



Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon / College of Science
Department of Chemistry

**Association of Afamin and Peroxiredoxin 4
Genes Polymorphisms with Antioxidant Status
and Diabetic Nephropathy**

A Thesis

**Submitted to the Council of College of Science
University of Babylon in Partial Fulfillment of the
Requirements for the Degree of Doctor in
Chemistry**

By

Tholfiqar Ahmed Hamza Kadhim
M.Sc. Chemistry 2014
(University of Babylon / College of Science)

Supervised by

Prof. Dr. Mahmoud Hussein Hadwan
(University of Babylon / College of Science)

2021 A.D

1443 A.H



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل / كلية العلوم
قسم الكيمياء

ترافق التناظر الجيني للأفامين والبيروكسي ريدوكسين 4 مع حالة مضادات الأكسدة و اعتلالية الكلية السكرية

أطروحة مقدمة إلى مجلس
كلية العلوم / جامعة بابل وهي جزء من متطلبات نيل درجة الدكتوراه- فلسفة
في علوم الكيمياء

من قبل
ذوالفقار أحمد حمزه كاظم
ماجستير كيمياء 2014
(جامعة بابل / كلية العلوم)

إشراف
أ.د. محمود حسين هدوان
(جامعة بابل / كلية العلوم)

Summary

Summary

Background: The prevalence of type 2 diabetic in Iraq has increased ,the medical expenditures of such a rise in prevalence are substantial . diabetic nephropathy is characterized by an increase in urine albumin excretion rate, as well as abnormal kidney function as evidenced by a typical serum creatinine and a decreased glomerular filtration rate. Diabetic nephropathy, too known as inter capillary glomerulonephritis or "nodular diabetic glomerulosclerosis" . Albuminuria (more than 300 mg/day or more than 200 µcg/min) is a clinical condition characterized by "nodular diabetic glomerulosclerosis." assured on at least two appropriateness three to six months apart, "Glomerular filtration rate (GFR)" decreases permanently and irreversibly . DN is a chronic complications , produce of beta-cell destruction (T1DM), or insulin resistance (T2DM).

Afamin is a glycoprotein 87-kDa (GPC87) is an 87-kDa mammalian glycoprotein encoded by chromosome 4 in humans that has 15 percent carbohydrate content and 55 percent amino acid composition and is considered similar to albumin. It is mainly expressed in the liver and eventually secreted into the circulation blood where it possibly acts as a transport protein for small, "hydrophobic molecule" Also, based on studies have previously, specific binding vitamin E to afamin based on possible chemical binding properties. Afamin has been thought to act as an antioxidant and thus protect the brain from high levels of oxidative stress.

Peroxiredoxins are a family of antioxidant proteins, ubiquitous protected in a variety of living organisms from eukaryotes to prokaryotes , the molecular size of antioxidant enzymes about 20 to 30 k Da , they modulate biological function to prevent organism from oxidative stress, therefore regulation the intracellular signal transmit . Based on numbers of a preserved cysteine residue , the member of the Prx family can be divided into six subtypes ; The only

Summary

isoform of Prx4 found in serum . Changes in Prx4 expression or oxidation status in pancreatic islet cells have been seen in animal models of DM .

Aims: To measure peroxiredoxin 4 and Afamin activity, and study the peroxiredoxin 4 and Afamin Gene polymorphisms association with DN and compared it with control individuals . Current method , development novel accurate method for determining catalase activity by new method, CAT is an important antioxidant enzyme that converts hydrogen peroxide to (H_2O and O_2). Other method , development novel precise method for assessment of peroxiredoxin activity by new method . This protocol used sodium azide (NaN_3) to eliminate catalase interference and avoided using high concentrations of strong acid to inhibit the Prx reaction.

Methods: The study consisted of 200 patients (100 of T2D patients and 100 of T2D with nephropathy) and 100 of control individuals . Biochemical measurements the included peroxiredoxin 4, Afamin, peroxiredoxin activity, serum lipid peroxidation, total antioxidant capacity, total oxidant status, serum catalase activity and vitamin E. Genotyping of rs 35233345 afamin Gene, Rs 564561 peroxiredoxin 4 Gene polymorphism are carried out by PCR-sequences.

Results: The estimation of biochemical data pointed out significant differences in, PRX4 (0.014) between (G1, G2) (0.013) between (G1, G3) (0.973) between (G2, G3) , Afamin (0.01) between (G1, G2), (0.01) between (G1, G3), (0.799) between (G2, G3), Vitamin E (0.01) between (G1, G2) (0.01) between (G1, G3), (0.801) between (G2, G3) , PRX using H_2O_2 as the Prx substrate (0.001) between (G1, G2) (0.001) between (G1, G3) (0.171) between (G2, G3) , PRX using t-BOOH as the Prx substrate (0.001) between (G1, G2) (0.001) between (G1, G3) (0.888) between (G2, G3) , TAO (0.001) between (G1, G2) (0.001) between (G1, G3), (0.544) between (G2, G3), TOS (0.030) between (G1, G2) (0.000) between (G1, G3) (0.103) between (G2, G3), Catalase (0.001) between (G1, G2) (0.001) between (G1, G3), (0.788) between (G2, G3), MDA, (0.001)

Summary

between (G1, G2) (0.001) between (G1, G3) (0.713) between (G2, G3), in T2D patients with and without DN when compared with those of the control group. The genotype distribution and allele frequencies of the Afamin (rs 35233345 G>A) polymorphisms and peroxiredoxin 4 (rs 564561 G> C) polymorphisms in all patients, and in the healthy controls were done.

In conclusion, Diabetic nephropathy associated with elevated afamin (0.01) between (G1, G2), (0.01) between (G1, G3), (0.799) between (G2, G3), and elevated PRX4 (0.014) between (G1, G2) (0.013) between (G1, G3) (0.973) between (G2, G3) . DN was associated with elevated ROS levels and MDA . Decrement TAO levels, DN associated with decrement CAT and vitamin E. to assessment of catalase activity , results were showed that this assay can be used for "routine health applications" because it is inexpensive, simple, and rapid and accurate . And to assessment of peroxiredoxin activity, the correlation coefficient of comparison ($r > 0.99$) was found between both the FAS and ferrithiocyanate method.

الخلاصة

لقد ازداد انتشار مرض السكري من نوع الثاني T2DM في العراق ، والنفقات الطبية لمثل هذا الارتفاع في الانتشار كبير. يتميز DN بزيادة في معدل إفراز الألبومين في الاضرار ، وكذلك وظائف الكلى غير الطبيعية كما يتضح من الكرياتينين في المصل وانخفاض معدل الترشيح الكبيبي . يُعد اعتلال الكلية السكري ، المعروف أيضًا باسم التهاب كبيبات الكلى بين الشعيرات الدموية أو "متلازمة كيميلستيل ويلسون أو تصلب الكبيبات العقدي السكري" متلازمات إكلينيكية تتميز بالبيلة الزلالية (< 300 مجم / يوم أو < 200 ميكروغرام / دقيقة) اثبتت على الأقل مرتين (ثلاثة - ستة) أشهر ، انخفاض دائم ولا رجعة فيه في "معدل الترشيح الكبيبي (GFR) ، وارتفاع ضغط الدم الشرياني". "اعتلال الكلية السكري" (DN) هو مضاعفات مزمنة لجميع مرض السكري من نوع الاول T1DM (نقص الأنسولين سبب تحطم خلايا بيتا) ، و T2DM (مقاومة الأنسولين أو نقص إفراز الأنسولين).

الأفامين هو بروتين سكري 87 كيلو دالتون (GPC87) للتديبات مشفر بواسطة الكروموسوم 4 في البشر الذي يحتوي على 15 % من الكربوهيدرات و 55 % من الأحماض الأمينية ويعتبر مشابهًا للألبومين. يتم التعبير عنه بشكل أساسي في الكبد ويتم إفرازه في الدورة الدموية حيث يعمل كبروتين ناقل "كارهة للماء" ، ويرتبط فيتامين E بالأفامين بناءً على خصائص الارتباط الكيميائي المحتملة. يُعتقد أن الأفامين يعمل كمضاد للأكسدة وبالتالي يحمي الدماغ من مستويات عالية من الإجهاد التأكسدي.

البيروكسي ريدوكسينات هي عائلة من البروتينات المضادة للأكسدة ، محمية في مجموعة متنوعة من الكائنات الحية من حقيقيات النوى إلى بدائيات النوى ، الحجم الجزيئي للأنزيمات المضادة للأكسدة حوالي 20 إلى 30 كيلو دالتون ، وهي تعادل الوظيفة البيولوجية لمنع الكائن الحي من الإجهاد التأكسدي ، وبالتالي تعمل على تنظيم الإشارة داخل الخلايا ، يمكن تقسيم عضو عائلة Prx إلى ستة أنواع ؛ الشكل الوحيد لـ Prx4 الموجود في المصل. لاحظنا وجود تغييرات من Prx4 في مرض السكري.

الأهداف: تم قياس نشاط البيروكسي ريدوكسين 4 ونشاط أفامين. ودراسة ارتباط جين بيروكسي ريدوكسين 4 وجين أفامين مع DN ومقارنته بأفراد مجموعة التحكم. وتم تطوير طريقة دقيقة جديدة لتقييم نشاط الكتاليز بطريقة جديدة ، الكتاليز هو إنزيم حيوي مضاد للأكسدة يفتك H_2O_2 الى الماء وجزئية الأكسجين. وكذلك تم تطوير طريقة دقيقة جديدة لتقييم نشاط البيروكسي ريدوكسين بطريقة جديدة. استخدم في هذا البروتوكول أزيد الصوديوم (NaN_3) للقضاء على تداخل الكتاليز وتجنب استخدام تركيزات عالية من حامض قوي لتثبيط تفاعل Prx.

الخلاصة

الطريقة: تكونت الدراسة من 200 مريض (100 مريض مصاب بالنوع T2DM غير مصاب باعتلال الكلية و 100 مريض مصاب بالنوع T2DM مع اعتلال الكلية) و 100 من أفراد التحكم الأصحاء. القياسات البيوكيميائية تشمل بيروكسيبريدوكسين 4 ، أفامين ، نشاط بيروكسي ريدوكسين ، المالون داي الديهايد ، مضادات الأكسدة الكلية ، الأكسدة الكلية ، نشاط الكتاليز في المصل وفيتامين E . وتم تطوير طريقة دقيقة جديدة لتقييم نشاط الكتاليز و يعتمد على تفكك بيروكسيد الهيدروجين ، يقيس الاختبار المطور التفاعل بين كاشف هيدروكينون / كبريتات الأنيلينيوم / موليبدات الأمونيوم وبيروكسيد الهيدروجين غير المتفاعل ، مما ينتج عنه مركب كينون أرجواني (امتصاصية عند طول موجي 550 نانومتر) . تطوير طريقة دقيقة جديدة لتقييم نشاط البيروكسيبريدوكسين ، تم تقدير أنشطة البيروكسي روكسين من خلال احتضان العينات بتركيزات مناسبة من (DTT) وبيروكسيد الهيدروجين (H_2O_2) أو (t-BOOH) ، كمادة اساس . تم منع التفاعل الأنزيمي بعد الحضنة باستخدام كاشف يحتوي على كبريتات الحديدوز الأمونيوم (AFS) و أمينوساليسيليك (ASA). نتيجة لذلك ، سوف يتفاعل البيروكسيد المتبقي مع الكاشف لتكوين مركب أمينوساليسيلات بني اللون (امتصاصية عند طول موجي 425 نانومتر). تم إجراء التنميط الجيني لـ rs 35233345 Afamin Gene ، rs 564561 peroxiredoxin 4 ، تعدد الأشكال الجيني بواسطة تسلسلات PCR.

النتائج: لاحظنا تقدير البيانات البيوكيميائية إلى وجود فروق ذات دلالة إحصائية في PRX4 (0.014) بين (G2 ، G1) (0.01) بين (G3 ، G1) (0.973) بين (G3 ، G2) ، أفامين (0.01) بين (G2 ، G1) (0.01) (G3،G1) (0.799) بين (G3 ، G2) ، فيتامين E (0.01) بين (G1 ، G2) (0.01) بين (G3 ، G1) (0.801) بين (G3 ، G2) ، PRX باستخدام H_2O_2 كمادة اساس ل Prx (0.001) بين (G2،G1) (0.001) بين (G1 ، G3) (0.171) بين (G2 ، G3) باستخدام t-BOOH كمادة اساس ل Prx (0.001) بين (G ، G1) (0.001) بين (G3 ، G1) (0.888) بين (G3 ، G2) ، TAO (0.001) بين (G2،G1) (0.001) بين (G3 ، G1) (0.544) بين (G3 ، G2) ، TOS (0.030) بين (G2،G1) (0.000) بين (G3 ، G1) (0.103) بين (G3 ، G2) ، Catalase (0.001) بين (G2 ، G1) (0.001) بين (G3 ، G1) (0.788) بين (G3 ، G2) ، MDA (0.001) بين (G2،G1) (0.001) بين (G3 ، G1) (0.713) بين (G3 ، G2) في المرضى المصابين بالنوع T2DM مع وبدون اعتلال الكلية بالمقارنة مع تلك الموجودة في مجموعة التحكم . و أوضحت النتائج أنه لا توجد ارتباطات ذات دلالة إحصائية بين تعدد الأشكال AA و AG و Afamin (rs 35233345 G> A) و GG

الخلاصة

تعدد الأشكال ، كما أوضحت النتائج أنه لا توجد ارتباطات معنوية وغير معنوية بين تعدد الأشكال CC و GC و GG للبيروكسي ريدوكسين.4 (rs 564561 G> C) تعدد الأشكال.

في الاستنتاج ، يرتبط اعتلال الكلية السكري بارتفاع نسبة الأفيامين (0.01) بين (G2 ، G1) ، (0.01) بين (G3 ، G1) ، (0.799) بين (G3 ، G2) ، وارتفاع PRX4 (0.014) بين (G1 ، G2) (0.013) بين (G3،G1) (0.973) بين (G3 ، G2). ارتبط DN بمستويات مرتفعة من ROS و MDA. انخفاض مستويات TAO ، المرتبط بانخفاض CAT وفيتامين E. لتقييم نشاط الكتاليز ، تم إثبات النتائج أن هذا الاختبار يمكن استخدامه للبحث العلمي والتطبيقات الصحية الروتينية لأنه غير مكلف وبسيط ودقيق وسريع. هذه الطريقة مناسبة للاستخدام في مختبرات علم الأمراض السريرية لأنها بسيطة وتنتج نتائج دقيقة وقابلة للتكرار. ولتقييم نشاط البيروكسي ريدوكسين ، وجد معامل الارتباط للمقارنة ($r > 0.99$) بين كل من طريقة FAS وطريقة ferrithiocyanate.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ
الْحَمْدُ لِلَّهِ الَّذِي
خَلَقَ السَّمَوَاتِ وَالْأَرْضَ
وَالَّذِي خَلَقَ الْمَرْءَ مِنْ
نُطْفَةٍ ثُمَّ يَرْجِعُهُ إِلَى
الْأَرْضِ أَوْ إِلَى سَائِرِ
الْمَوَاقِدِ

سَبِّحِ اسْمَ رَبِّكَ الْأَعْلَى * الَّذِي خَلَقَ فَسَوَّى * وَالَّذِي
قَدَرَهُ فَهْدَى * وَالَّذِي أَخْرَجَ الْمَرْعَى * فَجَعَلَ خُنُفًا
أُحْوَى *

صدق الله العلي العظيم

سورة الأعلى

(١ - ٥)

Acknowledgements

Acknowledgements

Thanks and praise be to Allah, and prayer and peace upon his prophet Mohammed, and his pure relatives and best colleagues.

Sincerely I feel deep gratitude to my supervisors Prof. Dr. Mahmoud Hussein Hadwan for steering the project. Their interest in my work and in particular their determination in never allow me to give up until the final draft was completed, is sincerely appreciated.

I would like to thank the University of Babylon, College of Science and my instructors in the department of Chemistry for providing me with the necessary facilities during this study.

I would also like to thank the Deanery of the College of Science, Babylon University for providing me necessary instruments for measurements

Sincere thanks are due to the head and all staff members of the chemistry Department at the College of Science, University of Babylon for helping me in this work.

I would like to thank Dr. Lamia Abdul Majeed Al-Mashhedy for her guidance, advice, and great support.

I like to extend special thanks to Dr. Mohaned Al. Hachamii in the University of Babylon, College of Science, Chemistry Department provided the most necessary chemical materials during the study.

I also wish to express my deep gratitude to my family; father, mother, brothers, sisters and wife for their patience, boundless love, understanding and support during my study.

Finally, I would like to thank all my friends who supported me to finish my research.

Tholfiqar

Dedication

I wish to express my deep gratitude to my family; father, mother, brothers, sisters and wife for their patience, boundless love, understanding and support during my study.

Finally, I would like to thank all my friends who supported me to finish my research.

Chapter One

Introduction And Literature Review

Chapter Two

Materials and Methods

Chapter Three

Results and Discussion

References

Contents

List of Contents

NO.	TITLE	PAGE
	CERTIFICATION	I
	Acknowledgement	II
	Contents	III
	List of Figure	VII
	List of Tables	IX
	List of abbreviations	XIII
	Abstract	XV
1.	Introduction	1
1.1.	1.1. Classification of Diabetes Mellitus	2
1.1.1.	Type (1) Diabetes	2
1.1.1.1.	immune-based diabetes	2
1.1.1.2.	idiopathic diabetes	3
1.1.1.3.	Fulminant (type I) diabetes	3
1.1.2.	Type II Diabetes Mellitus	4
1.1.2.1.	Genetics of Type II Diabetes	5
1.1.3.	Gestational Diabetes Mellitus	6
1.2.	Diagnosis of Type 2 Diabetes	7
1.2.1.	Glycated Hemoglobin	7
1.3.	Causes of Type II Diabetes Mellitus	8
1.4.	Complications of Diabetes Mellitus	9
1.4.1.	Diabetic nephropathy	10
1.4.2.	Microalbuminuria	13
1.5.	Oxidative stress	14
1.6.	Mechanisms of Production of ROS	15
1.6.1.	Endogenous Sources of ROS	16
1.6.1.1.	Mitochondria	16
1.6.1.2.	Peroxisomes	17
1.6.1.3.	Endoplasmic Reticulum	17
1.6.2.	Exogenous Sources of ROS	18
1.7.	Oxidative stress in Diabetes Mellitus	19

Contents

1.8.	Classification of Antioxidants	21
1.8.1.	Enzymatic Antioxidant	22
1.8.1.1.	Catalase	22
1.8.2.	Non-enzymatic Antioxidants	22
1.8.2.1.	Vitamin E	23
1.9.	Peroxiredoxins	24
1.9.1.	Peroxiredoxins 4	26
1.9.1.1.	Peroxiredoxins 4 gene	28
1.10.	Lipid Peroxidation	29
1.11.	Afamin	31
1.11.1.	Afamin gene	31
1.11.2.	Functional characterization of afamin	32
1.11.3.	Applications of afamin	33
1.12.	Statistical analysis	35
1.13.	Aim of the Study	35
2.1.	Materials	36
2.1.1.	Chemicals	36
2.1.2.	Instrument analysis and equipment	38
2.2.	Methodologies	39
2.2.1.	Collection of Blood and Serum Preparation	39
2.2.2.	Total Oxidant Status	40
2.2.2.1.	Principle	40
2.2.2.2.	Assay Reagents	40
2.2.2.3.	Procedure	41
2.2.3.	Total Antioxidant Capacity Assay	41
2.2.3.1.	Principle	41
2.2.3.2.	Reagent Preparation	41
2.2.3.3.	Procedure	42
2.2.3.4.	Calculation	43
2.2.4.	Assay of total tocopherol (vitamin E) in plasma	44
2.2.4.1.	Principle	44
2.2.4.2.	Reagents	44

Contents

2.2.4.3.	Procedure	44
2.2.4.4.	Calculation	45
2.2.5.	Determination of Serum AFM (Afamin) Activity	45
2.2.5.1.	Principle	45
2.2.5.2.	Preparation of Reagents	46
2.2.5.3.	Assay procedure	47
2.2.5.4.	Calculation	48
2.2.6.	Determination of Serum peroxiredoxine 4 Activity	48
2.2.6.1.	Principle	48
2.2.6.2.	Preparation of Reagents	49
2.2.6.3.	Assay procedure	50
2.2.6.4.	Calculation	51
2.2.7.	Determination of Serum Lipid Peroxidation	52
2.2.7.1.	principle	52
2.2.7.2.	Reagents	53
2.2.7.3.	Procedure	53
2.2.7.4.	Calculation	53
2.2.8.	Genetics Study	53
2.2.8.1.	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis Design	53
2.2.8.1.1.	SNPs Flanked Sequences Retrieving	54
2.2.8.1.2.	Restriction Enzyme Selection	54
2.2.8.1.3.	Primers Design for PCR-RFLP and SSCP	54
2.2.8.2.	DNA Extraction	54
2.2.8.3.	Agarose Gel Electrophoresis	56
2.2.8.4.	Photo Documentation	58
2.2.8.5.	Reconstituting and diluting primers	58
2.2.8.6.	Amplification by Polymerase Chain Reaction (PCR)	59
2.2.9.	Restriction Fragment Length Polymorphism (RFLP) for study the genotyping of peroxiredoxin 4 gene	60
2.2.9.1.	Principle	60
2.2.9.2.	Restriction Enzyme	61
2.2.9.3.	Procedure.	61
2.2.10.	Method of Sample preparation	62
2.2.10.1.	Polyacrylamide Gel Electrophoresis	62

Contents

2.2.11.	Methods of Sequencing	63
2.2.11.1.	DNA Sequencing of PCR amplicons	63
2.2.11.2.	Interpretation of sequencing data	64
2.2.12.	Materials and methods for assessment catalase activity	64
2.2.12.1.	Chemicals	64
2.2.12.2.	Principle	64
2.2.12.3.	Reagents	65
2.2.12.4 .	Erythrocyte Samples concentration	66
2.2.12.5.	Tissue Preparations	66
2.2.12.6.	Procedure	66
2.2.12.7.	Calculation	67
2.2.13.	Assessment peroxiredoxin activity	67
2.2.13.1.	Reagents and solutions	67
2.2.13.2.	Peroxiredoxin purification	68
2.2.13.3.	Tissue preparation	68
2.2.13.4.	Potassium thiocyanate method	69
2.2.13.5.	Aminosalicylic acid method	69
2.2.13.6.	Calculation	71
3.1.	Biochemical and clinical characteristics of the study subjects	72
3.2.	Total oxidant levels in sera of diabetic patients and control	73
3.3.	Total antioxidant levels in sera of diabetic patients and controls	75
3.4.	Antioxidant enzymes activities in blood of diabetic patients and controls	78
3.5.	Vitamin E levels in sera of diabetic patients and control	80
3.6.	Afamin activity in sera of diabetic patients and controls	82
3.7.	Peroxiredoxin activity in sera of diabetic patients and controls	85
3.8 .	Peroxiredoxin 4 activity in sera of diabetic patients and controls	88
3.9.	Lipid peroxidation levels in sera of diabetic patients with and without nephropathy	91
3.10.	Genetic Study	94
3.10.1.	Amplification of afamin and peroxiredoxin 4 genes primers using temperature gradient of PCR reaction	94

