of Obesity	0.047	0.641	-0.259*	0.009*

r: Pearson coefficient

*: Statistically significant at $p \leq 0.05$

Chaptar Five

(Discussion, Conclusion and Recommendations)

5.1: Discussion

Body fat distribution is a heritable trait that independently predicts T2DM and cardiovascular risk. Genome-wide association studies (GWAS) meta-analyses have identified sexually dimorphic associations, with greater effect in women, between loci within RSPO3 (e.g. rs9491696) and BMI-adjusted waist-to-hip ratio (WHR). RSPO3 is a LGR4 receptor ligand and a Wnt/β-catenin signaling agonist.

The aim of study was to investigate the possible correlation between mutation in RSPO3 gene, abdominal obesity and susceptibility to cardiovascular disease.

The study was including (300) participants (156 males and 144 females) classified into (3) groups (Table 4 -1). The first group was including (100) participants with abdominal obesity (obese), the second group was including (100) participants already diagnosed with CVD entangled with obesity (heart disease group as positive control group), while the third group was include (100) healthy lean volunteers (negative control group). All the participants their age group between (27 to 63 years) old. BMI and WHR were taken for all subjects too for detection the mutation in RSPO3 gene, Conventional PCR was done for control, obesity and heart subjects respectively and followed by Real Time PCR for all of them. The same of the (3) groups underwent for lipid profile. For measurement of fat distribution in clinical practice, WC and WHR were used to determine regional FD. CT and abdominal MRI scan were used for evaluating the adipose tissue which was considered as gold standard for that .To measure the visceral and subcutaneous abdominal areas in total abdominal area), a CT or MRI scan was taken at the level of L4–L5 or the umbilicus. The ratio of visceral to subcutaneous adipose tissue has been shown to be strongly correlated with RSPO3 gene in obese subjects and heart subjects respectively Abdominal sagittal diameter derived from CT or MRI images has also been used to determine abdominal FD. The CT and MRI were applied in 200 volunteers.

The conventional PCR was done to all of the three groups which showed clear variation in the mean value of conventional PCR ($P < 0.001$) in heart disease group subjects as compared to healthy controls and obese group (Table 4-1).

Of the 200 cases (obesity &heart) 19 (19%) were positive for mutation in RSPO3 gene all of them from heart disease group and the rest of heart group 81 (81%) were negative for mutation as well as obesity group all 0 (0 %) positive for mutation (Table 4 -1). This result was leads us to do real

time PCR to quantify the level of gene expressed. Comparison between the different studied groups according to gene expression showed clear variation in the mean value of gene expression with (P <0.001) in healthy group subjects was (1.0 ± 0.0), Obesity group was 2.44 ± 0.50 and heart disease group subjects was (4.54 ± 0.87) respectively (Table 4 - 2) Figure (4 - 1).This result was inagreement with the finding of N.Y. Loh [(288)]

In Correlation between gene expression and age in heart disease group and obesity group showed weak positive correlation with the r value of age in heart group was (0.034 and obesity group was (0.007) respectively (Table 4 - 3). These finidingd were in agreed with DoritSchleinitz. [(289)] who were carried study on heart and obesity group respectively.

Correlation between gene expression and BMI in heart and obesity group showed weak Negative correlation with the r value of age in heart disease group was (-0.259) and obesity group was (0.078) respectively (Table 4 - 3). This result was agreed with finding of Michael M. [(290)]

Correlation between gene expression and WHR in heart and obesity group showed weak Negative correlation with the r value of age in heart disease group was (-0.064) and obesity was (-0.145) respectively (Table 4 - 3). This result was agreed with Rajiv G. [(291)]

Comparison between gene expression and fat phenotype among the heart disease group showed significant association (P <0.001) mean value of gene expression among heart group subjects was (5.0 ± 1.05) visceral fat and (4.15 ± 0.36) subcutaneous fat respectively and comparison between gene expression and fat phenotype among the obesity group showed insignificant association (P = 0.663) mean value of gene expression among obesity group subjects was (2.45 ± 0.51)Visceral fat , (2.53 ± 0.51) gluteal fat and (4.15 ± 0.36) subcutaneous fat respectively (Table 4 - 4) Figure (4 -2) . This result was agreed with finding of Kalypso Karastergiou [(292)] .but study the obesity was diagnosed according to the Japanese obesity criteria by using CT & MRI Technologies.