Contents

3.10.2.	Sequencing of the 320 bp region within the afamin gene polymorphisms	97
3.10.3.	Distribution of genotype and allele frequencies of peroxiredoxin 4 gene polymorphisms	108
3.11.	Assessment Catalase Activity	114
3.12.	Assessment Peroxiredoxin Activity	122
3.13.	Conclusion	128
3.11.	Recommendations	128
	References	129
	Appendix I	192
	Appendix II	194
	Appendix III	196
	Appendix IV	199

List of Abbreviations

List of Abbreviations

DN	Diabetic nephropathy	TAO	Total antioxidant
DM	Diabetes mellitus	ESRD	End-stage renal disease
T1A	Immune-based diabetes	T1B	Idiopathic diabetes
GDM	Gestational Diabetes Mellitus	FBG	Fasting blood glucose
HbA1c	Glycated Hemoglobin	ADA	American Diabetes Association
GFR	Glomerular filtration rate	BUN	Blood urea nitrogen
ACR	Albumin to Creatinin Ratio	CKD	Chronic kidney disease
ROS	Reactive oxygen species	CFS	Chronic fatigue syndrome
CAT	Catalase enzyme	OS	Oxidative stress
G6PD	glucose-6-phosphate-dehydrogenase	MDA	Malondialdehyde
GPx	glutathione peroxidase	PUFA	Polyunsaturated fatty acids
GRD	glutathione reductase	ROS	Reactive oxygen species
GSH	reduced glutathione	SOD	Superoxide dismutase
GSSG	oxidized glutathione	TBARS	Thiobarbituric acid reactive substances
T2D	Type 2 diabetes	UV	Ultraviolet
IU	International Units	WHO	World Health Organization
LOOH	lipid peroxidation	Kg	Kilogram
O ₂ ⁻	Superoxide anion radicals	ANOVA	Analysis of Variance
ONOO ⁻	Peroxynitrate	PARP-1	poly-ADP-ribose polymerase-1
H ₂ O ₂	Hydrogen peroxide	Prx	Peroxiredoxin
LPO	Lipid peroxidation	GF	Growth factors
AFM	Afamin	HRP	Horseradish Peroxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate	<	Less Than
NOS	nitric oxide synthase	>	Greater Than
TOS	Total Oxidant Status	S.D.	Standard Deviation
OS	Oxidative stress	°C	Degrees Celsius
OH [•]	hydroxyl radical	gm	Grams
OD	Optical density	µl	Microlitre
PUFA	polyunsaturated fatty acids	µg	Micrograms
PCR-	Polymerase Chain Reaction-	nmol/l	Nanomoles per litre

List of Abbreviations

RFLP	Restriction Fragment Length Polymorphism		
ROO [•]	peroxyl radical	μmol/l	Micromoles per litre
SSCP	Single-strand conformation polymorphism	g.	Gravitational Force
TBA	thiobarbituric acid	TCA	trichloroacetic acid
cm	Centimetre	h	Hours
RFLP	Restriction Fragment Length Polymorphism	ml	Millilitre
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid	NO [•]	Nitric oxide radical
AFS	Ammonium ferrous sulfate	NO	Nitric oxide
ASA	Aminosalicylic acid	NOS	Nitric oxide synthase
DTT	4-dithio-DL-threitol	t-BOOH	t-Butyl hydroperoxide
BP	Blood pressure	TAC	Total antioxidant capacity
CRP	C-reactive protein	TBA	Thiobarbituric acid
IL-6	Interleukins 6	TBARS	Thiobarbituric acid-reacting substances
HWE	Hardy-Weinberg equilibrium	LOO [•]	Lipid peroxyl radical
RBC	Red Blood Cell	LOOH	Lipid hydroperoxides
min	Minute	LPO	Lipid peroxidation

List of Figures

List of Figures

FIGURE	TITLE	PAGE
1. 1.	Describes the significance and causes of CKD for those with and without diabetes	2
1.2.	A model that proposes a role in a pathogens of T2D for epigenetic mechanisms	4
1.3.	Diabetic Complication	9
1.4.	The processes that lead to specific clinical manifestations of diabetes	10
1.5.	Various diagnostic measures to determine the presence and degree of that interfere with kidney function. Urine Albumin to Creatinin Ratio: albumin to creatinin ratio	12
1.6.	Oxidative stress mechanism involved in cell injury in tissue injury	15
1.7.	Mitochondrial ROS production	16
1.8.	The oxidation of Glucose and stimulated oxidative stress in the hyperglycemic	20
1.9.	Antioxidants classified as enzymatic and non-enzymatic	21
1.10.	α -Tocopherol action as a radical-scavenging antioxidant against lipid peroxidation	23
1.11.	H ₂ O ₂ and 2-Cys Prxs in redox signaling	25
1.12.	Crystal structure of human peroxiredoxin 4 (PDB 2PN8)	27
1.13.	Antioxidant role of Prx4. (a) The antioxidant role of Prx4 in the blood and the endothelial cell surface interaction of Prx4	29
1.14.	Pathways of lipid peroxidation	30
1.15.	Presentation and visual depiction of the afamin structural map	33
1.16.	Patho-physiological functions of afamin	34
2.1.	The standard curve of AFM (Afamin) concentration	48
2.2.	The standard curve of Peroxiredoxin 4 concentration	52
2.3.	Scheme of the adduct MDA-(TBA) ₂	52
2.4.	Proposed reaction for the formation of purple -colored product of the disubstituted quinone compound	65
3.1.	PCR temperature gradient was used to detection optimum annealing T _m for afamin primer	94
3. 2.	The electrophoresis pattern of PCR product for afamin gene	95
3. 3.	PCR temperature gradient was used to detection optimum annealing T _m for peroxiredoxin 4 primer	95
3. 4.	The electrophoresis pattern of PCR product for peroxiredoxin 4 genes	96
3. 5.	Silver stained SSCP electrophoresis for afamin gene genotyping , lane L DNA ladder lanes 1,2,7,12,13,and 14 A	96

List of Figures

	pattern ; lanes 3 and 11 B pattern ; other lanes C pattern	
3.6.	The sequencing of amplified 320 bp gene in (SNP rs35233345) after using specific PCR primer to detect the single nucleotide polymorphism of afamin gene	97
3.7.	The precise location on chromosome 4 of the 320 bp amplicon that partially covered a section of the afamin gene	98
3.8.	The 320 bp amplicons of the afamin genetic DNA sequences were aligned with the appropriate reference sequences of 5 samples	100
3.9.	The pattern of the G119A SNP inside the DNA chromatogram of the AMF gene's targeted 320 bp amplicons	101
3.10.	The dbSNP server was used to check the novelty of afamin genetic single nucleotide polymorphisms. The detected SNP was highlighted in red	102
3.11.	Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) for peroxiredoxin 4 genes	108
3.12.	Catalase enzyme activity correlated with the spectrophotometric properties of the disubstituted quinone	115
3.13.	The comparison between catalase enzyme activity of RBC homogenates that assessed by utilize the disubstituted quinone method and peroxovanadate method	119
3.14.	The comparison of the disubstituted quinone method and peroxovanadate methods for assessment of catalase activities in (1-500) dilution of homogenate tissues	121
3.15.	Peroxiredoxin estimated reaction. The final product includes the production of the brown ferriaminosalicylate complex	123
3.16.	A spectrophotometric spectrum of the ferriaminosalicylate complex	123
3.17.	Bland-Altman plot indicating the mean relative bias the relative difference between the ferrithiocyanate and ferriaminosalicylate methods	126
3.18.	The Passing–Bablok analysis correlation of the ferriaminosalicylate method and the ferrithiocyanate method	127

List of Figures

List of Tables

List of Tables

TABLE	TITLE	PAGE
1.1.	Stage of diabetic nephropathy (DN) by the level of urinary albumin levels	13
1.2.	Reactive oxygen species producing enzymes are produced in peroxisomes	17
1.3.	Reactive oxygen species produced from the exogenous sources	18
2.1.	All chemicals were used as being supplied without further purification	36
2.2.	Instrument Analysis and Equipment	38
2.3.	Total subjects divided to the three group	39
2.4.	Components of DNA Extraction Kit	54
2.5.	Sequences of primers used for PCR amplification of Afamin and Peroxiredoxin 4 genes polymorphisms	59
2.6.	The starting PCR reaction ingredients concentration for each optimization processes of Afamin gene and peroxiredoxin 4 gene	59
2.7.	The program used for Afamin gene amplification sequence	60
2.8.	The program used for peroxiredoxin 4 gene amplification sequence	60
2.9.	The method of silver-staining	63
2.10.	Details of the procedure utilized to assess catalase activity	67
2.11.	The details of the method that used to measure prx activity when used hydrogen peroxide as the prx substrate	70
2.12.	The details of the method that used to measure prx activity when used t-BOOH as the prx substrate	71
3.1.	Patients clinical characteristics	72
3.2.	Total oxidant levels ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	74
3.3.	Total antioxidant levels ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	76
3.4.	Catalase activity (Katal/l) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	79
3.5.	Vitamin E (mg/dl) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	81
3.6.	Afamin ($\mu\text{g/ml}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	84
3.7.	Peroxiredoxin enzyme activity (U/L) was measured when using H ₂ O ₂ as the Prx substrate in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	86

List of Tables

3.8.	Peroxiredoxin enzyme activity (U/L) was measured when using t-BOOH as the Prx substrate in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	87
3.9.	Peroxiredoxin 4 ($\mu\text{g/ml}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	90
3.10.	malondialdehyde concentrations ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy	93
3.11.	The location and length of the 320 bp PCR amplicons utilized to amplify a segment of the "afamin gene on chromosome no. 4"	98
3.12.	In comparison to the NCBI reference sequences	102
3.13.	Response variable of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	103
3.14.	Allelic frequency of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	103
3.15.	genotype frequency of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	103
3.16.	Hardy-Weinberg equilibrium test of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	104
3.17.	Genotypic association of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	104
3.18.	Response variable of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	104
3.19.	Allelic frequency of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	105
3.20.	genotype frequency of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	105
3.21.	Hardy-Weinberg equilibrium test of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	105
3.22.	Genotypic association of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	105
3.23.	Biochemical characteristics of subject studied according to	106

List of Tables

	the afamin gene rs 35233345 genotype	
3.24.	Genotypic association of afamin polymorphisms for healthy controls ,Diabetic patients and Diabetic patients with nephropathy	107
3.25.	Response variable of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	108
3.26.	Allelic frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	109
3.27.	genotype frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	109
3.28.	Hardy-Weinberg equilibrium test of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	109
3.29.	Genotypic association of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls	109
3.30.	Response variable of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	110
3.31.	Allelic frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	110
3.32.	genotype frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	110
3.33.	Hardy-Weinberg equilibrium test of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	111
3.34.	Genotypic association of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients	111
3.35.	Biochemical characteristics of subject studied according to peroxiredoxin 4 genes Rs 564561 genotype	112
3.36.	Genotypic association of peroxiredoxin 4 polymorphisms for healthy controls ,Diabetic patients and Diabetic patients with nephropathy	113
3.37.	Correlation between incubation time and catalase activity	116
3.38.	The probable interference of selected chemicals on the assessment of catalase enzyme activity using the disubstituted	117

List of Tables

	quinone method	
3.39.	Precision of the disubstituted quinone method	117
3.40.	The statistical correlation between the catalase activity assessment that obtained by applied the disubstituted quinone method and the peroxovanadium method	118
3.41.	The recovery percentage of catalase enzyme activity after addition a suitable activities of catalase to enzymatic reaction solutions	119
3.42.	Comparison between the disubstituted quinone and peroxovanadate methods for the assessment of catalase activities (KU) in different types of bacteria	121
3.43.	The activity of peroxiredoxin enzyme measured using the ferrithiocyanate and ferriaminosalicylate protocols for tissues of a male albino rat	125
3.44.	Effect of interfering biomolecules on relative percentage errors during the measurement of prx activity, using the aminosalicylate protocol	126

1. Introduction

Diabetes mellitus (DM) has been one of the leading causes of death worldwide in the last century, becoming more urgent in recent decades due to the rapid growth in obesity (Glovaci *et al.* , 2019) . DM is a chronic condition marked by hyperglycemia and vasculature problems (macro and micro) (Ighodaro, 2018).

Currently, the rate of DM morbidity and mortality is on the rise, and by 2030 there will be more than half a billion people with DM (Shaw *et al.*, 2010). DM complications such as diabetic retinopathy, diabetic nephropathy, and (DN) is a most serious consequence of DM, and it's to the lead cause of chronic kidney failure beginning with norm albuminuria, micro albuminuria, macro albuminuria and ultimately leading to "end-stage renal disease" (ESRD) (Gross *et al.*, 2005). In addition, since the annual risk of developing (ESRD) among diabetic patients remains largely unknown around the world, a global atlas was created to compare the incidence of diabetes-related ESRD from 2000 to 2015 (Cheng *et al.* , 2021) . Complications due to diabetes can sometimes develop because of many influencing causes, such as diabetic microvasculopathy due to diabetic retinopathy or diabetic nephropathy (Sultana, 2020).

However, in type 2 diabetes (T2D), clinical markers for reliably separating diabetic nephropathy from non-diabetic renal disease are missing (Jiang *et al.* , 2020). It is Canada's leading cause of kidney failure (Ottawa, 2011). Neurological disorders may cause severe damage to brain functions and development (Foley *et al.* , 1998 ; Bell *et al.* , 2001). There are many types of renal problems in diabetes, some of them include diabetic nephropathy, myocardial nephropathy, hypertensive nephropathy, and other renal diseases due to unrelated diseases (Mazzucco *et al.* , 2002; Gambarara *et al.* , 1993) (Figure 1-1).

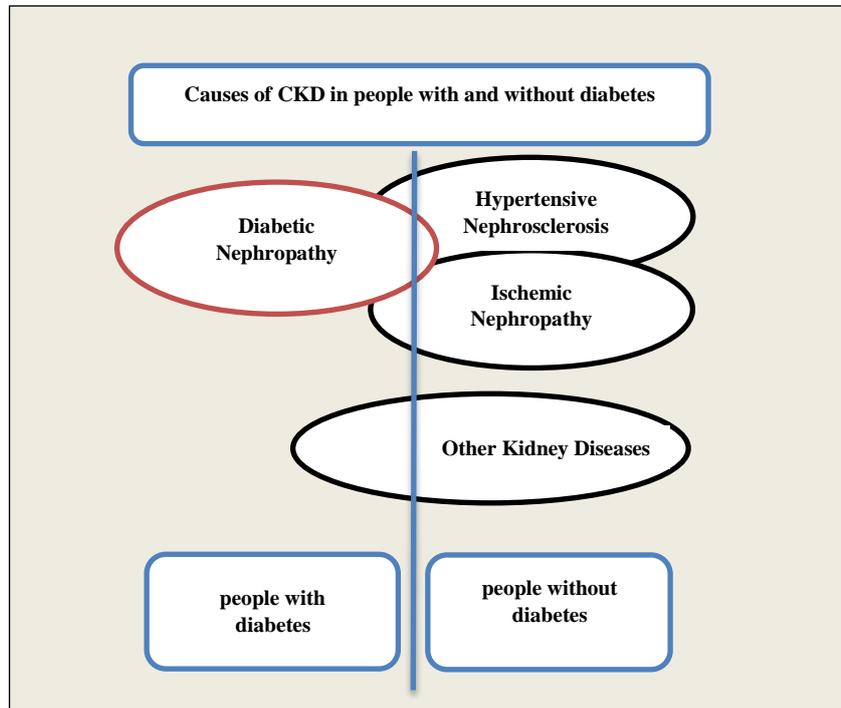


Figure (1-1): Describes the significance and causes of CKD for those with and without diabetes (Mazzucco *et al* ., 2002; Gambarara *et al* ., 1993).

1.1. Classification of Diabetes Mellitus

Diabetes can be divided into different categories, depending on a detailed clinical investigation.

1.1.1. Type (1) Diabetes

This type is a problem related to the defective secretion of insulin caused by damage to the β -cells of the pancreas.

1.1.1.1. immune-based diabetes (T1A)

The development of "Type 1A diabetes" (T1A) occurred due to T-cell destruction from the environment of the pancreas, which causes an autoimmune condition targeting tissue β -cells (Zheng *et al* ., 2018 ; Egan and Dinneen ,2019). In addition, exogenous destroying factors, such as pro - inflammatory cytokines or increased glucose, may induce an accumulating of damage-inducing reactive oxygen species in pancreatic β - cells during the pathogenesis of diabetes (Muralidharan *et al* ., 2021) .

Besides, other than immune therapy, beta-cell replacement therapy also should be considered, due to the presence of "necrotic and apoptotic" pathways (Herold *et al.*, 2013; Eizirik and Darville, 2001). Thus, stem cell techniques will be another option for treating people who suffer from diabetes (Melton, 2001).

1.1.1.2. Idiopathic Diabetes (T1B)

Type 1B diabetes, known as idiopathic, is known as type 1 diabetes with no autoimmune marker and where the causes of beta cell loss are unclear (ADA, 2009), idiopathic diabetes (T1B), It is minimal extreme, and occurs mostly in people with variegate levels of insulin deficiency of (African or Asian descent) between the intermittent episodes of African or Asian descent who have varying degrees of insulin deficiency between sporadic occurrences in the ketoacidosis (Abiru *et al.* , 2002).

1.1.1.3. Fulminant (type I) diabetes

Fulminant (type I) diabetes are a novel kind -1B diabetic subtype characterized by the sudden beginning of diabetic ketoacidosis, frequently associated with elevated serum pancreatic enzymes, the rapid development of a disease, and absence of autoantibodies linked to diabetes (Imagawa *et al.*,2000).It occurs in Japanese primarily with occasional occurrences recorded in other ethnicities (Taniyama *et al.*, 2004; Kawabata and Ikegami , 2020). Diabetes had been diagnosed as fulminant type 1 diabetes (Shiga *et al.* , 2018), there is the establishment of ketoacidosis soon after the hyperglycemic symptoms out from the previous day, glucose level 17.0 mmol / L (289 mg / dl) and HbA1c level b8.4 percent at the initial visit. There may also be other characteristics, such as gastrointestinal and cold-like symptom before the beginning, pregnancy, and pancreatic enzyme levels increase in serum (Hanafusa and Imagawa, 2007). Since the early development of micro vascular

complication has been described, special attention should be given to micro vascular complication (Sun *et al.*, 2021).

1.1.2. Type II Diabetes Mellitus (T2DM)

The primary cause of Type 2 diabetes is the intake of more calories than the person burns throughout the day (Zhao *et al.* , 2015) . T2DM or non-insulin-dependent diabetes is a heterogeneous disease produced by an interaction between environmental (modifiable) factors and genetics (no modifiable), reason as seen in this Figure (1-2) (De Rosa *et al.* , 2018) .

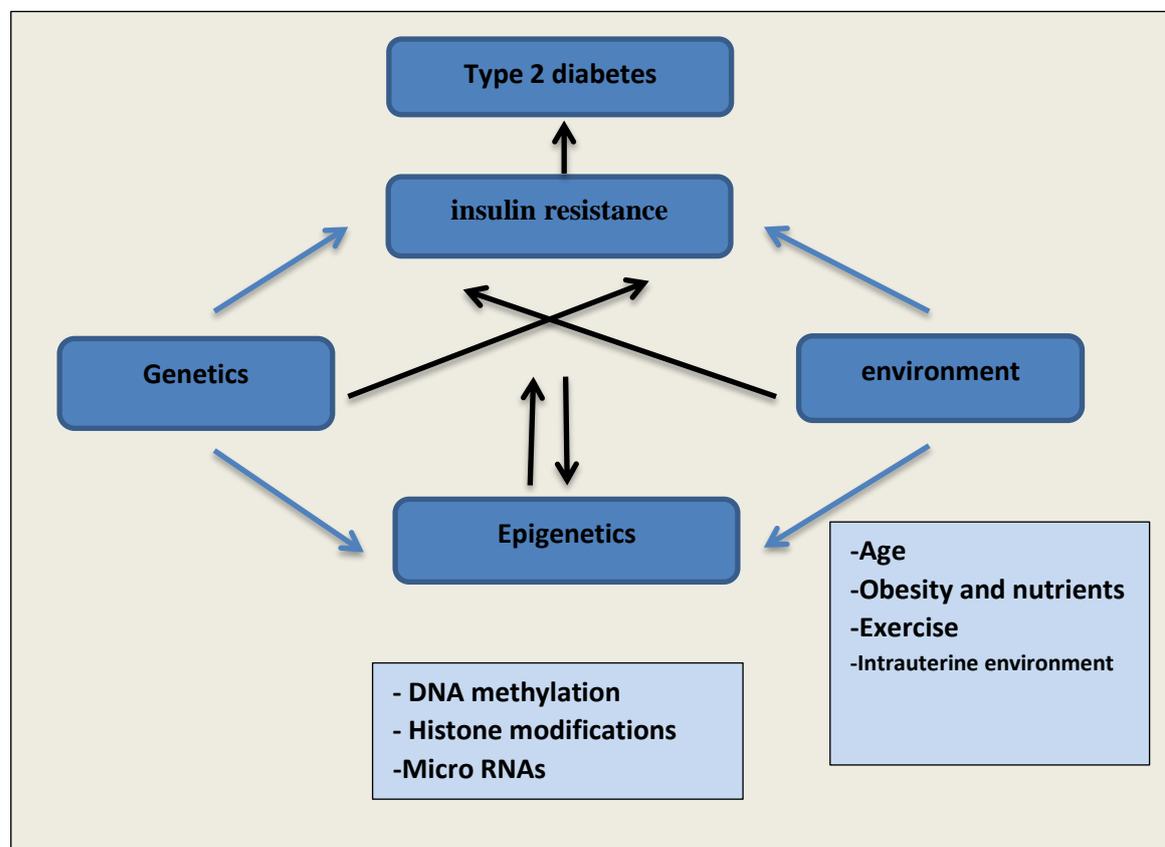


Figure (1-2): A model that proposes a role in pathogen of T2D for epigenetic mechanisms (De Rosa *et al.* , 2018).

The overall impact of this interaction will be insulin resistance and also will increase obesity and T2D, leading to the developed of metabolic syndrome (Ortega *et al.* , 2020; Sladek *et al.* , 2007; Zeggini *et al.* , 2007; Kahn *et al.* , 2006). Type 2 diabetes (T2D) is the most prevalent type of diabetes, accounting for more than 90% of cases worldwide (Sirdah and

Reading , 2020) . Also, Many individuals who suffer from overweight, due to insulin resistance, do not develop diabetes; they can sustain high blood glucose levels and compensate for their insulin resistance by increasing their insulin secretion (Mitsui *et al .* , 2006 ; Butler *et al .* , 2003). Normally, responding beta cells to insulin resistance, which takes place sometimes during disruptions in sleep and excessive anxiousness, leads to the greater secretion of insulin for the need of tissues (Santin and Eizirik,2013) . Hundreds of genetic variations linked to type 2 diabetes have been discovered in the last decade, many of which are associated with insulin secretion , However proof for single or cumulative impact on beta-cell activity, on the other hand, is based primarily on genetic linkage of the variants (Kettunen *et al .* , 2020).Therefore, the environmental risk factors are the primary etiology as well as treatment of T2D patients (Herold *et al .* , 2013) .

Two unique hypotheses for insulin destruction in type 2 diabetes address problems ranging from the weakening of organ systems due to glucose toxicity to the impact of lipotoxicity which decreases insulin secretion (Del Prato, 2009). Another hypothesis is the potentially high risk of pancreatic beta-amyloid accumulation in diabetics (Epstein *et al .* , 2000; Skog *et al .* , 2013). This higher risk is partly due to the heterogeneity of the underlying disease process in the Baltimore Longitudinal Study of Aging (Meigs *et al .* , 2003), it's concluded that post challenge hyperglycemia and fasting may represent Type II diabetes phenotypes (Tan *et al .* ,2010; Umpierrez *et al .* ,2006) . In a specific instance, the disease type ketosis-prone T2D, which also known as type 1.5 diabetes or diabetes type two complicated, is one of the more unusual examples of types of diabetes, a type 2 (Winter *et al .* , 1987; Mauvais *et al .* , 2004).

1.1.2.1. Genetics of Type II Diabetes

Type II diabetes is a difficult condition to manage, the development of this heterogeneous disease is caused by interactions between inherited variants and environmental signal (Cuschieri, 2019). Type II diabetes is strengthened by genetic linking in studies between monozygotic and dizygotic twins, and both genetic and environmental factors influence the different characteristics which contribute to a diabetic phenotype (β cell mass, insulin secretion, insulin action, and obesity) (Stumvoll *et al.*, 2005; Lee *et al.*, 2018). Type 2 diabetes can be explained as having multiple genetic influences, with some of these contributing to an increased likelihood of becoming type II diabetic (Melmed *et al.*, 2011). According to studies, about 72% of diabetes is genetically inherited (Willemsen *et al.*, 2015). Around 36 genes and 80 SNPs have been confirmed to contribute to an individual's risks of developing type II diabetes (Herder *et al.*, 2011; Fuchsberger *et al.*, 2016), these genes together do not account for more than 10 percent of the genetic heritability of the disease (Herder *et al.*, 2011). All of these factors have played a significant role in genetic susceptibility towards these kinds of diseases (Doria *et al.*, 2008). Therefore, physically-active activities improve insulin sensitivity, while high-fat diets and low fiber content lead to insulin resistance (Bonadonna, 2004), an association between type II diabetes and obesity, both conditions share susceptibility genes (Carmelli *et al.*, 1994).

1.1.3. Gestational Diabetes Mellitus (GDM)

Hyperglycemia in pregnancy is DM that appears during the resolve postpartum and pregnancy, it is important to distinguish for the gestational DM from type II diabetes, type I diabetes, polycystic ovary-related diabetes mellitus (DM), or LADA that was present before pregnancy if, whether (diagnosed or undiagnosed) (American Diabetes,

2014) . The incidence of Gestational Diabetes Mellitus is related to an individual being genetically predisposed to develop the condition and the body's less efficient response in pregnancy, the majority of those who have gestational diabetes have positive case status for abnormal b-cell function. There is a possibility that some of these women have a B-cell malfunction and they might become either T1DM or LADA patients (Lowe *et al .*, 2012) .

1.2. Diagnosis of Type 2 Diabetes

T2D was categorized on an international level by World Health Organization (WHO) as "a long-term condition which cannot be improved without medication"(WHO,2007). These changes are now adopted or promoted by numerous authorities including American Diabetes Association (ADA, 2018) , other more recent changes for diagnosis of diabetes include the change of cut point for FBG level from (141 mg/dl) to (124 mg/dl) and therefore , the introduction of non-"fasting plasma glucose" (FPG) measurement and addition of "HbA1c" as the diagnostic tool as follows:

- fasting blood glucose (FBG) - 140 mg/dL (7.0 mmol/L) or.
- two hour plasma glucose - 14.1 mmol/L (202 mg/dl) or
- HbA1c - 48 mmol/L (6.5%) or
- The diabetes symptom is observed along with "random plasma glucose" - 14.1 mmol/L (202 mg/dl).

1.2.1. Glycated Hemoglobin (HbA1c)

Glycated hemoglobin (HbA1c) is produced by the non - enzymatic glycation of hemoglobin, and more commonly used for the alternative tests for an identity of T2DM (Jelinek *et al .*, 2016). HbA1c is the reflection of the average glucose levels of the previous 2-3 months of your most recent blood profile(Vigersky and Going , 2019 ; Battelino *et al.*, 2019),which reflects the average length of RBC life (American

Diabetes,2015). Besides, HbA1c is an appropriate test for assessing a person's diabetes because of the following reasons. As HbA1c tests can be performed even without the help of tested subjects, these tests are easy and can be done at any time. The second important property of telepathy is its level of biological consistency (Lapolla *et al .*, 2011). Therefore, HbA1c is not influenced by sudden fluctuations of blood sugar level or psychological stress. The third, represents average "blood glucose levels" through the (three months) . Thus, glycated hemoglobin can be estimated approximate all 12 weeks to estimate whether the patient target for glycemic control has been preserved . The fourth epidemiology study terminated that the levels of glycated hemoglobin decreased by a percentage point , there is a 35% lowering in the risk of micro vascular complication, 25% lowering in diabetic associated deaths , 18% lower in combined "non-fatal and fatal myocardial infarctions" (Chin *et al .*, 2013) . The American Diabetes Association (ADA) in 2010, supported that HbA1 as a diagnostic standard for diabetic (ADA,2010).

1.3. Causes of Type II Diabetes Mellitus(T2DM)

Both genetics and environmental factors are responsible for diabetes type 2 development (Risérus *et al .*, 2009), obesity and diet are controllable factors for people, the increase in genetics, the "special situation" of the female gender, and old age are uncontrollable factors (Melmed *et al .*, 2011). In addition, low-income countries, such as Africa, type 2 diabetes mellitus is still a public health issue (Issaka *et al .*, 2018) .

It is believed that lack of sleep causes diabetes due to its effect on the metabolic rate (Touma *et al .*, 2011). There may be some interaction of fetal development on the nutritional status of the mother. In this case, the factors of DNA methylation are likely to play a role as well (Christian and Stewart, 2010). The intestinal bacteria *Bacteroides vulgatus* and *Prevotella copri* have been connected with T2D (Pedersen *et al .*, 2016).

1.4. Complications of Diabetes Mellitus

A complication of diabetes is due to retinopathy is a leading cause of visual disability and blindness (Sarwar *et al.* , 2010), the damage to the blood vessels in the retina is associated with diabetes mellitus, resulting in diseases such as glaucoma and cataract, which may be more common in people with diabetes than that in non-diabetics (O'Gara *et al.* , 2013). The major complications have been related long-term to damage to small blood vessels. Diabetics double the risk of macro vascular diseases and cardiovascular disease and approximately 75 percent of deaths in a diabetic are due to coronary artery disease and other macro vascular diseases include stroke and peripheral artery disease, diabetes is one of the most important causes of kidney failure in the developed world. But its incidence varies significantly across a population (Cukierman, 2005). Diabetic neuropathy, known as damage to the nerves of the body, is the common complication of diabetes, in addition to proximal diabetic neuropathy (DN), causes weakness and painful muscle atrophy (Yang *et al.* , 2016) .The complication of DM are represented in this Figure (1-3).

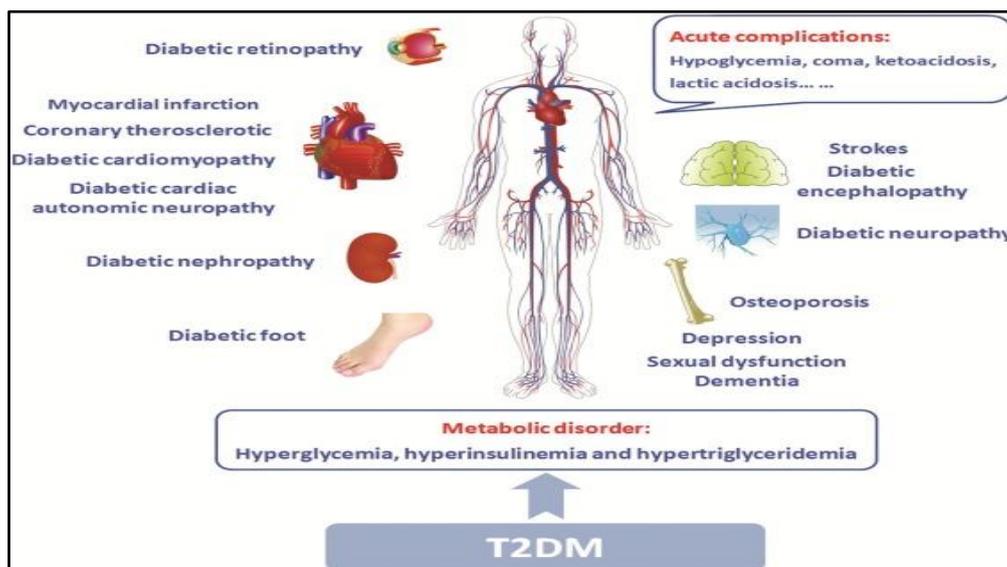


Figure (1-3): Diabetic Complication. (<https://pdb101.rcsb.org/global-health/diabetes-mellitus/monitoring/complications>)

1.4.1. Diabetic Nephropathy

"Diabetic nephropathy" (DN) is a chronic complications of all T1DM (beta-cell destruction absolute lack of insulin), and T2DM (insulin resistance or, decreased secretion of insulin) demonstrated in this Figure (1-4) (Pacifici *et al* 2014).

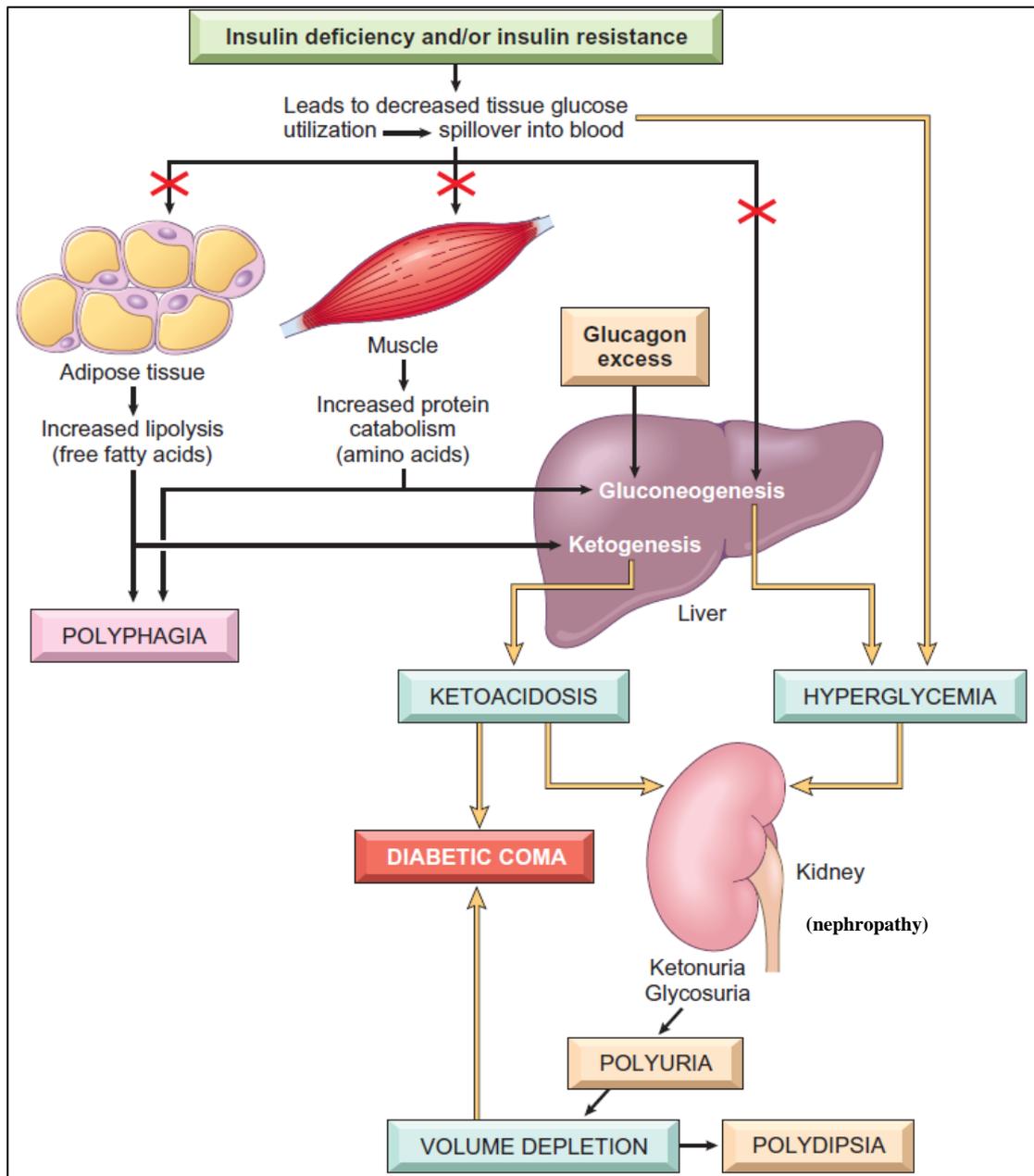


Figure (1-4): The processes that lead to specific clinical manifestations of diabetes. A normal insulin deficiency can lead to catabolic state, on the way that culminates in ketoacidosis. This effect can be extreme and result in "coma and eventual death" if untreated. Robbin and Cotran pathologic basis of diseases, 9th ed, Elsevier, Figure 24 – 33., p. 1114 .

Diabetic nephropathy, too known as inter capillary glomerulonephritis or "Kimmelstiel Wilson syndrome or nodular diabetic glomerulosclerosis" is a clinical syndromes characterized by albuminuria (>300 mg/day or >200 mcg/min) assured on at least two appropriateness (three - six) months apart, permanent and irreversible decrease in "glomerular filtration rate (GFR), and arterial hypertension" (Chen *et al* ., 2005). Synchronicity diagnosis was first observed by Clifford Wilson and Paul Kimmelstiel in 1936 (Styskal *et al* ., 2012). The progression of diabetic nephropathy can be categorized into five stages.

Stage I : In this particular stage. Hypertrophic hyper filtration involves either increasing glomerular filtration or maintaining the same degree. Stage I has lasted approximately 5 years , size of kidneys are increased approximate 20 percent, flow of renal plasma are increased by approximately 10 percent to 15 percent, while blood pressure stays normal, and albuminuria falls within normal limits.

Stage II : The tranquil stage of these disease start a little after the renal lesions appear, it's an early stage of damage to the kidney with an increase in mesangial neoplasm. There are yet do not have a signal of the disease, GFR is returned normal values. Many people remain in this stage of sickness or illness until they die.

Stage III : In the initial nephropathy or micro albuminuria stage, the amount of albumin measured (30-300 mg/dU) in the blood indicates the stage of glomerular damage. These changes are usually not detected before 5 to 10 years after the start of the disease. Approximately forty percent of the patients have identified these stage.

Stage IV : During this time. "Chronic kidney failure is irreversible". There is increased blood pressure, protein more than 300 mg/dU, and decreased kidney function.

Stage V : In the very late stage of renal failure, the glomerular filtration rate (GFR) is less than 20-30 ml/min/1.73 m². Some approximate fifty percent of patients such as renal failure require therapy of kidney replacement "hemodialysis, peritoneal dialysis" (Mogensen, 2008).

The description of diabetic nephropathy includes a progressive increase in albuminuria and an associated decrease in renal function among type 2 diabetic patients. Finally, lead to end-stage renal disease in this Figure (1-5) (de Boer *et al .*, 2007).

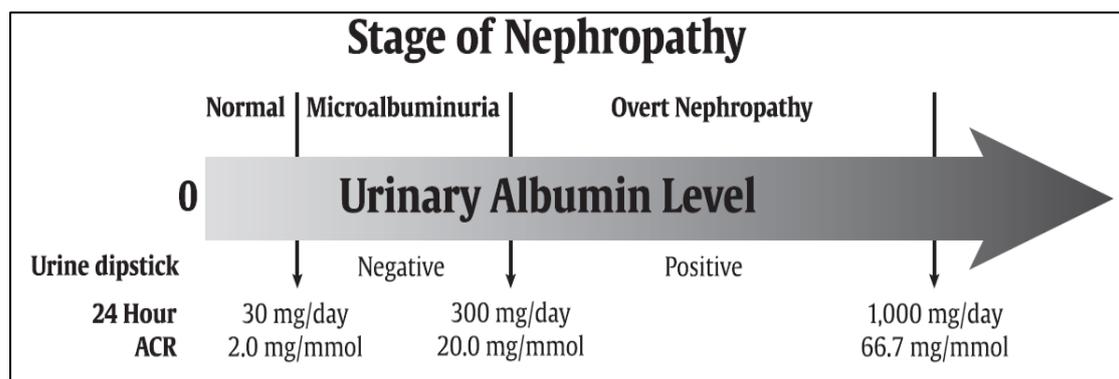


Figure (1-5): Various diagnostic measures to determine the presence and degree of (CKD) that interfere with kidney function. Urine Albumin to Creatinin Ratio (ACR): albumin to creatinin ratio; CKD: "chronic kidney disease". (de Boer *et al .*, 2007).

The risk factor includes a long period of diabetes: blood pressure, the non-optimal control of glycemic and plasma lipid levels: In regards to smoking, being overweight or diabetes, among many risk factors these are modifiable. Diabetes accounts for the first symptoms of diabetic nephropathy to come about as a result of low flow state that is known as the hyper filtration phase of the disease (MacIsaac *et al.*, 2014).

"Albuminuria" is considered the first clinical mark of (DN). At first, the amount of albumin is leaked, low than the detection onset of a urine dipstick (Molitch *et al .*, 2015). This stage has been referred to as micro albuminuria, Albuminuria will be worsening, so the amount of the

albumin excreted in urine will get too high that will be detectable by dipstick urinalysis, the details are shown in (Table 1-1) (McFarlane *et al* ., 2018).

Table (1-1): Stage of diabetic nephropathy (DN) by the level of urinary albumin levels.

Stage of Chronic Kidney Disease by Plasma Glomerular Filtration Rate.			
Stage of "nephropathy"	Urine dipstick for proteins	Urine ACR -mg/mmol	(24h)- urine collection for albumin
normal	negative	less than of 2	less than of 30mg/day
micro albuminuria	negative	2--20	30--300 mg/day
Over nephropathy	positive	more than of 20 less than of 67	more than of 300mg/day more than of 1,000mg/day

Kidney disease values are for albumin, not urinary protein, which will be higher than albumin levels. Higher than normal ACR values are unlikely if nephropathy were not present in the patient.

Albumin to creatinine ratio , ACR

1.4 .2. Micro albuminuria

Micro albuminuria is a major predictor of diabetic nephropathy and the spread of micro albuminuria in (T2DM) varies from (6.5% to 44%)(Satchell and Tooke, 2008 ; Bakris,2007). While, there is persuasive epidemiologic evidence that cardiovascular events and renal damage occur too soon in diabetes patients with micro albuminuria than those without it Reutens, 2013). So if the factors of micro albuminuria have been identified, they might clue provide to active intervention for not only advanced (DN) and micro albuminuria (Rawshani *et al* ., 2019). Therefore, it's well-known that destroyed β -cell function and insulin resistance is defect observed in type II diabetes (Egan *et al* ., 2020).

1.5 . Oxidative Stress

Oxidative stress reflects an imbalance between the systemic manifestations of the "reactive oxygen species" (ROS) and the biological system ability to readily detoxification the reactive intermediate or reform

the resulting harm, inhuman, Oxidative stress has been known to contribute to the formation of attention deficit hyperactivity disorder. (Joseph *et al* ., 2015), cancer (Halliwell and Barry, 2007), Parkinson's disease (Hwang ., 2013), laforae disease (Rome-Mateo, 2015). atherosclerosis (Valkeo *et al* .., 2007), heart failure (Bonomeni *et al* ., 2008), myocardial infarction (Raemond *et al* ., 2013; Dean *et al* ., 2011), sickle cell diseases (Amer *et al* ., 2006), lichen plan uses (Alyi *et al* ., 2010), vitiligo (Ariecan *et al* .., 2008), autism(Jamis *et al* ., 2004), chronic fatigue syndrome (CFS) (Kennidy *et al* ., 2005), and depression(Jimanez *et al* ., 2015), and seem to be characteristics of individual with Asperger's syndrome (Pareillada *et al* ., 2012) . Therefore, (ROS) can be advantageous, as they are used by immune systems as a way to attacks and kill pathogens demonstrated in Figure (1-6) (Segall, 2005; Gems and Partridge, 2008).

Oxygen is easily accepted for the production of ROS by free electrons produced under the normal aerobic metabolism of the cell, such as superoxide ($O_2^{\cdot-}$), singlet oxygen (Winteirbourn and Haimpton, 2008). The functions of reactive oxygen species in body are primarily dependent on the body's internal environment in which reactive oxygen species reside , including time, location, proximity, concentration, etc. (Auten and Davis, 2009). Through oxidative metabolism of the mitochondria and a component of utilized oxygen are reduced to H_2O , and the remaining oxygen is converted to oxygen free radicals which are an important ROS that is converted to other (RS) such as ($ONOO^{\cdot-}$), (OH) and hydrogen peroxide (Moussia , 2008).