In the Comparison between among heart group according to sex and gene expression showed insignificant differences (P = 0.926) mean value of gene expression among heart disease group subject was (4.53 ± 0.91) male and (4.55 ± 0.81) female. Comparison between among obesity group according to sex and gene expression showed insignificant differences (P = 0.154) mean value of gene expression among heart disease group subject was (2.52 ± 0.51) male and (2.38 ± 0.49) female (Table 4 - 5) Figure (4 - 3).This result was agreed with finding of Atzmon, G. [(86)]

In the Comparison between the heart disease group and obesity groups according to fat phenotypes showed significant differences (P <0.001) number (46 and 46 %) visceral fat, number 0 and percent 0 % gluteal fat and number (54 and 54 %) subcutaneous fat respectively among the heart group and number (31 and 31%) Visceral fat, number (17 and 17 %) Gluteal Fat and number (52 and 52 %) subcutaneous fat respectively among the obesity group respectively (Table 4 - 6). This result was agreed with finding of Ian J. Neeland [(292)] and Tobin M. Abraham. [(292)]

In the Comparison between the different studied groups according to sex showed significant differences (P = 0.035) number (54 and 54%) male and number (46 and 46 %) female among control group, number (60 and 60 %) male and number (40 and 40 %) female among heart disease group and number (42 and 42 %) male and number (58 and 58 %) female among obesity group respectively (Table 4-7). This result was agreed with finding of Ram LochanYadav. [(287)]

In the Comparison between the different studied groups according to age showed significant differences (P <0.001) mean value of age in heart disease group subjects (40.70 ± 4.81), Obesity group was (43.73 ± 8.85) and heart disease group subjects was (50.61 ± 8.65) respectively. Comparison between the control and heart disease group according to age showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to age showed significant differences (P_2 = 0.015). Comparison between the heart disease group and obesity groups according to age showed significant differences (P_3 = <0.001*) (Table 4-7). This result was agreed with finding of Kalypso. [(282)]

In the Comparison between the different studied groups according to BMI showed significant differences (P <0.001) mean value of BMI in healthy group subjects (40.70 ± 4.81), Obesity group was (43.73 ± 8.85) and heart disease group subjects was (50.61 ± 8.65) respectively. Comparison between the control and heart groups according to BMI showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to BMI showed significant differences (P_2 = 0.015). Comparison between the heart and obesity groups according to BMI showed insignificant differences (P_3=0.131) (Table 4-7). This result was agreed with finding of Mitchell BM [(217)] who concluded the obese and hearts subjects having higher BMI when compared with healthy subjects.

In the Comparison between the different studied groups according to WHR showed significant differences (P <0.001) mean value of WHR in healthy group subjects (40.70 ± 4.81), Obesity

group was (43.73 ± 8.85) and heart disease group subjects was (50.61 ± 8.65) respectively. Comparison between the control and heart disease group according to WHR showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to WHR showed significant differences (P_2 = 0.015). Comparison between the heart disease group and obesity groups according to WHR showed insignificant differences (P_3=0.316) (Table 4-7) .This result was agreed with finding of Adamska M [283] who concluded the obese and hearts subjects having higher WHR when compared with healthy subjects.

In the Comparison between the different studied groups according to cholesterol showed significant differences (P <0.001) mean value of cholesterol in healthy group subjects (134.4 ± 31.15), Obesity group was (210.8 ± 75.23) and heart disease group subjects was (235.4 ± 91.24) respectively. Comparison between the control and heart disease group according to cholesterol showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to cholesterol showed significant differences (P_2<0.001). Comparison between the heart and obesity groups according to cholesterol showed insignificant differences (P_3 = 0.061) (Table 4-8) Figure (4 - 4). These findings were agreed with Steven E 8 and Takatoshi Kasai 9 and this is result was disagreed with finding of RyuichiKawamoto. [218] the study result was suggested that cholesterol levels were greater in obesity subjects than heart subjects.