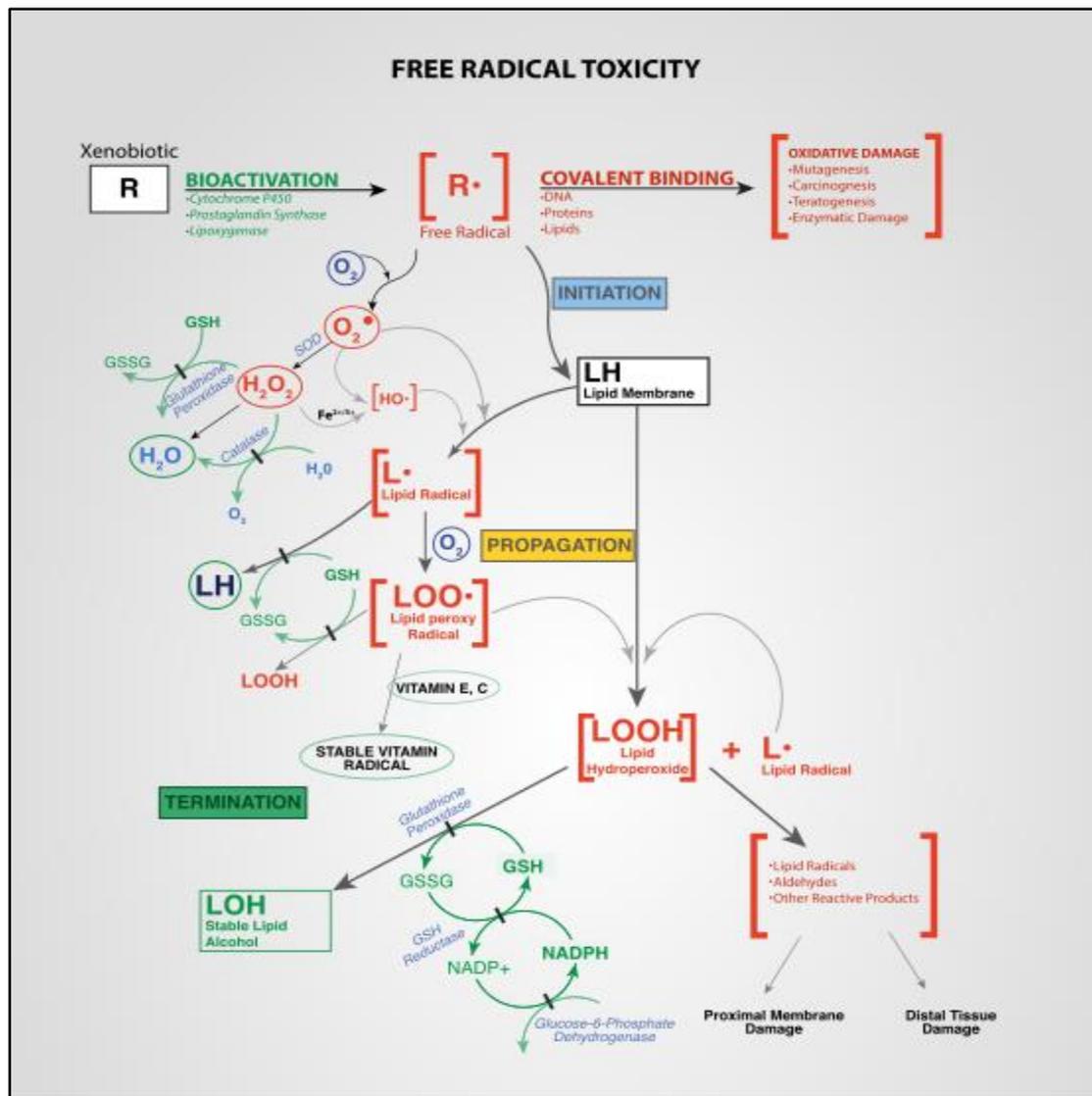


Figure (1-6):Oxidative stress mechanism involved in cell injury in tissue injury. Xenobiotic-mediated free radical formation and its subsequent removal (termination) (Gems and Partridge, 2008) .

1.6. Mechanisms of Production of ROS

Cells and organs in their normal aerobic metabolism are subjected to continuous exposure to oxidant, sources could be categorized into exogenous and endogenous due to the impact of both lifestyle on both sources.

1.6.1. Endogenous Sources of ROS

The reactive oxygen species can be generated from endogenous sources and the endogenous sources of reactive oxygen species involve

various cellular organs , wherever the oxygen consumptions are high such as peroxisomes, endoplasmic reticulum's and mitochondria.

1.6.1.1.Mitochondria

General , of the intracellular reactive oxygen species are produce from the mitochondria demonstrated in Figure (1-7), The superoxide radical is produced in the electron transfer chain at two main sites, namely complex I of "NADH dehydrogenase" and complex III of "ubiquinone cytochrome reductase ", the transport of an electron of complex I or complex II to ubiquinone – Q or coenzyme - Q result in the formation of reduced forms of coenzyme Q (QH₂), In the Q-cycle, the reduced forms of QH₂ regenerate coenzyme Q via an unstable semi-quinone intermediate anion ($\cdot\text{Q}^-$). Since the produced $\cdot\text{Q}^-$ transfers electrons immediately to molecular oxygen, it catalyzes the formation of superoxide radicals, leading to superoxide production (Ishii *et al.* , 2011).

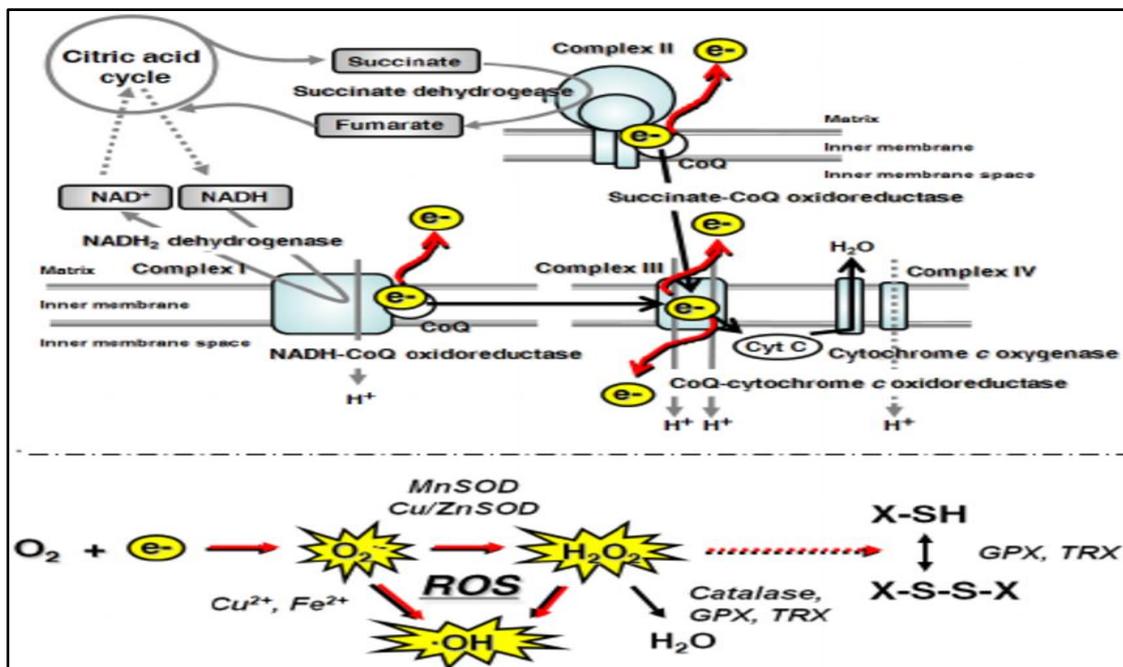


Figure (1-7) :Mitochondrial ROS production (Ishii *et al.* , 2011) .

1.6.1.2.Peroxisomes

In peroxisomes, the decomposition pathway in respiratory involve the transfer of electrons from different metabolites which produce hydrogen

peroxide (Duvee and Bauduhuin , 1966), but is not oxidized to result in ATP instead a byproduct is given off in the form of heat, Furthermore, there are other free radicals of peroxisome including (hydrogen peroxide, NO^{\bullet} , $\text{O}_2^{\bullet-}$ and OH^{\bullet} .The beta-oxidation of fatty acid is a major function of peroxisomes, the various peroxisome enzymes of urate oxidase, L- α -hydroxy oxidase, D-amino acid oxidase , and D-aspartate oxidase has all been shown to create various diverse of reactive oxygen species shown in Table (1-2) (Schrader and Fahimi , 2006).

Table (1-2): Reactive oxygen species producing enzymes are produced in peroxisomes (Schrader and Fahimi , 2006).

Enzymes	ROS	Substrate
Acyl CoA-oxidases (enzymes of β -oxidation)	H_2O_2	Fatty acids
D-amino acid oxidase	H_2O_2	d-proline
L- α -hydroxy oxidase	H_2O_2	Glycolate
Urate oxidase	H_2O_2	Uric acid
D-aspartate oxidase	H_2O_2	D-aspartate
Xanthine oxidase	$\text{O}_2^{\bullet-}$, H_2O_2	Xanthine

1.6.1.3.Endoplasmic Reticulum

The endoplasmic reticulum contains enzymes such as diamine oxidase and cytochrome p-450 that form harmful and toxic compounds, the most important of these is oxy radical that destructs biological tissue and operates as a carcinogen (Cheeseman and Slater 1993). As well there is another important thiol oxidase enzyme-like Erop1p which catalyzes molecular oxygen-based electron transfer resulting in the production of hydrogen peroxide (Gross *et al* .,2006).

There are several endogenous sources are include "auto-oxidation of adrenalin," "prostaglandin synthesis," "immune cells activation," "reduced riboflavin," "FMNH₂," "FADH₂," "immune cell activation," "cytochrome P 450," "inflammation," "ischemia," "aging," and so on, are also a source of reactive oxygen species (Cheeseman and Slater 1993) .

1.6.2. Exogenous Sources of ROS

The formation of ROS can be stimulated by a diversity of agents such as heavy metals and pollutants, smoke, drugs, radiation, or xenobiotic (Nachiappan *et al .*, 2010), the reactive oxygen species or ROSs is generated in the human body by different external sources shown in Table (1-3) (Pham *et al .*, 2008).

Table (1-3):Reactive oxygen species produced from the exogenous sources (Pham *et al .*, 2008).

water and Air pollution	Ultraviolet (UV)light
Alcohol	Cooking (used oil - smoked meat - fat) Drugs such as Halothene -Paracetamol, Bleomycine - Doxorubicin, Metronidazole - Ethanol. CCl ₄
Tobacco smoke	
Heavy metals - Cd-Pb- Hg - As	
Transition metals - Cu- Fe - Cr -Co	
Industrial solvents	
Pesticides	
High temperature	

1.7. Oxidative Stress in Diabetes Mellitus

In both the development and continuity of diabetes, oxidative stress plays a significant role (Ighodaro , 2018). Several studies indicate that oxidative stress is a major component of diabetes development and its related complications (Biró *et al .* , 2020), in addition, oxidative stress occurs when cells have a distorted redox balance, causing membrane harm and important biomolecule such as proteins , lipids, and DNA (Koltover and Skipa , 2021) , and development of oxidative stress compromises both the main mechanisms of failure that are impaired insulin action and secretion (Giacco and Brownlee , 2010). Experiments provided support for the direct effects of reactive oxygen species (ROS) on islet cells involved in insulin production causing beta-cell damage, inflammation, and autoimmunity (Prattichizzo *et al .* , 2018). In addition , hyperglycemia enhances oxidative stress during free radicals production and inhibition of the antioxidants defense system (Volpe *et al .* , 2018). In the chronic hyperglycemic condition, production of reactive oxygen species is continue , and in different tissues, enzymatic and non-enzymatic antioxidants are therefore strongly suppressed and oxidative stresses are further intensified (Rains and Jain , 2011) . Under normal conditions of physiological , the cellular process (involving glucose oxidation) results in the mitochondrial production of superoxide anion radicals (O_2^-), although the grid can deal with the body's antioxidant protection at levels (Yan *et al .* , 2020).

Therefore, in a hyperglycemic situation, there is overproduction of reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-) which results in the damages to the body's cell walls, including the blood-brain barrier and damage to nuclei (DNA)and other biomolecules (Robertson *et al* 2004 ; Rolo *et al .* , 2006) . As a result of DNA harm, a DNA repair enzymes, "poly-ADP-ribose polymerase-1

(PARP-1)" are activated, these enzymes poly-ADP-ribose polymerase-1 inhibits "GAPDH" , producing an increased level of glycolytic intermediate such as (fructose-6-P and glucose -6-P) as well as glucose, aggregations of this molecules in the cells induces other pro-oxidative pathway such as PKC and AGE pathway cause to increased levels of GAP; polyol and hexamine pathways due to increased level of fructose -6-P and glucose respective Figure (1-8) (Makino *et al .*, 2010 ; Styskal *et al .*, 2012). Besides, GAP accumulation can be causes free radical production that can lead to reactive oxygen species formation, thus increasing oxidative stress (Chung *et al .*, 2003), the auto-oxidation of glucose by itself in the absence of dietary factors can be initiated from glucose accumulation by cells. These products induce the production of glyoxal, and that is a source for advanced final products of glycation and often leads to oxidative cell stress demonstrated in this Figure (1-8) (Cho *et al .*,2007).

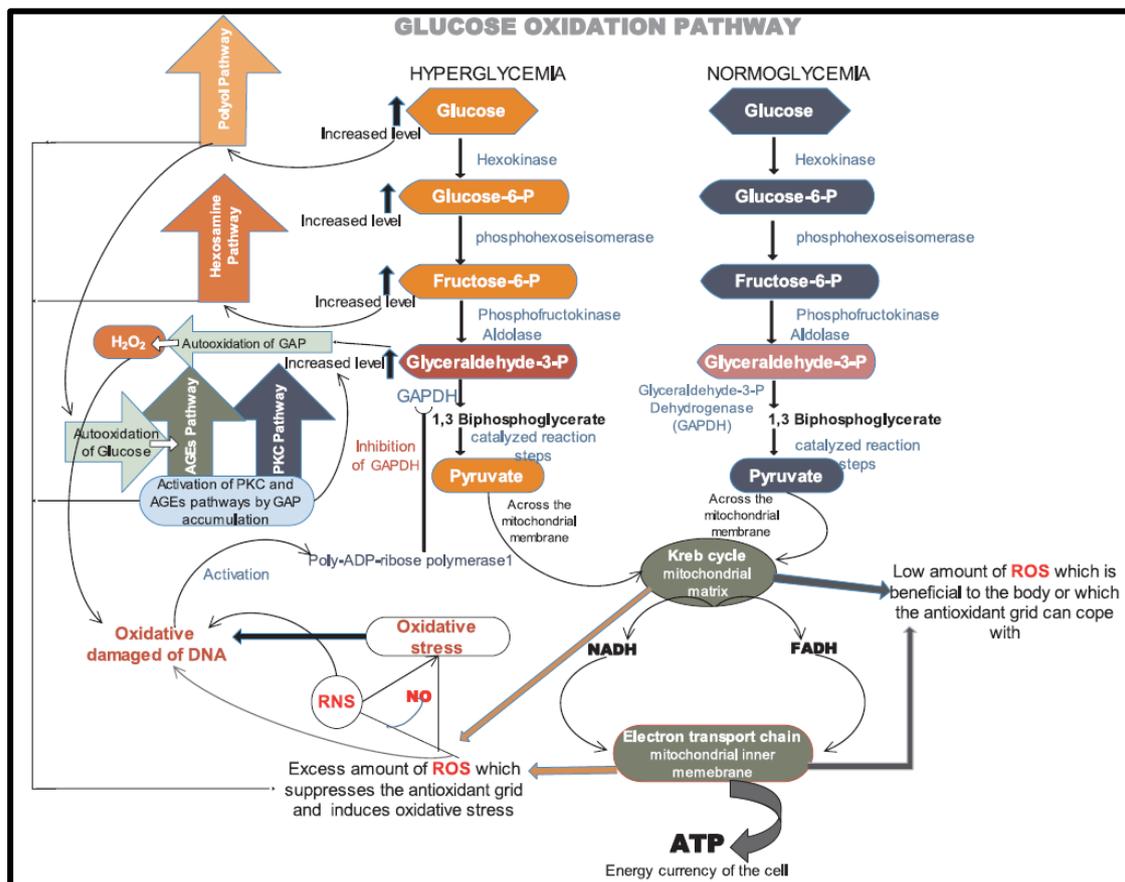


Figure (1-8): The oxidation of Glucose and stimulated oxidative stress in the hyperglycemic (Cho *et al* ., 2007) .

1.8. Classification of Antioxidants

Antioxidants are compounds that prevent oxidation, oxidations are a chemical reaction that can yield free radicals and lead to chain reactions that harm the cells of organisms (Jayedi *et al* ., 2018) . Antioxidants such as vitamin C , vitamin E, and thiols, terminate this chain reaction (Aune *et al* ., 2018) , and balance the oxidative stresses ,animals and plants maintain on the complex systems of overlapping antioxidant such as enzymes (e.g., superoxide dismutase and catalase) and glutathione, generated internally or the dietary antioxidant vitamin E and vitamin C (Rees *et al* ., 2013) . The defense system (ROS/RNS) consists of non-enzymatic and enzymatic antioxidants demonstrated in this Figure(1-9) (Misra *et al* ., 2014).

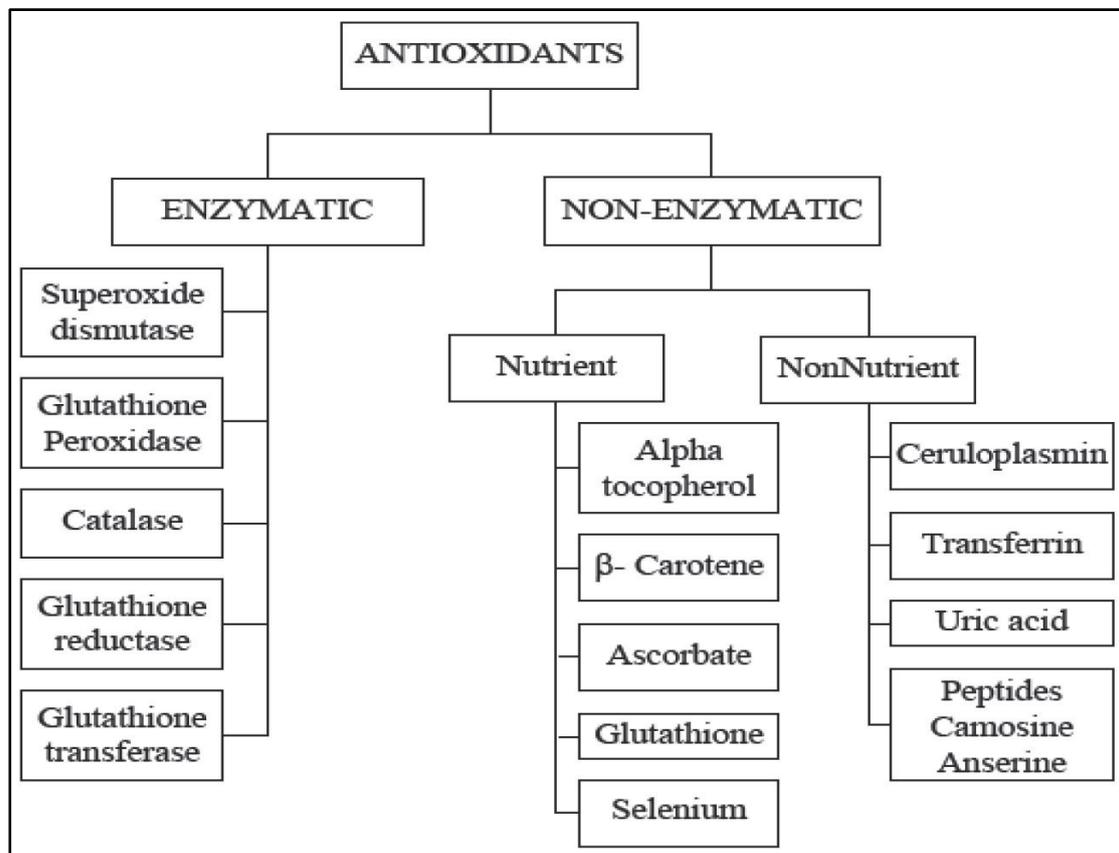


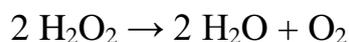
Figure (1-9) :Antioxidants classified as enzymatic and non-enzymatic (Misra *et al* ., 2014).

1.8.1. Enzymatic Antioxidant

An enzymatic antioxidant is uniquely generated in the human and can be divided into primary and secondary antioxidants, the primary antioxidant is involved (GPx) , (SOD) and (CAT) as described below, the secondary antioxidants include "glutathione reductase" and glucose-6-phosphate dehydrogenase, G6PDH generates "NADPH" .glutathione reductase (GR) is required to recycle the reduced (GSH) using NADPH and secondary enzyme glutathione reductase (Misra *et al* ., 2014).

1.8.1.1. Catalase

Catalase (EC 1.11.1.6) is an antioxidant enzyme , which exists in each living things such as animals, plants and bacteria. It accelerates the reaction of decaying hydrogen peroxide (H₂O₂) to oxygen and water (Karakus , 2020).



The catalase enzyme is responsible for the removal of hydrogen peroxide to a biologically inactive water molecule (Sharma *et al* ., 2012) . It is an important enzyme in protecting the cells oxidative stress by reactive oxygen species, catalase has one of the highest turnover numbers of each enzyme, Approximately one molecule of catalase can convert millions of H₂O₂ into oxygen and water all the second (Tasaki *et al* ., 2017) . Catalase enzyme is composed of four polypeptide chains of over 500 amino acids each (Hansberg *et al* ., 2012) . It has four iron-containing heme group that allows catalase to react with H₂O₂ , pH optimum for human catalase about 7 (Heit *et al* ., 2017).

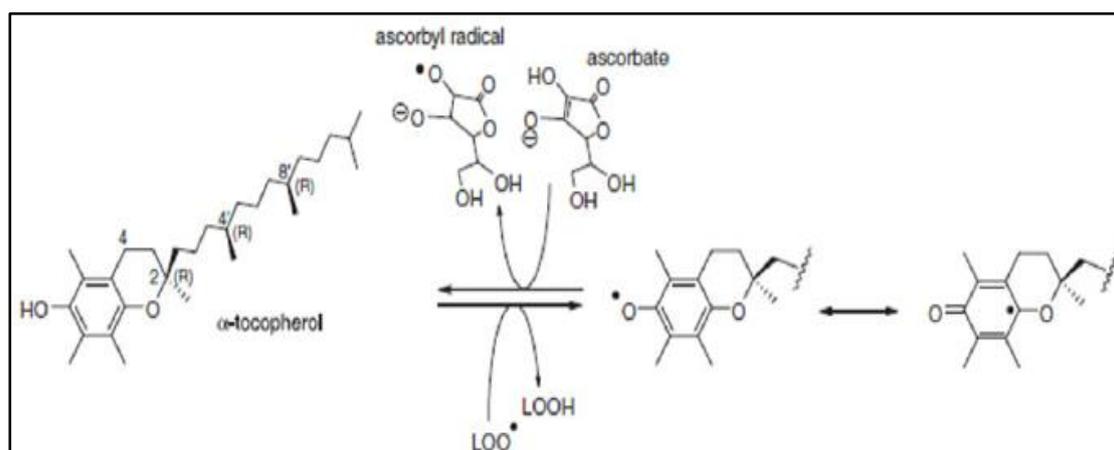
1.8.2. Non-enzymatic Antioxidants

Non-enzymatic antioxidants are class of the antioxidant, which are not found in the body natural but are required to be completed for the suitable

metabolism (Jakubczyk *et al.* , 2020) ,Some of the known non-enzymatic antioxidants are carotenoids, vitamins, minerals, polyphenols and other antioxidants in Figure (1-9) (Misra *et al.* , 2014).

1.8.2.1. Vitamin E

Vitamin E is a potent antioxidant that helps to protect the human from cell damage (Yang *et al.* , 2020; Handan *et al.*, 2007; Abdaella, 2009). Vitamin E rapidly transfers H-atom of phenolic to lipid peroxy radical and converting into vitamin E radical and lipid hydro peroxide (Bashir *et al.*, 2004) , Vitamin E, tocopherols 30% and tocotrienols 70% are provided antioxidant activity, that helps maintain of carotenoids and other qualities of oil stability (Nesma *et al.*, 2010). Vitamin E will protect "Poly Unsaturated Fatty Acid" found in cell membranes against lipid peroxidation because it scavenges intermediate of peroxy radical in lipid peroxidation (Khallouki *et al.* , 2020). In addition , tocopherols are considered as scavengers of lipid radical and ROS , tocopherol is considered as main antioxidants in bio membrane, its considered antioxidant for protection of membranes stability in lipid peroxidation demonstrated in this Figure (1-10) (Niki , 2014).



Figure(1-10): α -Tocopherol action as a radical-scavenging antioxidant against lipid peroxidation (Niki , 2014) .

Tocopherols has four isomers of (α -, β -, γ -, δ -) presence in the plants, α -tocopherols have high antioxidant activity caused to the found three groups of "methyl" in its molecular structure (Sarvajiet and Narindra, 2010). vitamin E has Protective roles (Shastri *et al* ., 2016) :

1. Scavengers of free radicals.
2. It maintains the immune functions and integrity of membranes.
3. It prevents the cancer cell's growth and differentiation.
4. Has roles in inhibition of mutagenic and formation of nitrosamine.
5. In cancer cells it prevents RNA ,DNA and protein synthesis.
6. "Cytotoxicity".

1.9. Peroxiredoxins

Peroxiredoxins are a family of antioxidant proteins, ubiquitous protected in a variety of living organisms from eukaryotes to prokaryotes (Kim and Jang , 2019), the molecular size of antioxidant enzymes about 20 to 30 k Da , they modulate biological function to prevent organism from oxidative stress, therefore regulation the intracellular signal transmit (Wood *et al* .., 2003; Sue *et al* ., 2001) . Based on numbers of a preserved cysteine residue , the member of the Prx family can be divided into six subtypes: Prx 1, Prx 2 and Prx 6 are found in the "cytosol", Prx 3 in the "mitochondrial matrix", Prx 4 in the "endoplasmic reticulum" and Prx 5 in "peroxisomes, mitochondria, and the cytosol".

It has been proven that at present, the makeup from 1 to more than 1 percent of the cellular protein and contributes to reducing 99 percent of "cytosolic peroxides" and also 90 percent of "mitochondrial peroxides" in humans (Winterbourn,2008).

In addition , there is an indication that the roles of Prx in antioxidants defense are more complex than the elimination of hydro peroxide, peroxiredoxins can be readily inactivated by H_2O_2 , which reduce peroxidase activation and therefore limiting their abilities to act as an

antioxidant (Yang *et al.* , 2002). Eukaryote peroxiredoxin is more exposed to oxidative stress inactivation by pathogenic microbial entities than is prokaryotic peroxiredoxin family members, suggesting a gain-of-function gene that has been selected by evolution (Mailloux , 2020). peroxiredoxin also has an oligomer structure that is affected by the redox of a protein (Barranco *et al.*, 2009; Zeida *et al.* , 2020). In addition, studies have suggested that peroxiredoxins work as redox sensors and regulate signaling activities in oxidative response (Fourquet *et al.*, 2008; Pastor-Flores *et al.* , 2020) , and serve as the primary H_2O_2 receptor, further interaction of the oxidized peroxiredoxin with other protein is main and determinant for the transmit of the signal demonstrated in this Figure (1-10) (Morinaka *et al.* , 2011).

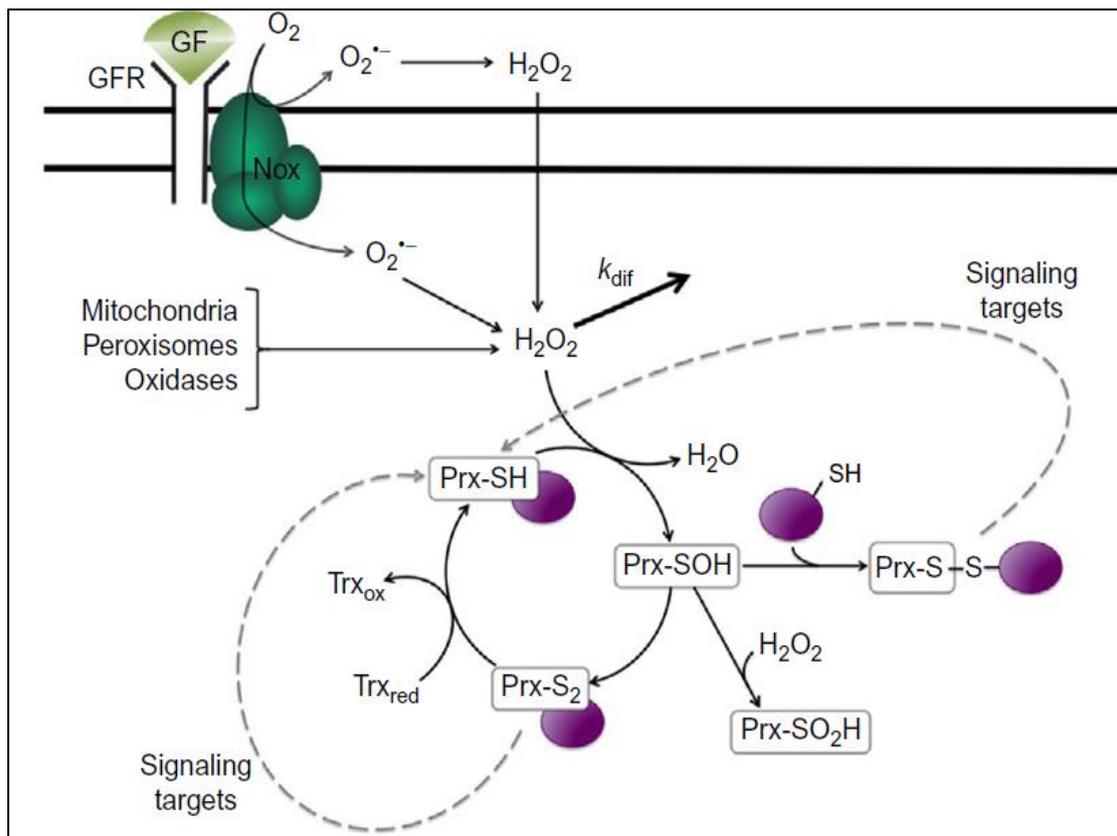


Figure (1-11) : H_2O_2 and 2-Cys Prxs in redox signaling (Morinaka *et al.* , 2011).

1.9.1. Peroxiredoxins 4

Peroxiredoxins 4 are a member of a peroxiredoxins family of antioxidant enzyme and it's localized to the cytoplasm and human peroxiredoxins 4 (PDB 2PN8) have Crystal structure demonstrated in this Figure (1-11) (Jin *et al.* , 1998). The peroxiredoxins family of protein scavenge H_2O_2 and plays role in the cellular response to the oxidative stress, intracellular signal transmit and peroxidases of the peroxiredoxins family reduce alkyl hydro peroxide and H_2O_2 to alcohol and water with the use of reduction equivalent derived from thiol containing donor molecule (Mishra *et al.* , 2013). Peroxiredoxins 4 are major localized in the endoplasmic reticulum and but are also present in the lysosome, cytosol, and nucleus (Jia *et al.* , 2019). The antioxidant of Prxs 4 may play a major role in the redox balance of the endoplasmic reticulum (Mishra *et al.* , 2013). The Cysteine residue of Prxs 4 is initially oxidized to sulfenic acid forms and then form an intermolecular disulfide bond with other Prxs molecules, that can be reversed by the reduction activity of the "thioredoxin-thioredoxin reductase system" by oxidative stress condition, in addition, the Cysteine of peroxiredoxins 4 are undergo more oxidation to forms of the sulfonic acid that can only be reduced by sulfiredoxin (Jeong *et al.*, 2012). The over oxidized form of peroxiredoxins 4 loses its antioxidant property but maybe functions as a molecular chaperone to simplify protein folding (Rhee and Woo, 2011; Zito *et al.*, 2010). Prxs 4 seem to have cytokine similar properties, as peroxiredoxin 4 exposure of the cell induces iNOS (NADPH oxidase) by receptors mediated mechanism (Haridas *et al.*, 1998). It also induces proliferations in fibroblast and activates "c - Jun N terminal kinase" and it can bind to "heparan sulphate", which is located on the cells surface, which Prxs 4 are anchored on extracellular surfaces, therefore its

functions are regulated by an extracellular signal such as redox status (Okado-Matsumoto *et al.*, 2000).

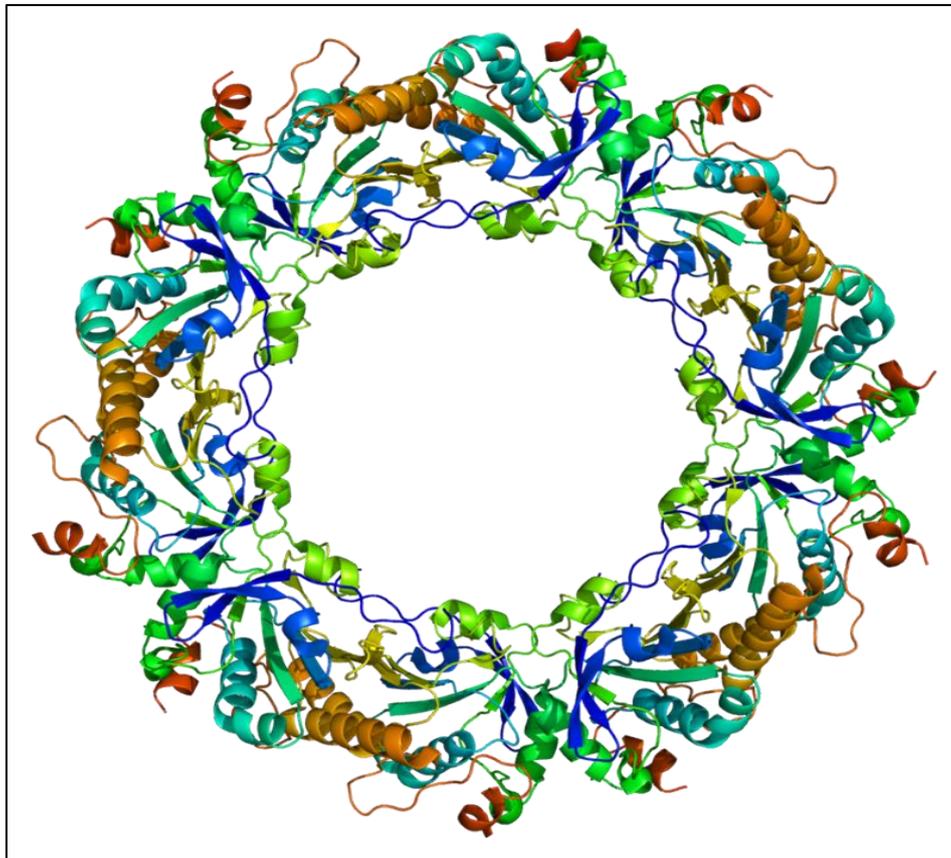


Figure (1-12) :Crystal structure of human peroxiredoxin 4 (PDB 2PN8).

1.9.1.1. Prx 4 Gene

Peroxiredoxins 4 are present in many organs and human tissues, being most prevalent in muscles, liver, blood vessels, ovary, and ovary, the peroxiredoxins 4 genes were first cloned in 1997 from the human HeLa S3 cDNA library (the proteins product of the gene were known as the antioxidants enzymes 372 (AOE372) at that time) (Jin *et al.* , 1997). Its protein which in human is encoded by the peroxiredoxin 4 gene, the proteins encoded by this gene are antioxidants enzymes of the peroxiredoxin family, these proteins have been found to play a regulatory role as a mediator in transcriptional signal transduction (NF-kappaB) (Jin *et al.* , 1998) . Human peroxiredoxins 4 genes are present on chromosome

X at (p22) location and transcription of peroxiredoxin 4 gene produce five various transcripts, the length (1005 bp) contain seven exons (Mishrae *et al.* , 2013). Peroxiredoxin 4 gene encodes (271) of the amino acid (A.a), it's may found in the biological systems of decameric and dimeric state, the found of decameric and dimeric of "peroxiredoxin 4" be redox-regulated (Wood *et al.*., 2002). Peroxiredoxin 4 occurs all within the cells in extracellular space, cleavable signal sequences are in the N-terminal region of peroxiredoxins 4 and are essential for peroxiredoxin 4 secretion, reduced peroxiredoxin 4 binds to the endothelial cells surface by interaction with "heparan sulfate ", oxidized peroxiredoxins 4 are incapable of this interacting possibly due of conformational change include the heparan sulfate - binding region (Okado *et al.* , 2000). Therefore, secreted peroxiredoxin 4 play a role in protection endothelial cell from extracellular ROS and function as membranes associated peroxidase demonstrated in this Figure (1-13) , In amino acids sequences, secreted mature peroxiredoxins 4 are a (27- k Da) processed protein forms, that lacks a 36 N - terminal amino acids residue encoded in mRNA, , the high molecular weight of peroxiredoxins 4 (31 k Da) are non-processed polypeptide, that is able of anchoring in cells membrane

via its N-terminal "hydrophobic region" (Sharapov *et al.* , 2014).

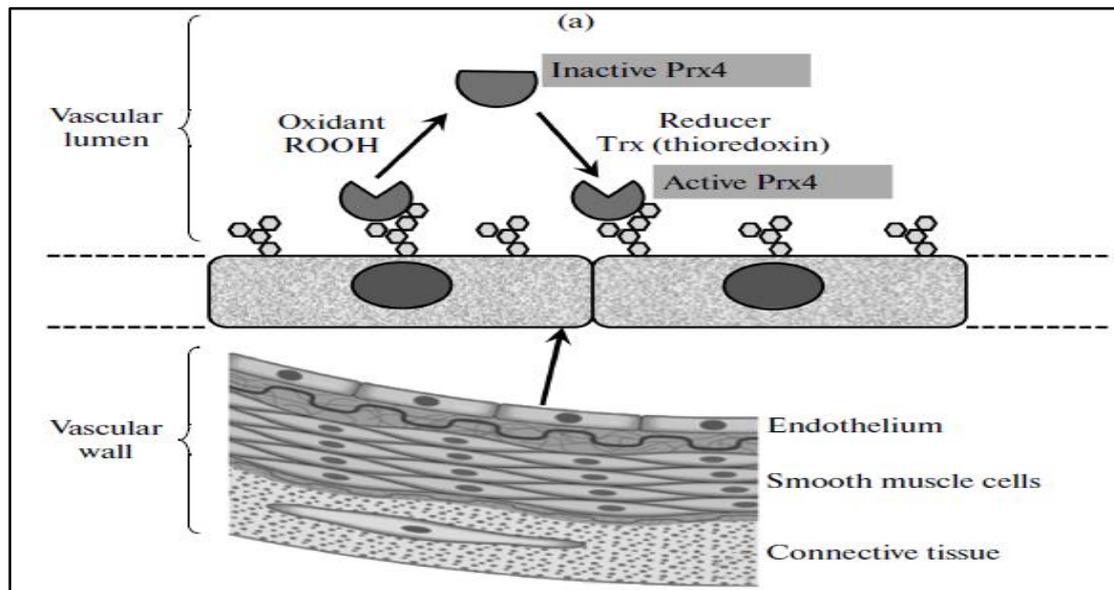


Figure (1-13) : Antioxidant role of Prx4. (a) The antioxidant role of Prx4 in the blood and the endothelial cell surface interaction of Prx4 (Sharapov *et al.* , 2014).

1.10. Lipid Peroxidation

Lipid peroxidations are considered as the most damage process known that membrane damaging in every living organism, lipid peroxidation is formed from polyunsaturated results of oxidative stresses (Yaman and Ayhanci , 2021) . Oxidative stresses damage cellular macromolecules including lipids, nucleic acids, and proteins, one particular target, the peroxidation of lipids, is especially more harmful due to its formation of lipid peroxidation (Breitenbach and Eckl , 2015; Zielinski and Pratt , 2017) .An attack on polyunsaturated fatty acids is initiated by the formation of carbon radicals by the abstraction of the hydrogen atom at one of the double bonds of the fatty acids (Wang, 2005). Oxidation of Lipid Peroxidation is the most extensively studied phenomenon of oxygen activation in biological, and most extensive aspect of oxygen activation in the study of PUFA is Lipid Peroxidation which is the process of “oxidative deterioration of PUFA” (Maneesh and

Jayalekshmi, 2006). Free radicals and lipid peroxidation can result in both "indirect" and "severe" damage to lipids by a chain reaction (Devasagayam *et al.*, 2004), Lipid peroxidation is a preliminary process followed by the producing of Malondialdehyde (MDA) demonstrated in Figure (1-14) (Niedworok and Bielaszka., 2007) , this is an end product that helps in determining, the amount of free radicals, genetic damage, lipid peroxidation, and is an indicator of oxidative stress (Atip *et al.*, 2010). It was shown to be a precise biomarker of lipid peroxidation (Adriano *et al.*, 2004). Malonaldehyde can react with free amino groups of nucleic acids, proteins and phospholipids which causes structural modification of these compounds, that "vitamin E" supplementation has been determined to be advantageous to the decrease of "malonaldehyde" concentration (Ashok and Sushil., 2005).

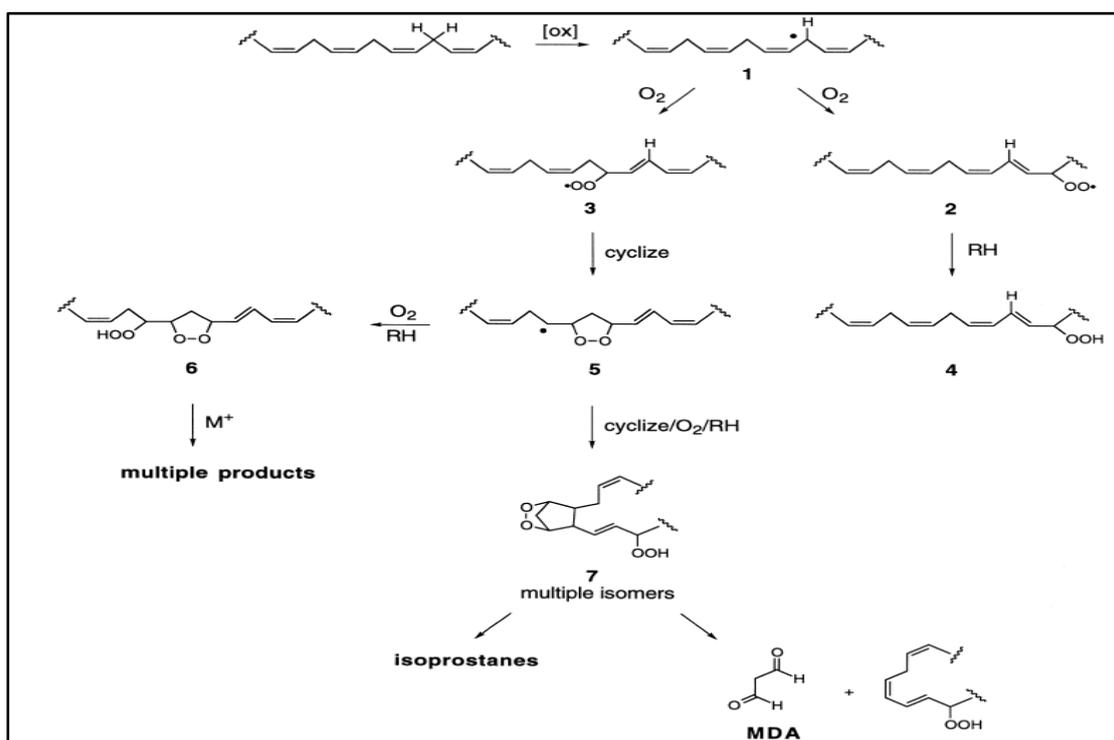


Figure (1-14) :Pathways of lipid peroxidation

1.11. Afamin

Afamin was first discovered as the fourth member of the human "albumin gene" family, also known as albumin family proteins, which

consists of human serum albumin, vitamin D-binding protein, and Alfa-fetoprotein (Schett *et al.* , 2006) , it's a glycoprotein 87-kDa (GPC87) is an 87-kDa mammalian glycoprotein encoded by chromosome 4 in humans that has 15 percent carbohydrate content and 55 percent amino acid composition and is considered similar to albumin. It is mainly expressed in the liver and eventually secreted into the circulation blood where it possibly acts as a transport protein for small, "hydrophobic molecule" (Aon *et al.* , 2015) . Also, based on studies have previously, specific binding vitamin E to afamin based on possible chemical binding properties (Flohé , 2016). Afamin has been thought to act as an antioxidant and thus protect the brain from high levels of oxidative stress (Filomeni *et al.* , 2002) . Even found in plasma at a concentration of 65mg/l, it can also be found in the blood-brain barrier, placenta, indicating a new role for afamin in neurological and fertile purposes (Funk and Cyrus , 2001).

1.11.1. Afamin Gene

All four genes in the albumin genes family are transcribed from a different region of "chromosomal 4q11–q22" are attached together in region "5'ALB–5'AFP–5'AFM–5'DBP3'-centromere" (Nishio *et al.* , 1996; Liu *et al.* , 2011). More recently, an additional 5th member of the albumin gene family have been discovered, situated adjacent to the 3' end of the afamin gene, but structurally attached to AFP, being named α -fetoprotein AF-LP-attached gene (ARG) (Naidu *et al.* , 2010). Nucleotide sequences were discovered in the afamin gene a distance of 24.454bp was first revealed by Nishio *et al.*, which was composed of at least fifteen exons, and was further divided into a locus composed of 14 introns (Nishio and Dugaiczky , 1996) . Notably, the homologous α -albumin gene and the α -fetoprotein (AFP) have widely (200 kb)

contiguous loci in the (4q) sub-centromeric regions of the human genome (Jin *et al.* , 2009).