In the Comparison between the different studied groups according to TG showed significant differences (P <0.001) mean value of triglyceride in heart disease group subjects (94.31 ± 43.16), Obesity group was (558.6 ± 311.5) and heart disease group subjects was (589.2 ± 303.4) respectively. Comparison between the control and heart disease group according to triglyceride showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to TG showed significant differences (P_2<0.001). Comparison between the heart and obesity groups according to TG showed insignificant differences (P_3 = 0.061) (Table 4-8) Figure (4 - 4). These findings were agreed with Steven E [247] and Takatoshi Kasai. [189]

In the Comparison between the different studied groups according to HDL showed significant differences (P <0.001) mean value of HDL in healthy group subjects (42.42 ± 9.70), Obesity group was (28.45 ± 9.99) and heart disease group subjects was (30.26 ± 14.69) respectively. Comparison between the control and heart disease group according to HDL showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to HDL showed

significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to HDL showed insignificant differences (P_3 = 0.899) (Table 4-8) Figure (4 - 4). This result was agreed with finding of He W. [273]

In the Comparison between the different studied groups according to LDL showed significant differences (P <0.001) mean value of LDL in heart disease group subjects (81.27 ± 25.38), Obesity group was 113.9 ± 31.18 and heart disease group subjects was (108.4 ± 36.19) respectively. Comparison between the control and heart disease group according to LDL showed significant differences (P_1<0.001). Comparison between the control and obesity groups according to LDL showed significant differences (P_2<0.001). Comparison between the heart disease group and obesity groups according to LDL showed insignificant differences (P_3 = 0.346) (Table 4-8) Figure (4 - 4). This result was agreed with finding of Maryam Tohidi. [293]

5.2 Conclusion: -

The results of the current study revealed that there is correlation between mutation in R-SPONDIN3 gene and abdominal obesity which is all the mutations showed only in heart disease group so the mutation of RSPO3 has significant effect in obese patients which are having CVD. Also the amount of RSPO3 gene expression among the obese and CVD patients is show up significant different and the amount of gene expressing among the CVD patients is higher than obese patiens. Also the results showed the amount of gene expression in individual with visceral fat is more than individuals with subcutaneous fat. Also the results showed weak correlation between the amount of gene expressed among obese and CVD patients with BMI, WHR and age. Also the results showed the amount of gene expressed has significant effected on the fat phenotype (visceral fat and subcutaneous fat) and have insignificant on gluteal fat.

5.3 Recommedndation: -

1. Further studies with more subjects are required to investigate the association between visceral fat, SNPs and metabolic traits.
2. Further advanced studies, like genome Sequencing and genotyping are required to investigate R-SPONDIN3 gene and fat phenotype to increase the chance for more detection of genes.
3. Further studies should be done to Addressing intra-abdominal adiposity should play a central role in future strategies aimed at improving cardiovascular outcomes in patients with abdominal obesity and its associated cardiometabolic risk in Sudan
4. To avoid, whenever possible, the risk factors which may accelerate the development of obesity such as overeating, physical inactivity, unhealthy lifestyle habits and environments, etc.
5. To introduce educational programs for the enhancement of the patient's awareness regarding the obesity diseases, complication and risk factors.
6. To exchange information between local, regional and international research centers with respect to R-SPONDIN3 gene and fat phenotype and association with cardiovascular diseases.
7. More support - in terms of medical, psychological and social care - is needed for the patient's obesity and cardiovascular disease.
8. More studies are strongly needed to shed light on the genetics of the obesity as no clear information is available regarding this.
9. This study was carried in Khartoum state; other study should be carried to include all Sudanese regions
10. This study was carried in adult volunteers; other study is required to include other age groups.
11. Other study should be done to demonstrate the effect of abdominal treatment on visceral fat.
12. To identify novel loci associated with central or peripheral fat distribution.
13. To identify the genetic determinants obesity that interacts with environmental variables in complex ways.

Chapter six

References& Appendices

6.1 References:

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6.2 Appendix I

Gantt chart

Tasks	**Responsible person**	**Month 1-2**	**Month 3-6**	**Month 7-8**	**Month 9-10**	**Month 11-13**	**Month 14-24**	**Month 25-30**
Meeting faculty authorities and present the study proposal	Researcher							
Data collection	Patients and controls attending the study areas							
Biochemical Analysis	Researcher							
Data analysis	Researcher							
Writing of the results &Submit article for publishing	Researcher							
Finalizing the study	Researcher							
Submission of the final project	Researcher							

بسم الله الرحمن الرحيم
جامعة شندى
كليه الدراسات العليا

استمارة مشاركه في بحث مقدم لنيل درجه الدكتوراه في الكيمياء ألسريريه بعنوان :- دور الطفرة في جين ار سبوندين 3 في تحديد ظواهر السمنة / وتوزيع الدهون وإمكانية الاصابه بأمراض القلب والاوعيه الدموية لدي المرضي السودانيين بولاية الخرطوم .

السيد/السيدة: -.....