1.11.2. Functional Characterization of "Afamin"

plasma afamin's were shown to be specificity binding proteins for high amounts of vitamin E (Kollerits *et al.* , 2017) , the radio ligand binding followed by Hill and Scatchard tests showed in vitro afamin binding affinity are two of the most important forms of vitamin E for each β -tocopherol and α -tocopherol, the maximum (18) binding site for vitamin E for each afamin molecule has been determined (Jin *et al.* , 2009). Therefore, afamin's were found to have a binding constant of 18 μ M indicating that afamin's might play roles as vitamin E in other body fluids (Dieplinger and Dieplinger , 2015) . In addition , studies show that Hill coefficients of 1.8 were found which signifies mutualness because incoming hydrophobic α -tocopherol molecules increased the hydrophobicity of the proteins–ligand complex thereby making this complex more accessible to follow-up ligands (Naidu *et al.* , 2010). Due to the large capacity of afamin for vitamin E supplementation may interfere with vitamin E transport in the body fluids under certain conditions, while the binding of vitamin E is also confirmed utilizing surface plasma on resonance technology with afamin immobilized on "carboxyl methyl dextran surface chips". (Fanali *et al.* , 2013). Previously studies, Calculation, modeling, and homology procedures were also performed on the designed tertiary motif of an afamin and demonstrated in Figure (1-15) (Jerkovic *et al.* , 2005).

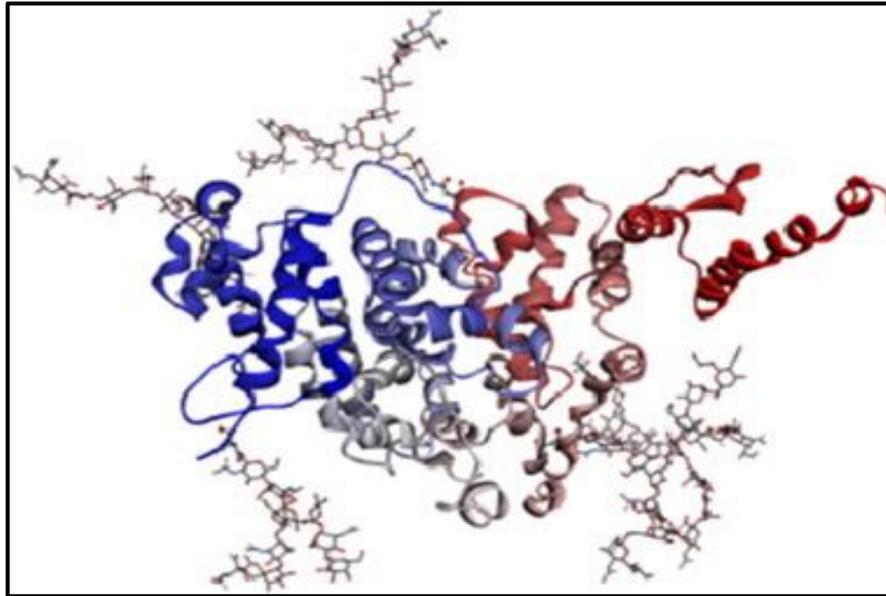


Figure (1-15) : Presentation and visual depiction of the afamin structural map

The images Above show the image blue of the N-terminal and red of the C-terminal poles colored while the putative glycan's images showing a ball-and-stick-like model (Jerkovic *et al .*, 2005).

1.11.3. Applications of "Afamin"

Various studies are in progress in look for the pathophysiological function of afamin in proteomics as a potential marker for different diseases (Hasan *et al .*, 2020). A recent study by international medical literature about the pathophysiological actions of afamin was confirmed, Through a quantitative immunoassay and immunoblotting for afamin in a population-based epidemiological study (Kronenberg *et al .*, 2014).

Previous studies, their analyses on the correlation between afamin mice and it is found to cause the same association in humans, one study of selected aspects of afamin's protein with on patients with afflicted of hypoglycemia and hyperlipidemia (Kratzer *et al .*, 2009). More study is needed to ascertain the function of the afamin gene in the development of cardiovascular diseases, including cardiovascular problems, particularly

those mediated by obesity and diabetes, and demonstrated in figure (1-16) (Dieplinger and Dieplinger, 2015).

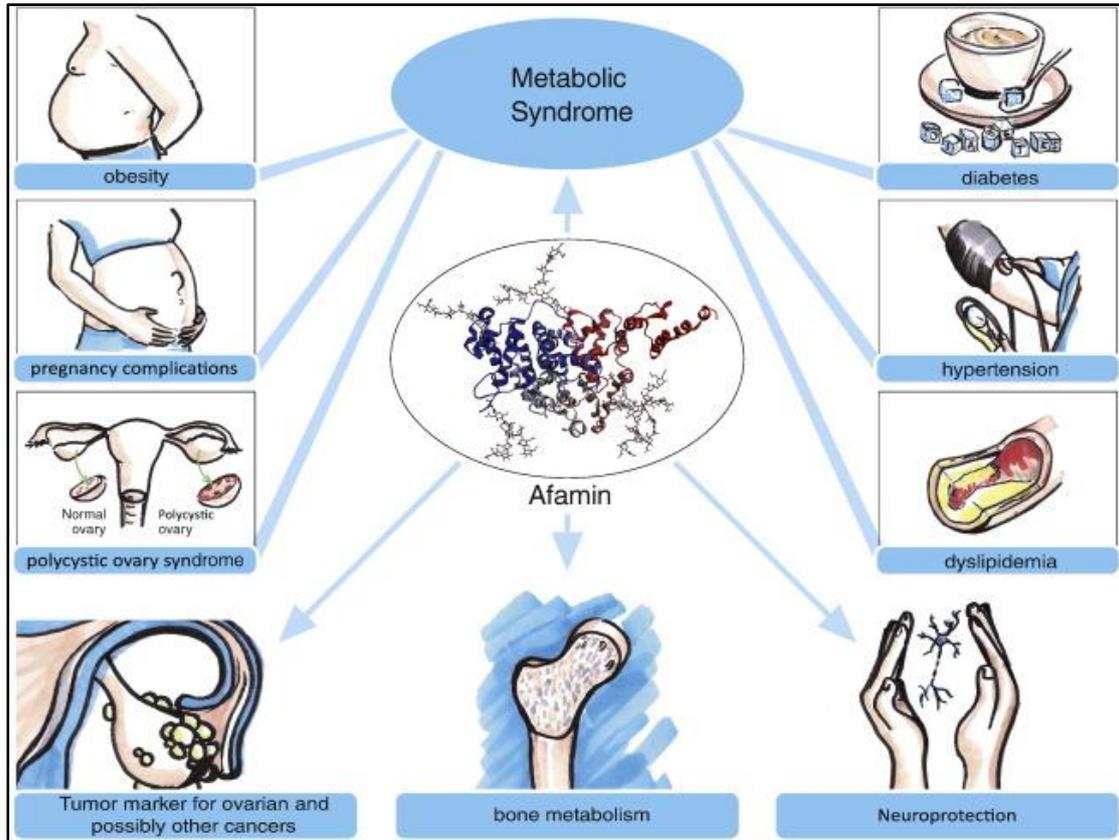


Figure (1-16) Patho-physiological functions of "afamin"

1.12.Aim of the study:

This study aims to :

A) Study the levels of the following parameters and the association with diabetic nephropathy :

- Assessment peroxiredoxin 4 activity and afamin levels.
- Assessment total antioxidant capacity (TAC), total reactive oxidant status (ROS) , vitamin E and lipid peroxidation levels .
- Measure total peroxiredoxin activity.
- Assessment hydrogen peroxide scavenging enzyme activity (Catalase activity).
- development a novel precise methods for assessment of catalase activity and peroxiredoxin activity and compared it with ordinary methods.

B) Study the peroxiredoxin 4 and afamin gene polymorphisms relationship with diabetes mellitus and compared it with that of healthy control

2. Materials and Chemicals

2.1. Materials

2.1.1. Chemicals

All chemicals were used as being supplied without further purification.

Table 2-1 chemicals used in the present study

Chemicals	Purity %	Supplied company
100 bp DNA ladder	-----	Bioneer (Korea)
1,4-dithio-threitol	98	Sigma
Thiobarbituric acid	99.0	BDH
Sodium hydroxide	99.0	BDH
Sodium acetate	99.0	BDH
α,α' -dipyridyl	99.0	BDH
Ferric chloride hexahydrate	99.0	BDH
Ammonium ferrous sulfate	99	Sigma
Catalase enzyme powder	-----	Hi Media India
α -tocopherol	99.0	BDH
SDS	99.0	BDH
Hydrochloric acid	99.0	BDH
Tri chloroacetate	99.0	BDH
Hydroquinone	99.0	BDH
Anilinium sulfate	99.0	BDH

Ammonium molybdate	99.0	BDH
Aminosalicylic acid	99.0	BDH
Potassium thiocyanate	99.0	BDH
DNA Extraction kit	-----	Favorgen Biotech, Taiwan
Absolute ethanol	99.98	Fluka
Propanol	99.8	Fluka
Ethylene diamine tetra acetic di- hydrate (EDTA).2 H ₂ O	99.5	Fluka
Ferrous ammonium sulfate	98	Sigma
Glycerol	99.0	Sigma chemicals
H ₂ SO ₄	98	BDH
Hydrogen peroxide	----	Fluka
KH ₂ PO ₄	99.0	Sigma chemicals
K ₂ HPO ₄	99.0	Sigma chemicals
Methanol	99.8	Fluka
Na ₂ HPO ₄	99.0	BDH
NaH ₂ PO ₄	99.0	BDH
Sodium chloride	98	Sigma
O-dianisidine dihydrochloride	95	Sigma
PCR Master Mix Kit	-----	Promega, USA
Peroxiredoxin 4 ELISA Kit	-----	Elabscience Biotechnology Inc.
Xylene		

Afamin ELISA Kit	-----	Elabscience Biotechnology Inc.
Sodium azide	99	Sigma
Sorbitol	99	Sigma
HEPES buffer	-----	Promega, USA
Tert- butylhydroperoxide	98	Sigma
acetic acid	99.0	BDH
Uric acid	97	Sigma
sodium benzoate	99.0	BDH
xylenol orange	98.0	Sigma chemicals

2.1.2. Instrument analysis and equipment

Table (2.2): Instrument Analysis and Equipment

Instrument	Supplied company
Centrifuge	Heraeus (Germany)
Centrifuge, Cooling Centrifuge	Hettich / Germany
Deep Freez	GFL / Germany
Horizontal Gel electrophoresis unit	Cleaver scientific / UK
Magnetic stirrer	Gallin kamp (England)
Micro-centrifuge	Hettich / Germany
Microplate reader	BioTek (USA)
Oven	Hearson (England)
PCR(Biometra)	Germany

pH meter	Jenway (Germany)
Photo-documentation	Cleaver Scientific / UK
Sensitive balance	Stanton 461 AN (Germany)
Spectrophotometer	Shimadzu 1800 spectrophotometer
Vortex mixer	Karlkole (Germany)
Water bath	Karlkole (Germany)

2.2. Methodologies

2.2.1. Collection of Blood and Serum Preparation

The blood samples were drawn from patients and control subjects. 5ml of blood were drawn from each participant by vein puncture, two ml was placed into EDTA-tubes and the residual 3ml was put into disposable tubes. Blood in the EDTA tubes was stored in - 20°C in order to be used later in genetic part of the study, while blood in the gel containing tubes was centrifuged at $3000 \times g$ for approximately 10 minutes then the sera were obtained stored at -20°C until analysis.

This study included 300 subjects with T2DM [100 Diabetic patients without nephropathy (male) and 100 Diabetic patients with nephropathy (male)] and (100) volunteers . The subjects under study were classified as shown in Table (2-3) :

Table (2- 3) : Total subjects divided to the three group.

The group symbols	Explanation	Numbers
G ₁	It means the control subjects (male).	100
G ₂	It means the diabetic patients without nephropathy (male).	100

G3	It means the diabetic patients with nephropathy (male).	100
----	---	-----

The study has also included a questionnaire about the criteria : Type II diabetes, Males, 30th to 60th average age, History and family history of diabetes mellitus, Types of treatment and drugs if use, Fasting blood sugar test .

Table (2- 4): The selection of patients in this study

Included	Excluded
Type II diabetes	Type I diabetes
Males	Females
30th to 60th average age	Insulin injection depended patient
History and family history	Smoking or alcoholic patient
Types of treatment and drugs if use	Hepatitis and thyroid disorders
Fasting blood sugar test	All types of cancer

2.2.2. Total Oxidant Status

2.2.2.1. Principle:

The TOS of serum was determined using a novel method that described by Erel. Oxidants found in the sample acts to oxidize the ferrous ion – O - dianisidine complex to ferric ion O- dianisidine. The glycerol molecule acts to enhance the oxidation reaction that is richly found in the reaction solution. Ferric ion reacts with xylenol orange to create a colored complex in an acidic medium. The color intensity was measured spectrophotometrically at 570 nm, which is correlated with the total amount of oxidant molecules that found in the measured sample. The standard curve was drawn using hydrogen peroxide and the outcome are expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2\text{Eq/L}$) (Erel , 2005).

2.2.2.2. Assay Reagents

Reagent 1 was prepared by dissolving 3.17 g of O-dianisidine dihydrochloride and 1.96 g of ferrous ammonium sulfate in 1000 mL of H₂SO₄ solution, 25 mM. The ending reagent was composed of 10 mM O-dianisidine dihydrochloride and 5 mM ferrous ammonium sulfate. This reagent is stable for as a minimum six months at 4°C.

Reagent 2 was prepared by dissolving 8.18 g of NaCl and 114 mg of xylenol orange in 900 mL of 25 mM H₂SO₄ solution. The solution was completed to 1L by **add one hundred milliliters of glycerol**. The final concentrations of components in the reagent are 150µM xylenol orange, 140 mM NaCl and **1.35 M glycerol**. The pH value of the equilibrium reagent was 1.75. The stability of the reagent is reaching to 6 months at 4°C.

Hydrogen peroxide (100 µmol/L) was standardized daily and freshly diluted depending upon a molar extinction coefficient at 240 nm, which equal to 43.6 M⁻¹ cm⁻¹.

2.2.2.3. Procedure

	Blank	Standard	Sample
Distilled water	50 µl	-----	-----
Serum	-----	-----	50 µl
Hydrogen peroxide	-----	50 µl	-----
R1	2 ml	2 ml	2 ml
Test tubes were mixed by vortex, and then add:			
R2	2 ml	2 ml	2 ml

Quietly mix the content of each tube after addition, allow standing at room temperature for 3 minute, read spectrophotometrically at 560 nm.

$$\text{Total oxidants status} = \frac{A.\text{test}}{A.\text{STD}} * \text{Conc.of STD}$$

2.2.3. Total Antioxidant Activity Assay

2.2.3.1. Principle :

A standardized solution of Fe–EDTA complex reacts with hydrogen peroxide by a Fenton type reaction, leading to the formation of hydroxyl radicals . These reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of human fluid cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically and the inhibition of color development defined as the AOA (Gutteridge *et al .*, 1990; Yamazaki and Piette , 1990).

2.2.3.2. Reagent Preparation

1- Sodium phosphate buffer (100 mmol/litre, pH 7.4) :This buffer was prepared by dissolving 16.394 g in 100 ml of distilled water.

2- sodium benzoate (10 mmol/litre) was prepared by dissolving 1.441 g in 100 ml of distilled water.

3- NaOH (50 mmol/litre) was prepared by dissolving 1.999 g in 100 ml of distilled water.

4- (Ethylene diamin tetra acetic acid) EDTA 2 mmol/litre was prepared by dissolving 584.48 mg in 100 ml of distilled water, in phosphate buffer (solution 1)

5- Fe(NH₄)₂SO₄ (2 mmol/litre) was prepared by dissolving 568.09 mg in 100 ml of distilled water.

6- Fe–EDTA complex (prepared freshly by mixing equal volumes of solutions 4 and 5, left to stand 60 minutes at room temperature)

7- H₂O₂ (10 mmol/litre) was prepared by dissolving 340.147 mg in 100 ml of distilled water.

8- acetic acid: 20%

9- thiobarbituric acid (TBA): 0.8% (wt/vol) in 50 mmol/litre NaOH was prepared by dissolving 1.999 g in 100 ml of distilled water

10- uric acid (1 mmol/litre) was prepared by dissolving 168.11 mg in 100 ml of distilled water, with NaOH (5 mmol/litre) was prepared by dissolving 199.98 mg in 100 ml of distilled water.

Solutions 4–9 should be prepared immediately before use. The sodium phosphate buffer and sodium benzoate should be kept in a refrigerator (0–4°C) and the uric acid solution in a deep freeze (–20 to –30°C).

2.2.3.3. Procedure

Each sample (A_1) should have its own control (A_0 , sample blank) in which the Fe–EDTA mixture and H_2O_2 should be added after 20% acetic acid. For each series of analysis a negative control (K_0 and K_1) should be prepared (at least in triplicate), containing the same reagents as A_1 or A_0 , except that serum (or other human fluid) is replaced with phosphate buffer. Standards containing 1 mmol/litre uric acid (UA_1 and UA_0) are used for calibration.

	A_1	A_0	K_1	K_0	UA_1	UA_0
Serum	10 μ l	10 μ l	-----	-----	-----	-----
Uric acid	-----	-----	-----	-----	10 μ L	10 μ L
Buffer	490 μ l	490 μ l	500 μ l	500 μ l	490 μ l	490 μ l
Na-benzoate	500 μ l					
Acetic acid	-----	1000 μ l	-----	1000 μ l	-----	1000 μ l
Fe-EDTA	200 μ l					
H_2O_2	200 μ l					
Incubate for 60 minutes at 37°C, then add:						
Acetic acid	1000 μ l	-----	1000 μ l	-----	1000 μ l	-----

TBA	1000 μ l					
-----	--------------	--------------	--------------	--------------	--------------	--------------

2.2.3.4. Calculation

Antioxidant activity should be calculated as follows:

$$\text{AOA (mmol/litre)} = (\text{CUA}) \times (\text{K} - \text{A}) / (\text{K} - \text{UA})$$

where

K = absorbance of control ($K_1 - K_0$)

A = absorbance of sample ($A_1 - A_0$)

UA = absorbance of uric acid solution = ($UA_1 - UA_0$)

CUA = concentration of uric acid (in mmol/ litre).

2.2.4. Assay of Total Tocopherol (vitamin E) in Plasma:

2.2.4.1. Principle

Plasma total tocopherol was assayed by the method of Quaife et al. (Quaife *et al.*, 1949), It involves the Emmerie- Engel color reaction with ferric chloride and α, α' -dipyridyl to give a red color. As in equation below:

2.2.4.2. Reagents

1- absolute ethanol.

2- α, α' -dipyridyl was prepared by dissolving 0.120 gm of α, α' -dipyridyl in 100 ml of n-propyl alcohol.

3- Ferric chloride hexahydrate was prepared by dissolving 0.120 gm of Ferric chloride hexahydrate in 100 ml of absolute ethanol. this solution was kept in a dark brown or red glass bottle.

4- α -tocopherol standard ($1 \mu\text{mol/L}$) was prepared by dissolving 2.0 mg of α -tocopherol in 100 ml of absolute ethanol.

2.2.4.3. Procedure

Reagents	Test	STD	Blank
Absolute ethanol	0.6 ml	0.6 ml	0.6 ml
Sample	0.6 ml	-----	-----

D.W.	-----	-----	0.6 ml
STD	-----	0.6 ml	-----
Xylene	0.6 ml	0.6 ml	0.6 ml
Mixed well and centrifuged for 10 min at 3000 rpm.			
xylene supernatant layer	0.4 ml	0.4 ml	0.4 ml
α,α' -dipyridyl was added and vortexed	0.4 ml	0.4 ml	0.4 ml
The 0.6 ml of this mixture was then pipetted into a cuvette and the absorption was measured spectrophotometrically at 460 nm against deionized water.			
ferric chloride	0.13 ml	0.13 ml	0.13 ml
Mixed thoroughly and absorption was again read at 520 nm spectrophotometrically exactly 1.5 min after addition of ferric chloride.			

2.2.4.4. Calculation

$$\text{Conc. of test} = \frac{(A_{520} - 0.29 A_{460})_{\text{test}}}{A_{520\text{STD}}} * \text{Conc. of STD}$$

2.2.5. Determination of Serum AFM (Afamin) Activity (Lequin , 2005; Engvall and Perlmann , 1972).

2.2.5.1.Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human AFM. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human AFM and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human AFM, biotinylated detection antibody and Avidin-HRP conjugate will appear blue

in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The OD value is proportional to the concentration of Human AFM. You can calculate the concentration of Human AFM in the samples by comparing the OD of the samples to the standard curve.

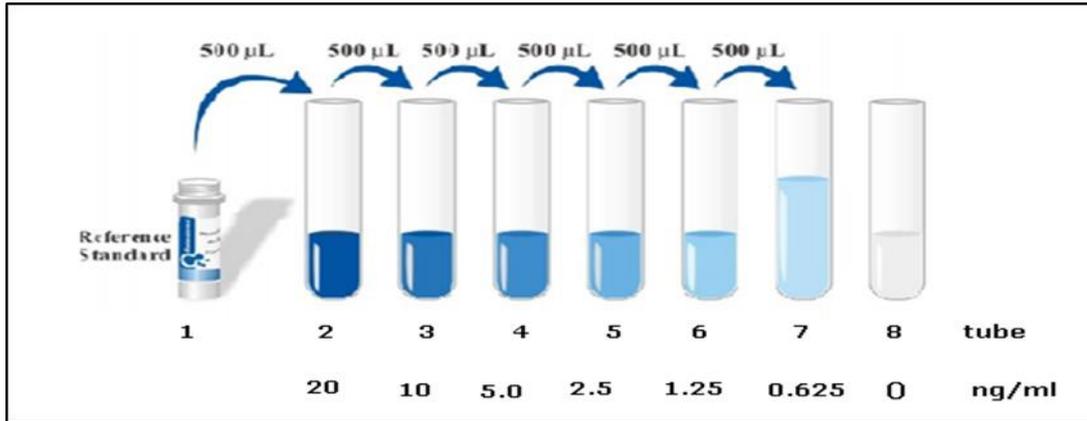
2.2.5.2. Preparation of Reagents

The reagents were brought to room temperature (18-25 °C) immediately before use.

Wash Buffer - Diluted 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, it in a 40°C water bath and mixed it gently until the crystals have completely dissolved.

Standard – Centrifuge the standard at $10,000 \times g$ for 1 min. was added 1.0 mL of Reference Standard & Sample Diluent, let it stand for 10 min and it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 200ng/ml. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 200 , 100 , 50 , 25 , 12.5, 6.25 ,3.13 , 0 ng/ml.

Dilution method: Take 7 EP tubes, add 500uL of Reference Standard and Sample Diluent to each tube. Pipette 500uL of the 200ng/mL working solution to the first tube and mix up to produce a 100ng/mL working solution. Pipette 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



2.2.5.3. Assay procedure:

All solutions were brought to room temperature before use.

1. the **Standard working solution was added** to the first two columns: Each concentration of the solution was added in duplicate, to one well each, side by side (100 µL for each well). the samples were added to the other wells (100 µL for each well). Covered the plate with the sealer provided in the kit. Incubate for 90 min at 37°C.
2. Removed the liquid out of each well, **don't wash** . 100 µl of **Biotinylated Detection Ab working solution was added** to each well. Covered with the Plate sealer. The plate was spread with sealer and incubated for 1 hour at 37° c.
3. **Washed**: 350 µl of **buffer was added** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeated this wash step 3 times. Note: a micro plate washer can be used in this step and other wash steps.
4. 100 µl of **HRP Conjugate working solution was added** to each well. The plate sealer was incubated for 30 min at 37°C.
5. **Washed**: The wash process was repeated for five times as directed in step3.

6. 90 μl of **Substrate Reagent was added** to each well. Covered with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.

7. **Stopped:** 50 μl of stop solution was added for every well. At that point, the shading swings to yellow quickly. The request to include stop arrangement ought to be the same as the substrate arrangement.

8. Absorbance Determination: estimate the absorbance for every well at 450 nm.

2.2.5.4. Calculation

By using the suitable equation that resulted from the plotted the standard curve, all the measurements were calculated.

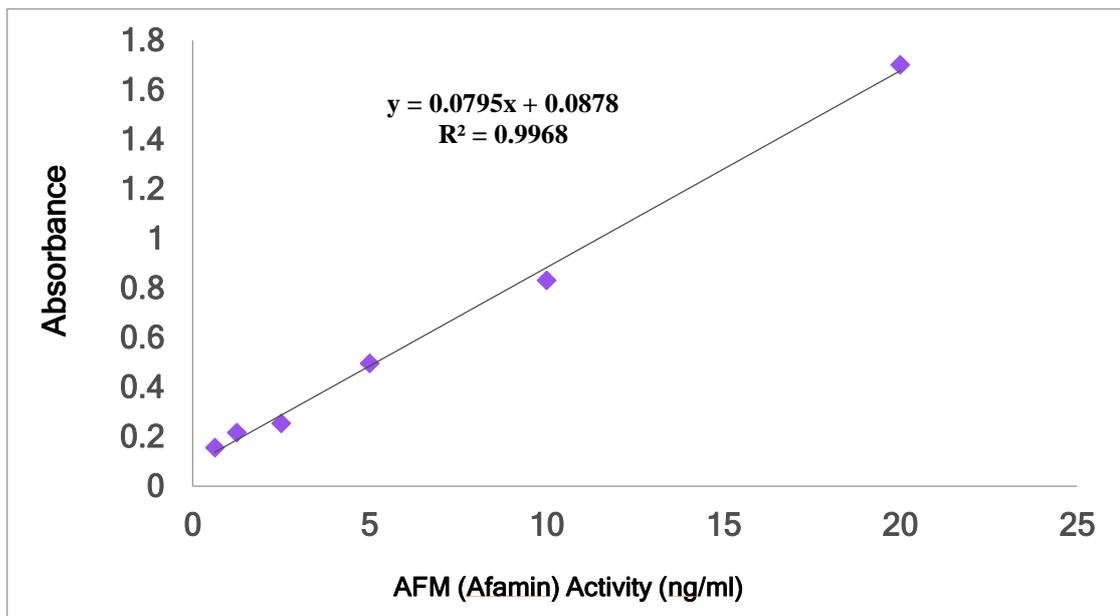


Figure (2-1): The standard curve of AFM (Afamin) concentration.

2.2.6. Determination of Serum peroxiredoxin 4 Activity :

PRX-4 activity was assessed by using ELISA Kit (Elabscience Biotechnology Inc) (Lequin , 2005; Engvall and Perlmann , 1972).

2.2.6.1. Principle

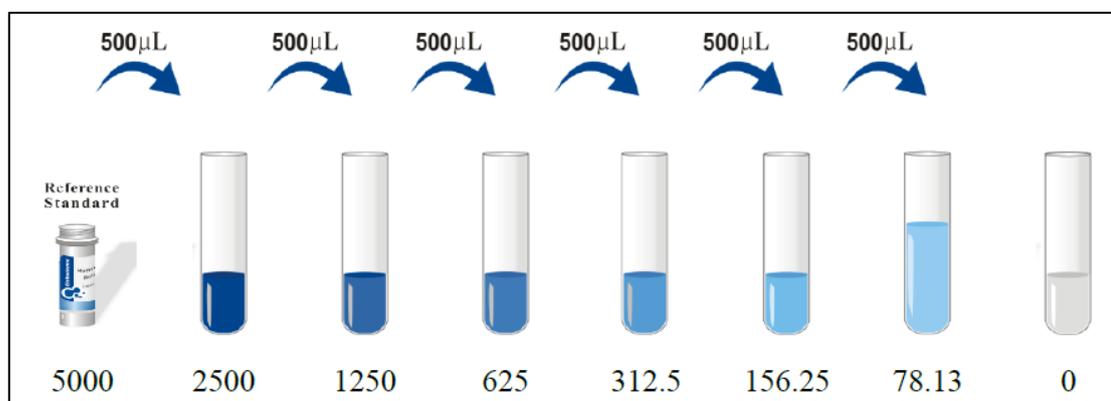
This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human PRDX4. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human PRDX4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human PRDX4, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of $450 \text{ nm} \pm 2 \text{ nm}$. The OD value is proportional to the concentration of Human PRDX4. You can calculate the concentration of Human PRDX4 in the samples by comparing the OD of the samples to the standard curve.

2.2.6.2. Preparation of Reagents

1. all reagents to room temperature ($18\sim 25^{\circ}\text{C}$) before use. the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.
2. **Wash Buffer**- 30 mL of Concentrated Wash Buffer was diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40°C water bath and mix it gently until the crystals have completely dissolved.
3. **Standard working solution** - the standard at $10,000\times\text{g}$ for 1 min. was added 1.0 mL of Reference Standard and Sample Diluent, it stand for 10 min and it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 5000pg/

μl . Then serial dilutions as needed. The recommended dilution gradient is as follows: 5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 0 pg/ml .

Dilution method: Taken 7 EP tubes, **was added** 500 μL of Reference Standard and Sample Diluent to each tube. Pipette 500 μL of the 5000 pg/mL working solution to the first tube and mixed up to produce a 2500 pg/mL working solution. 500 μL of the solution from the former tube into the latter one according to this step. The illustration below is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube.



4. **Biotinylated Detection Ab working solution** - the required amount before the experiment (100 μl /well) was Calculated. In preparation, slightly more than calculate should be prepared. Centrifuge the stock tube before use, dilute the 100 \times Concentrated Biotinylated Detection Ab to 1 \times working solution with Biotinylated Detection Ab Diluent.

5. **Concentrated HRP Conjugate working solution** - the required amount before the experiment (100 μl /well) was Calculated. In preparation ,slightly more than calculated should be prepared. Dilute the 100 \times Concentrated HRP Conjugate to 1 \times working solution with Concentrated HRP Conjugate Diluent.

2.2.6.3. Assay procedure

All solutions were brought to room temperature before use.

1. **Standard working solution was added** to the first two columns: Each concentration of the solution was added in duplicate, to one well each, side by side (100 μ l for each well). Added the samples to the other wells (100 μ l for each well). Covered the plate with the sealer provided in the kit. Incubate for 90 min at 37°C.
2. Removed the liquid out of each well, **don't wash** . 100 μ l of **Biotinylated Detection Ab working solution was added** to each well. Covered with the Plate sealer. The plate was spread with sealer and incubated for 1 hour at 37° c.
3. **Washed**: 350 μ l of **buffer was added** to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeated this wash step 3 times. Note: a micro plate washer can be used in this step and other wash steps.
4. 100 μ l of **HRP Conjugate working solution was added** to each well. The plate sealer was incubated for 30 min at 37°C.
5. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 3.
6. 90 μ l of **Substrate Reagent was added** to each well. Covered with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
7. Stopped: 50 μ l of stop solution was added for every well. At that point, the shading swings to yellow quickly. The request to include stop arrangement ought to be the same as the substrate arrangement.
8. Absorbance Determination: estimate the absorbance for every well at 450 nm.

2.2.6.4. Calculation

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

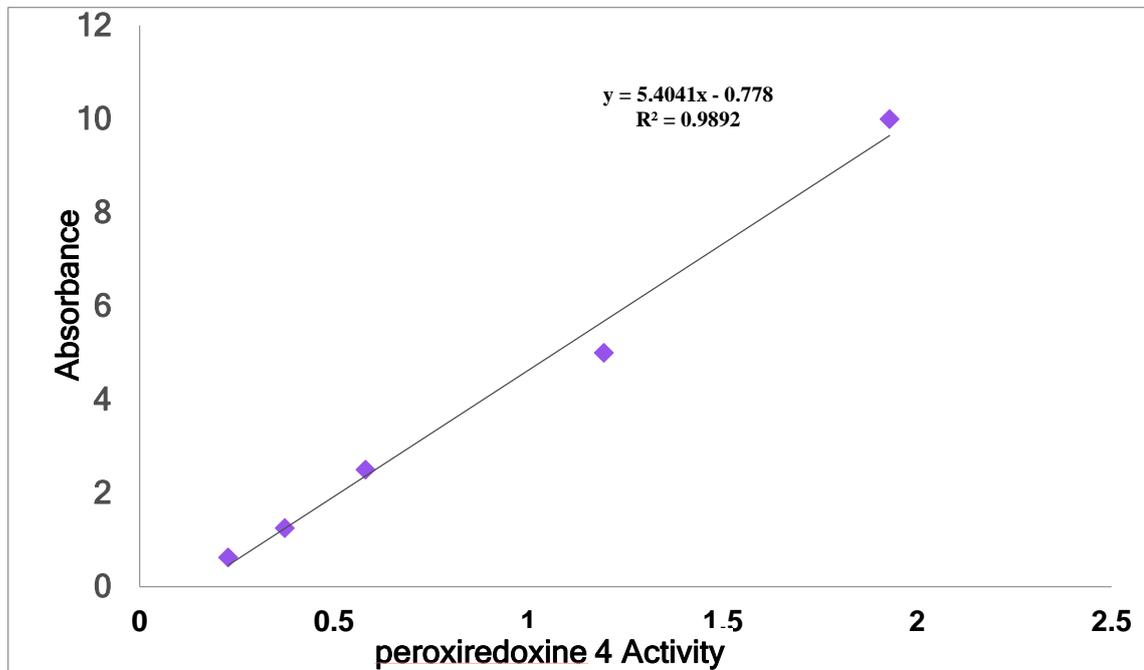


Figure (2- 2): The standard curve of Peroxiredoxin 4 concentration

2.2.7. Determination of Serum Lipid Peroxidation:

2.2.7.1. principle

Lipid peroxidation in sera was evaluated by thiobarbituric acid reative substances (TBARS). TBARS test gives a basic, reproducible, and standardized tool for measuring lipid peroxidation in serum. The MDA-TBA adduct designed by the response of MDA and 1,3-Diethyl-2-thiobarbituric acid (DETBA) under high temperature (90-100°C) at acidic

conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 515 nm and an emission wavelength of 555 nm. This reaction has a much higher sensitivity when measured fluorometrically (Jo *et al.* , 1998; Yagi , 1976; Kei , 1978; Ohkawa *et al.* , 1979; Hegazy , 2011) .

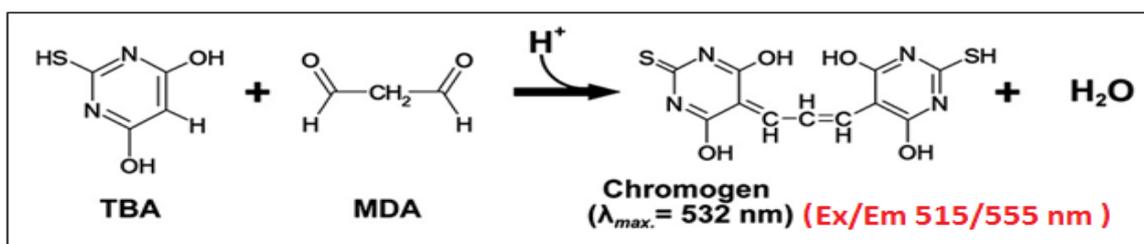


Figure (2- 3): Scheme of the adduct MDA–(TBA)₂.

2.2.7.2. Reagents

1. SDS
2. 1 M HCl
3. 1,1,3,3-tetramethoxypropane
4. TCA (tri chloroacetate)

2.2.7.3. Procedure:

1-100 μ l of serum was added to the test tube ,and 2ml of working solution which prepare as following :

0.514 of TBA , 25 g of TCA and 0.5 ml of 1M HCl mixed with 190 ml of D.W. Then we added 5 g of SDS and completed the volume to 200 ml .

2- The sample was vortexed and heated in a 90° C water bath for 50 min, and then allowed to cool.

3-The sample was then centrifuged for 5 min at 5000 rpm then measure the absorbance spectrophotometrically of the supernatant at the wave length 532 nm against a reagent blank. The preparation of reagent blank was the same procedure above except change the sample with DW.

2.2.7.4. Calculation

$$\text{serumMDA} = \frac{\text{Absorbance}}{d \times \zeta} \times D.F$$

$d = 1\text{cm}$, $\varepsilon = \text{extinction coefficient} = 1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$

D.F = dilution factor

2.2.8. Genetics Study

2.2.8.1. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis Design :

The following steps briefly describe the designing process of PCR-RFLP genotyping method .

2.2.8.1.1. SNPs Flanked Sequences Retrieving :

The flanked sequence for each SNP was retrieved from dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/snp>) according to human genome assembly GRCH38.p2 as a FASTA format.

2.2.8.1.2. Restriction Enzyme Selection

The selection of the suitable restriction enzyme was performed by the aid of WatCut online software (<http://watcut.uwaterloo.ca/template>) , we selected the restriction enzyme according to several criteria such as: the lesser primer mutations needed , the distance of mutation from the variant , compatibility of the produced primers , cost and availability .

2.2.8.1.3. Primers Design for PCR-RFLP and SSCP

The primers were designed by the aid of NCBI-primer BLAST online software(http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LC=BLASTHome) , at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome , then the primers pair was selected according to the

demand criteria such as : product length , the similarity of melting temperature , primers length , specificity , etc.

2.2.8.2. DNA Extraction

The manufacturer protocol (Favorgen Biotech/Taiwan) was followed for extraction the DNA from frozen blood samples by using some components of the extraction kit as following:

Table (2-4): Components of DNA Extraction Kit

Item	Quantity
GT Buffer	30 ml
GB Buffer	40 ml
W1 Buffer	45 ml
Wash Buffer	25 ml
Elution Buffer	30 ml
RBC Lysis Buffer	135 ml
GD Column	100 pcs
2 ml collection tube	200 pcs

The Basic steps for DNA Extraction could be summarized as follows:

Step 1: RBC Lysis.

- 1-Blood sample was collected in EDTA collection tubes.
- 2-About 200µl of blood was added to a 1.5ml micro-centrifuge tube.
- 3-About 40µl of Proteinase K (10mg/ml) was added to the 1.5ml micro-centrifuge tube and mixed briefly. The mixture then incubated at 60 °C water bath for 15 minutes.

Step 2: Cell Lysis.

- 1- GB buffer (200 µl) was added to the 1.5ml micro-centrifuge tube and mixed by vortex.
- 2- The mixture was incubated in a 70°C water bath for 15 minutes. The tube was inverted and mixed every 3 minutes during incubation.

Note: The required volume of elution buffer was pre-heated (100µl/sample) to 70°C (For step 5 DNA Elution).

Step 3: DNA Binding.

1- Absolute ethanol (200 µl) was added to the sample lysate and directly vortexed for 10 seconds. If precipitate formed, it was broken up by pipetting.

2- AGD column was put in a 2ml collection tube.

3- The entire mixture that contains any precipitate was added to the GD column.

4- Then, the samples were centrifuged at 15,000 x g for 5 minutes and the 2ml collection tube containing the flow-through was discarded and the GD column placed in a new 2ml collection tube.

Step 4: Washing

1- W1 buffer (400 µl) was transferred to the GD column and centrifuged at 15,000 x g for 30 seconds and the flow-through was discarded and the GD column was placed back in the 2ml collection tube.

2- Wash buffer (600 µl) was transferred to the GD column and centrifuged (15,000 x g for 30 seconds) and the flow-through was discarded and the GD column was placed back in the 2ml collection tube and centrifuged again (15,000 x g for 3 minutes) to dry the column matrix.

Step 5: DNA Elution.

Standard Elution volume was 100µl. If higher DNA yield is required, the DNA elution step was repeated to increase DNA recovery and the total elution volume to approximately 200µl.

1- The dried GD column was transferred to a clean 1.5ml micro-centrifuge tube.

2- To the center of the column matrix; 100µl of pre-heated elution buffer was added.

3- Stand for at least 3 minutes to ensure that the Elution Buffer absorbed completely by the matrix.

4- To elute the purified DNA; the tube was centrifuged at 15,000 x g for 30 second.

2.2.8.3. Agarose Gel Electrophoresis

Preparation of TBE (10X) Stock Solution/Liter.

TBE (1X) 1000ml was prepared by adding 50ml of stock solution (TBE (10X) to a final volume of 950 ml of DW (Sambrook and Russell , 2001).

Gel Electrophoresis Protocol.

1- The gel-casting tray was placed in plastic tray and checked that the teeth of the comb are 0.5mm above the gel bottom. Position the comb 1.5 cm from the edge of the gel.

2- TBE (1X) (40ml) was placed into a 250ml flask and 0.4 of agarose was added and then melt the agarose by heating the solution on hot plate for approximately 10 min. The agarose solution was swirled carefully to ensure that the agarose was completely dissolved and the solution became clear.

3- The agarose solution was cooled to approximately 60°C and adds 2-3µl of Red safe , and slowly pours the agarose into the gel-casting tray. If there is any air bubbles must be removed by tip.

4- Agarose was left solidify for about 20-30 minutes. After the agarose has been frozen the comb has been removed with the back and forth stirring, careful to prevent rupture of the gel.

5- The gel-casting tray was removed and placed the tray on the central supporting platform of the gel box.

6- Electrophoresis buffer was added to the buffer chamber until it reaches a level of 0.5-1 cm above the surface of the gel.

7- The samples were loaded in to the wells using white tips under the surface of the electrophoresis buffer just above the well. The sample was expelled slowly to allow it to sink to the bottom of the well.

8- Ladder molecular weight marker (5 μ l) was loaded to one side of the gel (flanking and sample line) and 5 μ l of DNA specimen in the other well.

9-The lid was placed on the gel box and connected the electrodes. DNA will travel towards the positive (red) electrode positioned away from the well. Turn on the power supply.

10–Electrophoresis was continued until the tracking dye moves at least 10 cm of the gel length.

2.2.8.4. Photo Documentation.

Agarose gel was visualized in a UV trans illuminator provided with gel documentation unit, agarose gel was placed above the UV trans illuminator device, the gel was exposed to UV light and the photo was captured using canon digital camera.

2.2.8.5. Reconstituting and diluting primers.

Primers were commonly shipped in a lyophilized state. The units of a lyophilized primer were given as a mass, in Pico-moles. In order to create a stock of primers, one would reconstitute the primer in sterile, nuclease free H₂O to be added to each primer to obtain a master stock that would be used again to obtain a working stock.

The following steps were followed for reconstituting and diluting the primers:

- 1- The tube was spined down before opening the cap.
- 2- The desired amount of water was added according to the oligos. Manufacturer to obtain a 100 Pmoles/ μ l (Master Stock).
- 3- The primers were vortexes properly to re-suspend evenly.
- 4- A 10 μ l of the master stock was transferred to a 0.2ml Eppendorf tube that contained 90 μ l of sterile, nuclease-free H₂O (Working Stock).

5- The master stock and working stock were stored at -20 °C.

6- The working stock was thawed on ice and vortex before using in PCR and then stored at -20 °C. Sequences of primers used for PCR amplification this study were shown in table (2-5).

Table (2-5) : Sequences of primers used for PCR amplification of Afamin and Peroxiredoxin 4 genes polymorphisms .

Primer gene name	Sequences
Afamin Rs 35233345	F:5'-GGCTGTGGTACATATATCCTCCT-3'
	R:5'-ACTCAGTTTGGATTCTCTCTGTCC-3'
Peroxiredoxin 4 genes Rs 564561	F:5'- GAGGGACGAACAGTAGGTGC -3'
	R:5'- GTAGTCGGCTTCGGTGATCC -3'

2.2.8.6. Amplification by Polymerase Chain Reaction (PCR)

PCR optimization was done as a first step using gradient temperature for each set of primers. After the determination of optimum annealing temperature, the PCR reaction mixture as shown in table (2-6).

Table (2-6) : The starting PCR reaction ingredients concentration for each optimization processes of Afamin gene and peroxiredoxin 4 gene

No	Ingredient	Concentration	Added volume
1	Go tag green mix	2.5x	8 µl
2	DNA	50ng / µl	2 µl
3	Primers	10 µM	2 µl
4	Molecular grad water		7.5 µl
5	MgCl ₂	3 mM	0.5 µl

Amplification reactions were carried out by Series thermocycler (Clever Scientific/UK) apparatus. The following program was set in the thermocycler after determination of the optimum annealing temperature to amplify Afamin gene as shown in table (2-7).

Table (2-7): The program used for Afamin gene amplification sequence

	step	Temp.(C°)	Time (min)	No. of cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	30 sec	35
3	annealing	62	30 sec	
4	Elongation	72	30 sec	
5	final elongation	72	5 min	1

The followed program was set in the thermocycler after determination of the optimum annealing temperature to amplify peroxiredoxin 4 gene as shown in table (2-8).

Table (2-8): The program used for peroxiredoxin 4 gene amplification sequence

	step	Temp.(C°)	Time (min)	No. of cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	30 sec	34
3	annealing	66	30 sec	
4	Elongation	72	30 sec	
5	final elongation	72	10 min	1

2.2.9. Restriction Fragment Length Polymorphism (RFLP) for study the genotyping of peroxiredoxin 4 gene

2.2.9.1. Principle

RFLP is a distinction in homologous DNA sequences that can be identified by the nearness of fragments of various lengths after digestion of the DNA tests being referred in question with specific restriction endonucleases. RFLP, as a molecular marker, is explicit to a single clone/restriction enzyme combination (De Vrieze et al ., 2018) . Most RFLP markers are co-dominant (the two alleles in heterozygous example will be identified) and profoundly locus- specific. A RFLP probe is a marked DNA sequence that hybridizes with at least one fragments of the processed DNA test after they were isolated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single-or low-duplicate genomic DNA or cDNA clones are normally utilized as RFLP probes. The RFLP tests are much of the time utilized in genome mapping and in variety investigation (genotyping, crime scene investigation, paternity tests, inherited disease diagnostics, etc.) (Jaber et al ., 2018)

2.2.9.2. Restriction Enzyme

Restriction Enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA at specific recognition nucleotide sequences known as restriction sites. Such enzymes, found in bacteria and archaea, are thought to have evolved to provide a defense mechanism against invading viruses (Sriphairoj et al .,2018) .