إنني أقوم بدراسة لمعرفه دور الطفرة في جين ار سبوندين 3 في تحديد ظواهر السمنة / توزيع الدهون وامكانيه الإصابة بأمراض القلب والأوعية الدموية عن طريق اختبار البلمرة التسلسلي و الزمن الحقيقي وهي تفيد في الكشف عن مسببات السمنة وتلافيها في المستقبل إن شاء الله لذا أمل منكم المشاركة معي في هذا البحث وذلك بالسماح لي بأخذ عينات دم وسوف تكون بياناتكم لغرض البحث فقط وسوف أقوم باتخاذ جميع الاجراءت التي تضمن خصوصية وسريه المعلومات الشخصية المتعلقة بكم ولكم مني جزيل الشكر والتقدير واسأل الله إن يتم عليكم بنعمه الشفاء العاجل.

موافق ( ) غير موافق ( )

Shandi University

Application of genetic polymorphism & gene experience of R-SPONDIN3 as biomarker of cardiometabolic traits associated with or without obesity in sample ofsudaneese pateins in Khartoum state

QUESTIONNAIRE

|

1. General Information

Name:……No……

Age:……Years Sex: M ☐ F ☐

Address:……..Tel. No:……

Duration of Obesity: ……

2. Complications of obesity: ……...

……

3. Tall: …….m. **Weight:** …….kg **BMI:** …….

4. Sagittal abdominal diameter (SAD): ……

Statement of other health condition:

o CVD	Yes ☐	No ☐
o Inflammation or infection	Yes ☐	No ☐
o Diabetes Mellitus	Yes ☐	No ☐
o Hypertension	Yes ☐	No ☐
o Pregnancy	Yes ☐	No ☐

o Cancer	Yes ☐	No ☐
o Hormonal problems	Yes ☐	No ☐
o Paralysis	Yes ☐	No ☐
o Bone problems	Yes ☐	No ☐
o Hypothyroidism	Yes ☐	No ☐
o Liver Disease	Yes ☐	No ☐
o Autoimmune Disease	Yes ☐	No ☐
o Gout	Yes ☐	No ☐
o Kidneydisease	Yes ☐	No ☐
o Cushing's syndrome	Yes ☐	No ☐
o Smoking	Yes ☐	No ☐

6. Statement of drugs used at this moment:

……

……

……

……

……

……

Results

- **Serum - Cholesterol:**……mg/dl
- **HDL – Cholesterol:**……mg/dl
- **LDL – Cholesterol:**……mg/dl

- **Serum Triglyceride:**……mg/dl
- **Waist-to-hip ratio (WHR):**……
- **BMI:** lean ☐ normal ☐ overweight ☐ obese ☐ fatal obesity ☐

Result of PCR: - Positive ☐ **Negative** ☐

PROGRESSION OF WEIGHT GAIN PATTERN:

☐ No pattern Steady,

☐ Gradual increase of weight over the years

☐ Sudden increases of weight with pregnancies

☐ Variable weight gain/loss due to intermittent diet and exercise (regained weight when stopped program

جامعه شندي

تطبيق تعدد الأشكال الوراثية وتجربة جينات ار سبندوين 3 كمؤشر حيوي لسمات القلب والأوعية الدموية المرتبطة أو غير المرتبطه بالسمنة في عينة من البطينات السودانية في ولاية الخرطوم

الاستبيان

1-المعلومات الأساسية :-

الاسم :- ..... الرقم :-.....

العمر بالسنوات :-..... النوع :- ذكر □ أنثي □

العنوان :-..... رقم الهاتف :-.....

مده السمنة :- .....

2- مضاعفات السمنة:-.....

.....

3- الطول :- .....بالمتر الوزن :- ..... بالكيلو مؤشر كتلة الجسم :- .....

4- قطر البطن السهمي :-..... سنتيمتر

5- بيان حالة صحية أخرى:-

- **الأمراض القلبية الوعائية نعم □ لا □**
- **التهاب أو عدوى نعم □ لا □**
- **السكري نعم □ لا □**
- **ارتفاع ضغط الدم نعم □ لا □**
- **حمل نعم □ لا □**
- **سرطان نعم □ لا □**
- **مشاكل هرمونية نعم □ لا □**
- **شلل نعم □ لا □**
- **مشاكل العظام نعم □ لا □**
- **قصور الغدة الدرقية نعم □ لا □**
- **مرض الكبد نعم □ لا □**

- **مرض يصيب جهاز المناعة نعم □ لا □**
- **نقرس نعم □ لا □**
- **مرض الكلية نعم □ لا □**
- **متلازمة كوشينغ نعم □ لا □**
- **التدخين نعم □ لا □**

6- العلاجات التي تستخدم في هذه اللحظة :-

.....
.....
.....
.....
.....

النتائج :-

- **نسبة الكولسترول: ..... ملغ / دل**
- **نسبة الكولسترول النافع: ..... ملغ / دل**
- **نسبة الكولسترول الضار: ..... ملغ / دل**
- **نسبة الدهون الثلاثية : ..... ملغ / دل**
- **نسبة الخصر إلى الورك : .....**
- **مؤشر كتلة الجسم :- نحيف 5 عادي 5 وزن زائد 5 بدين 5 سمنة قاتلة 5**

7- نتائج تحليل البلمرة التسلسلي :- ايجابيه ☐ سالبة ☐

نمط اكتساب الوزن :-

☐ **لا يوجد نمط ثابت.**

☐ **الزيادة التدريجية للوزن على مر السنين.**

☐ **زيادة مفاجئة في الوزن مع حالات الحمل.**

☐ **زيادة أو خسارة في الوزن المتغير بسبب إتباع نظام غذائي متقطع وممارسة الرياضة أو استعادة الوزن عند توقف البرنامج.**

Appendix II

Reagents and Primers

Agarose gel

Preparation

Amount of 2 gm of agarose powder dissolved by boiling in 100 ml 1X TBE buffer, then was cooled to 55°C in water bath, then, 1.5 µl of Ethidium bromides stock (10 mg/ml) per 100 ml gel solution for a final concentration of 0.5 ug/ml were added, mixed well and poured on to the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature. After solidification, the comb was gently removed and the spacer from the opened sides was removed.

Primer Design

Degenerate oligonucleotide primers from conserved regions of the R Spondin 3 gene was designed by primer3plus (www.bioinformatics.nl/primer3plus) from the R Spondin 3 gene sequences in the GenBank database (NCBI) and synthesized by Macrogen (South Korea).

No	Oligo Name	Sequence (5' -> 3')	Yield {OD}	Yield {ug}	Yield {umol/ul}	Vol for 100 pmol/ul	Tm {C}	MW {G/MOL}	CG – Content	Synthesis scale	Purifications
1	Rspo3 F	CGGGATCCGCCGCCGCCATGCACTTGCGACTGATTTC (37)	7.27	220	19.5	195	>75	11278	64.9%	0.01 umol	HPSF
2	Rspo3 R	CCTCTAGAGTGTACAGTGCTGACTGATACCG(31)	3.59	102	10.7	107	69.5	9511	51.6%	0.01 umol	HPSF

Table (6-1) oligonucleotide primers

Figure (6-1): PCR machine

Figure (6-2): q PCR Machine

Maxime PCR PreMix Kit (*i*-Taq)

for 20㎕ rxn / 50㎕ rxn

Cat. No. 25025(for 20㎕ rxn, 96 tubes) **Cat. No. 25026**(for 20㎕ rxn, 480 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. *Maxime* PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20㎕ rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50㎕ rxn) 96 tubes

Component in	20 ㎕ reaction	50 ㎕ reaction
i-Taq™ DNA Polymerase(5U/㎕)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3㎕~9㎕
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/㎕ each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20㎕ or 50㎕ . Do not calculate the dried components

Example **Total 20㎕ or 50㎕ reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2㎕	2 ~ 4㎕
Primer (F : 10pmol/㎕)	1㎕	2 ~ 2.5㎕
Primer (R : 10pmol/㎕)	1㎕	2 ~ 2.5㎕
Distilled Water	16 ~ 17㎕	44 ~ 41㎕
Total reaction volume	**20 ㎕**	**50 ㎕**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

• Comparison with different company kit

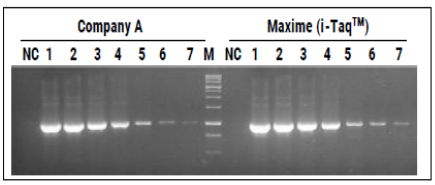

Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .

After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product. **Lane M,** SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC,** Negative control

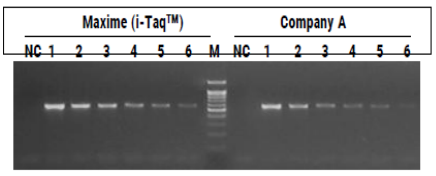

Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).

Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.

lane M, SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC,** Negative control