Such specific enzyme, found in microscopic organisms and archaea, are thought to have developed to give a protection component against attacking infections (Irshaid and Birmingham ,2018) .

2.2.9.3. Procedure

This procedure was done by : preparing volume of each amplified products with each of restriction enzyme BanII (Cardoso et al.,2018) .

NO	Materials	Added volume
1	Enzyme	0.25 μ l
2	Buffer	1 μ l
3	water	4 μ l
4	Pcr product	5 μ l

were put into the tubes and incubated at 37 C° overnight. Digested amplified DNA fragments were electrophoresed on 2% agarose (2 h at 100 V), and the bands visualized pre staining with Red Safe under UV light. A 100 base -pair ladder (England biolab - UK) (were used as a size marker for estimation of fragment sizes.

2.2.10.Method of Sample preparation

We take 2.5 μ l of buffer SSCP with 2.5 μ l of PCR product and incubate 95c° up for 5 a few minutes and then placed with ice.

Samples denatured with SSCP loading buffer (NaOH and form amide)and heated 95c° up for 5 minutes to be separated and then placed in the ice directly to prevent the formation of double chain and then electrophoresed by native polyacrylamide.

2.2.10.1. Polyacrylamide Gel Electrophoresis

The electrophoresis was carried out according to (Sambrook and Russell ,2001) , briefly as the following :

1- To prepare 10 ml of 10% polyacrylamide gel in 0.5X of TBE buffer , a 3.3 ml of polyacrylamide stock solution (30%) , 1 ml of 5X TBE buffer and 5.7 ml of double distilled water was mixed and filtrated by 0.45 μ m filter then the mixture was degased under vacuum .

2-The two glasses of the electrophoreses device was washed and dried by alcohol, then reassembled to the device according to the manufacture directions .

3-One hundred microliters of ammonium per sulfate (10%) and 10ul of TEMED was added and mixed briefly by swirling .

4-As quickly as possible the mixture was poured to the device by disposable Pasteur pipette, and the wells forming comb was inserted gently to its location .

5-The gel was incubated in 37C for 45 minutes to polymerize .

6-After the polymerization , the gel was submerged in 0.5 TBE buffer , and the comb was removed, then the wells were washed by the buffer .

7-A pre-run was carried out for 30 minutes under a constant current 20 mA and 200 V.

8-After the pre-run the a 5 μ l of the sample were loaded by mechanical pipet

9-The electrophoresis run was carried out until the bromophenol blue dye reach to the tow third of the gel , under a constant current **20 mA and 150 V for each gel** .

10-The gel then dissembled and stained by 1 μ g/ml silver for 35 minutes as shown in table (2- 9) .

11- The gel then imaged and the image analyzed by CS analyzer software .

Table (2-9): The method of silver-staining (Byun *et al.* , 2009)

Step	method
Stain	10% ethanol, 0.5% acetic acid, 0.2% AgNO ₃ (3–20 min).
Wash	Water (rinse,1 time).
Develop	3% NaOH, 0.1% HCOH (_55 °C, 5– 10 min).
Stop	10% ethanol, 0.5% acetic acid (1 min).

2.2.11.Methods of Sequencing

2.2.11.1.DNA Sequencing of PCR Amplicons

Two random samples of each SSCP pattern were selected and subjected to sequencing . The resolved PCR amplicons were commercially sequenced from forward termini according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

2.2.11.2.Interpretation of sequencing data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome. The highlighted SNPs were visualized to the dbSNP database to check their originality. Each particular SNP was re-positioned according to its place in the reference genome Subsequently, the determination of the presence of the detected

SNP was performed by viewing its corresponding dbSNP position. Then, the dbSNP's position for the detected SNP was documented.

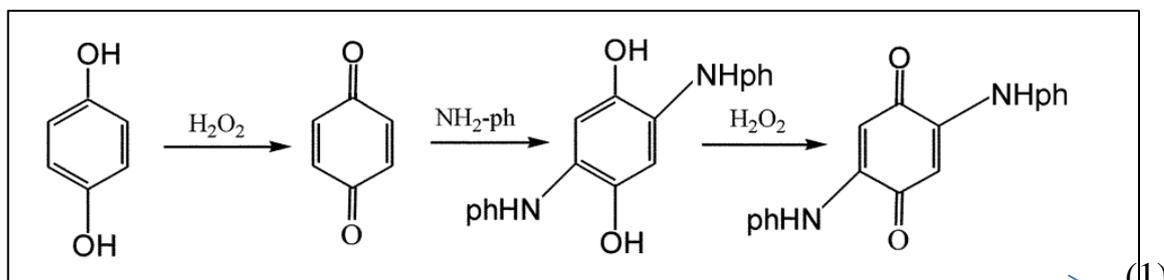
2.2.12. Materials and methods for assessment catalase activity

2.2.12.1. Chemicals

All chemical substances were attained from standard commercial suppliers.

2.2.12.2. Principle

The current method utilized the reaction between a hydroquinone/ anilinium sulfate / ammonium molybdate reagent and unreacted hydrogen peroxide, which forms a purple, disubstituted quinone compound with a



maximum absorbance at 550 nm. The reaction between hydroquinone and aniline in the presence of H_2O_2 is shown in Figure (2- 4): (Elnemma , 2004).

Figure (2- 4): Proposed reaction for the formation of purple –colored product of the disubstituted quinone compound.

The system, H_2O_2/ MoO_4^{2-} , could act as a peroxidizing agent *via* the generation of singlet oxygen (1O_2) (Nardello et al ., 1997) , as shown in equations 1 and 2 (Wahlen et al ., 2005) . Subsequently, 1O_2 molecules react with reduced hydroquinone to form the oxidized form.

2.2.12.3. Reagents

- 1- Hydroquinone solution (0.25 mol/L) was composed of 2.75 g hydroquinone in 100 ml of distilled water (DW).
- 2- Anilinium sulfate solution (0.125 mol/L) was composed of 3.554 g anilinium sulfate dissolved in 100 ml DW.

3- Ammonium molybdate solution (0.05%) was composed of 0.5 g ammonium molybdate dissolved in 100 ml DW.

4- The working reagent was prepared freshly by mixing anilinium sulfate (200 ml), hydroquinone (300 ml), and ammonium molybdate (100 ml). The sequence of addition for the component solutions was very important to the achievement of accurate results.

5- Phosphate buffer (pH 7.4, 50 mM): solution (a) was composed of 6.81 g KH_2PO_4 dissolved in 1 l of DW, and solution (b) was composed 8.90 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ dissolved in 1 l of DW; freshly prepared phosphate buffer was prepared by mixing (a):(b) at a 1:1.5 ratio.

6-Hydrogen peroxide solution (10 mM) was freshly prepared by mixing 114 μl H_2O_2 (30%) with 100 ml phosphate buffer.

2.2.12.4 .Erythrocyte Samples

Three milliliters of whole blood were used to prepare erythrocyte lysates. The blood was drawn from a researcher in the Department of Chemistry at the University of Babylon (Iraq). The heparinized whole blood was centrifuged at $400 \times g$ for 10 min, and then the buffy coat and plasma were removed and disposed of. Subsequently, aliquots of the resulting red blood cells (500 μl) were washed three times with 5 ml NaCl solution (0.9%). The samples were centrifuged after each wash at $500 \times g$ for 10 min. The test tubes were vortexed for five seconds after the addition of 2 ml ice-cold DW. Then, the test tubes were incubated at 4°C , for 15 min in the dark. Stock hemolysates were resuspended in phosphate buffer solution (0.05 M), at a dilution factor of 500. The resulting erythrocyte lysates were utilized as a suitable source of catalase enzyme activity.

2.2.12.5.Tissue Preparations

The central animal house of the College of Science at the University of Babylon, Iraq provided male albino mice and rats. Broiler chickens were

obtained from the central market (Hilla City, Iraq). Liver tissues were surgically excised immediately after the animals were sacrificed. Livers were washed with 0.9% NaCl solution (w/v) to eliminate blood and contaminants, and then the liver tissues were homogenized in 1.15% (w/v) cold KCl. After filtration, the resulting sample was diluted with 0.05 M phosphate buffer solution, at a ratio of 1:500, and utilized as a suitable source of catalase enzyme activity.

2.2.12.6. Procedure

The procedure utilized to assess catalase activity is shown in Table (2-10).

Table (2-10) : Details of the procedure utilized to assess catalase activity.

Reagents	Test	Standard	Blank
Sample	1000 μ l	-----	-----
Distilled water	-----	1000 μ l	3000 μ l
Hydrogen peroxide	2000 μ l	2000 μ l	-----
After mixing, test tubes were incubated for 2 min at 37 °C and the following reagent was added thereafter:			
Reagent working	6000 μ l	6000 μ l	6000 μ l

Test tubes were vortexed and incubated at room temperature for 10 min, at 25 °C and absorbance was read at 550 nm.

2.2.12.7. Calculation

Catalase enzyme activity was determined according to the rate constant of a first-order reaction equation (k):

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * \log \frac{S^{\circ}}{S} \quad \text{--- (1)}$$

where t is time, S° is the absorbance of the standard test tube,

and S is the absorbance of the sample test tube.

2.2.13. Assessment peroxiredoxin activity

2.2.13.1. Reagents and solutions

1-Hydrochloric acid solution (182 mM) was prepared by dissolved 1.5ml HCl in 100 ml of distilled water.

2- HEPES buffer (pH 7.0; 25 mM) was prepared by dissolve 39.3 mg of diethylenetriaminepentaacetic acid and 6 g of HEPES in 800 ml of distilled water. 1.0 M NaOH was used to adjust the pH buffer to 7.0. The volume was completed to 1000 ml with DW.

3-Hydrogen peroxide (320 μ M) was prepared by dissolve suitable quantity of hydrogen peroxide in HEPES buffer (25 mM; pH 7.0). the final concentration was standardized to required concentration using a molar extinction coefficient of hydrogen peroxide at 240 nm ($43.6 \text{ M}^{-1}\text{cm}^{-1}$).

4- t-BOOH (1M) was prepared by mix 130 μ l of 70% t-BOOH (7.7M) with 870 μ l of 25 mM HEPES buffer (pH 7.0).

5- t-BOOH (1mM) was prepared by mix 100 μ l of t-BOOH (1M) to 99.9 mL of 25 mM HEPES buffer (pH 7.0).

6- t-BOOH (320 μ M) was prepared by mix 320 ml of t-BOOH (1mM) to 680 ml of 25 mM HEPES buffer (pH 7.0).

7- 1,4-dithio-DL-threitol (2.1mM) was composed of 0.0323g of DTT that dissolved in 100 ml of 25 mM HEPES buffer (pH 7.0).

8- Sodium azide (320 μ M) was prepared by diluting 10 ml of the 10mM NaN₃ in 312.5 ml of 25mM HEPES buffer (pH 7.0).

9- Working solution was prepared freshly by mix 50 ml of solution A with 50 ml of solution B. Solution A (2 Mm) ammonium ferrous sulfate ((AFS)) was composed of 0.04 g AFS that dissolved in 50 ml of 182 mM hydrochloric acid solution. Solution B was composed of 0.2175 g aminosalicic acid (ASA) that dissolved in 50 ml 182 mM hydrochloric acid solution.

2.2.13.2. Peroxiredoxin purification

Human Peroxiredoxin II was prepared as documented by (Lim et al ., 1994).

2.2.13.3. Tissue preparation

The male albino rat was purchased from the central animal house / University of Babylon, Iraq, College of Science. Animal organs such as kidney and liver were surgically excised. The organs were washed with 0.9% (w/v) sodium chloride solution to eliminate blood and other contaminants. The next step was included tissues homogenizing using a cold potassium chloride solution (1.15% w/v). Finally, the ratio of 1:500 of HEPES buffer (25 mM) was used to dilute the homogenous solution. The resulting homogenate solution was used for the assessment of peroxiredoxin activity.

2.2.13.4. Potassium thiocyanate method

The method depends upon monitor the ferrous ion oxidation in the presence of potassium thiocyanate, with slight modification as described by (Netto et al ., 1996) . PRX activity was estimated by incubating 25 μ l peroxiredoxin enzyme sample in 25 Mm of HEPES buffer (pH 7.0), containing mM1,4-dithio-DL-threitol for 10 min, at 37°C. After incubation, t-Butyl hydro peroxide or peroxide hydrogen (final concentration 100 μ M) was added to the reaction solution. The enzymatic reaction solution was incubated for 10 min at 37°C. After stop the enzymatic by using 8% of trichloroacetic acid, the centrifugation (12,000 \times g for 5 min) was used to remove the precipitated protein. Then, 400 μ l volumes of 10 mM ferrous ammonium sulfate and 200 μ l of 2.5M potassium thiocyanate was added. The absorbance was measured at 480 nm.

2.2.13.5. Aminosalicylic acid method

Two protocols were used to assess prx activity. Table (2-11) was applied when the analyst use hydrogen peroxide as the prx substrate. Table (2-12) was applied when using t-BOOH as the prx substrate. Sodium azide was not necessary in Table (2-12) because the catalase enzyme does not use t-BOOH as a substrate.

Table (2-11):The details of the method that used to measure prx activity when used hydrogen peroxide as the Prx substrate.

Reagents	Test	Standard	Blank
HEPES buffer	525 μL	550 μL	1.050 μL
Sodium azide (NaN ₃)	50 μL	50 μL	50 μL
1,4-dithio-DL- threitol (DTT) *	500 μL	500 μL	500 μL
Sample containing peroxiredoxin enzyme (serum, RBC and homogeneous tissues)	25 μL	-----	-----
Incubate all test tubes for 10 min at 37°C			
Reaction is started with the addition of 500 μL 320 μM H ₂ O ₂ , yielding an initial concentration of 100 $\mu\text{mol/L}$, followed by vigorous mixing.			-----
Mix all test tubes with vortex, incubate at 37 °C for 10 min, after that, add:			
Working	1 ml	1 ml	1 ml

solution			
After 2 minutes, changes in absorbance were measured at 425 nm and against the reagent blank.			

* the final concentrations for DTT and peroxide were 100 $\mu\text{mol/L}$.

Table (2-12): The details of the method that used to measure prx activity when used t-BOOH as the Prx substrate.

Reagents	Test	Standard (STD)	Blank
HEPES buffer	575 μL	600 μL	1100 μL
1,4-dithio-DL-threitol (DTT) *	500 μL	500 μL	500 μL
Sample containing peroxiredoxin enzyme (serum,RBC,and homogeneous tissues)	25 μL	-----	-----
Incubate all test tubes for 10 min at 37°C			
Reaction is started with the addition of 500 μL 320 μM t-BOOH, yielding an initial concentration of 100 $\mu\text{mol/L}$, followed by vigorous mixing.			-----
Mix all test tubes with vortex, incubate at 37 °C for 10 min, after that, add:			
Working solution	1 ml	1 ml	1 ml
After 2 minutes, changes in absorbance were measured at 425 nm and against the reagent blank.			

* the final concentrations for DTT and peroxide were 100 $\mu\text{mol/L}$.

2.2.13.6. Calculation:

The residual peroxide in test tube = $\frac{A.test}{A.STD} \times \text{STD of Conc.}$

Peroxiredoxin activity (μmol of peroxide utilized/min) =

$$\frac{\text{Conc. of peroxide in STD} - \text{Conc. of peroxide}}{\text{time (10 min)}} \times \frac{\text{Total Volume}}{\text{Volume of Sample}} \times \text{Dilution Factor}$$

2.3. Statistical analysis

The statistical analysis by ANOVA test

3.Results and Discussion

3.1. Biochemical and clinical characteristics of the study subjects

The present study includes of 300 individuals (male) (100 T2DM, 100 DN and 100 control subjects)

G1: Healthy subjects

G2: Diabetic patients

G3: Diabetic patients with nephropathy.

The biochemical and clinical characteristics of the study participants are shown in Table 3-1. In diabetic patients with and without levels of nephropathy, glycated hemoglobin (HbA1c) values were significantly higher in diabetic patients with and without nephropathy than in healthy controls. In diabetic individuals with nephropathy, however, the concentration of micro albumin urea was substantially higher than in diabetic patients without nephropathy.

Table (3-1): Patients clinical characteristics.

Group Parameter	Healthy subjects	Diabetic Patients	Diabetic Patients with Nephropathy
Number	100	100	100
Age (yrs)	41.4±6.1	48.4±7.3	48.4±6.6
BMI (kg/m ²)	26.6 ± 1.6	27.2 ± 1.5 NS	26.5 ± 1.6 NS
HbA1c	5.08 ± 0.53	7.607±1.995*	8.24± 2.082*
Micro albumin (mg/L)	11.42±5.345	14.66± 6.63 NS	100.55± 37.25*

NS: non-significance versus healthy donors.

*: significance versus healthy donors.

"Type 2 diabetes mellitus" has been the most frequent metabolic disorder, "chronic hyperglycemia" and a lack of insulin responsiveness in peripheral tissues, culminating in insulin resistance. The pathophysiology of insulin resistance is complex (Yaribeygi et al., 2020). The most common cause of "end-stage renal disease" (ESRD) is diabetic kidney disease (DKD) (Chen et al., 2020).

Diabetic nephropathy is one of the most common problems in people with diabetes (DN). It is currently a main cause of (ESRD) as well as "cardiovascular and morbidity" among these individuals (Donate-Correa et al., 2020). Micro albuminuria is the first sign of (DN) and a strong predictor of renal failure (ESRD). The goal of this study was to how numerous risk factors affected the presence of micro albuminuria in type 2 diabetes (Indriani et al., 2020).

3.2. Total oxidant levels in sera of diabetic patients and control

When the system's ability to neutralize and remove free radicals is exceeded, "oxidative stress" occurs. Therefore, a unifying link between a numerous molecular disorders underlying the development of insulin resistance, Beta-cell dysfunction, and impaired glucose tolerance contributing to the onset of T2D have been suggested to be OS, which produces ROS (Palekar and Ray, 2016). It's also linked to the development of long-term diabetes complications including "micro vascular and macro vascular dysfunction". Furthermore, when glucose reacts with plasma proteins, advance glycation end products are produced, resulting in the development of "reactive oxygen species" (Wright *et al.*, 2006). A result, oxidative is thought, to be caused by NADH-induced reductive stress, and therefore will be transient (Teodoro *et al.*, 2013). Reduced hyperglycemia-induced reductive stress can provide therapeutic options for diabetes prevention and diabetic complications (Yan, 2014). In addition, It is critical to understand the function of oxidative stress in hyperglycemia-

induced cellular damage. The formation of free radicals can be stimulated by a high glucose levels. An imbalance between ROS and their safety occurs as a result of the body's inadequate defense system's failure to counteract excessive ROS generation, leading to OS dominance (Pandey et al ., 2010). Table (3-2) shows levels of the total oxidant in the present study. When compared to control groups, Diabetic individuals with and without nephropathy had considerably higher total oxidant levels .The generation of (ROS) in DM patients with nephropathy was assessed.

Table (3-2): Total oxidant levels ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean ($\mu\text{mol/l}$)	SD	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	1.4136	0.24783	0.02478	1.3644	1.4628	1	2	0.030*
							3	0.000*
G2	1.5816	0.52120	0.05212	1.4782	1.6850	2	1	0.030*
							3	0.103
G3	1.7076	0.74666	0.07467	1.5595	1.8558	3	1	0.000*
							2	0.103

*. The mean difference is significant at the 0.05 level.

There is evidence which OS plays a "critical" role in systemic inflammation, which leads to the pathophysiology of a variety of "macro and micro vascular complications" (Lugrin *et al* ., 2003). DM patients are at a higher risk for a variety of cardiovascular diseases, according to a growing body of data , which are the leading cause of diabetes-related incidence and death (Orasanu and Plutzky , 2009). In addition , Foot disease, which involves inflammation, ulceration, or loss of foot tissues, affects about 6% of patients with diabetes (Lazzarini *et al* ., 2015). Therefore , Atherosclerosis, obesity ,diabetic and hypertension have all been linked to free radicals (Sastre *et al* ., 2000) . Excessive OS is often

product of long-term hyperglycemia (Bikkad *et al.* , 2014), Increased development of free - radical or a reduced antioxidant defense mechanism are two potential causes of increased oxidative stress, free radicals are boosted One of the main mechanisms of glucotoxicity in a chronic hyperglycemic state may be forming accompanied by oxidative stress in diabetic (Yan , 2014). In T2D, hyperglycemia causes an increase in ROS production, which depletes plasma TAC (Mohieldein *et al.* , 2015; Pieme *et al.* , 2017) , It also causes oxidative stress, which promotes cell damage and diabetes complications (Gunawardena *et al.* , 2019) . Hyperglycemia that is uncontrolled due to insulin secretion can cause a number of problems including nephropathy, ,retinopathy, morbidity. Therefore, ROS /oxidative stress appear to play a significant role in the develops of chronic diseases, according to a large body of research (Tiwari *et al.* , 2013) . ROS generated by non-enzymatic protein glycation, glucose oxidation, and elevated lipid peroxidation in diabetes cause damage to enzymes (Maritim *et al.* , 2003) , and the development of ROS, such as high superoxide anion radical generation, the existence of toxins, and more, excessive activation of natural systems for producing reactive species, such as phagocytic cell activation in chronic diseases, as seen in Diabetes mellitus (Halliwell and Gutteridge , 2007) . Therefore , patients with diabetes have an increased oxidant levels than healthy ones (Pan *et al.* , 2009).

3.3. Total antioxidant levels in sera of diabetic patients and controls

The total antioxidant status represents the number of both exogenous and endogenous antioxidants, providing a comprehensive image of antioxidants (Rani and Mythili , 2014) . Antioxidants play a protective function in the development of "T2D" by reducing oxidative stress caused by glucose oxidation, and "lipid peroxidation" (Thakur *et al.* , 2018; He *et.al.* , 2017) . On the other hand, Diabetics have a lower anti-oxidant defense, resulting in a mismatch between the oxidative stress (OS) and the body's defense

against it (Gezginci et al ., 2009). TAC is a metric used to assess the health of all antioxidants found in serum, plasma, and other bodily fluids (Rubio et al ., 2016). In addition, (ROS) induce tissue oxidation in tissue, and "total antioxidant capacity" provides overall knowledge about their capacity. It inhibits macromolecule metabolism (lipids, carbohydrates, and proteins, for example) and leads to noncommunicable diseases like diabetes, cardiovascular disease and neurodegenerative diseases (Görlach et al ., 2015). There's a lot of evidence that diabetics' biological antioxidant defences are weakened, in lead to greater ROS output (Bajaj and Khan 2012).

In humans, a complex antioxidant mechanism has evolved in different biological fluids, dependent on "enzymatic and non-enzymatic antioxidants" such as SOD and GPx (Manafa et al ., 2017), TAC is the biological marker which indicates the presence of all antioxidants in different biological fluids (Ojeda et al ., 2016; Kiran et al ., 2016). Table (3-3) showed that TAC significantly decreases among type 2 diabetic and with nephropathy compared with control groups. Our findings have revealed that lower TAC levels have an impact on the period of diabetes.

Table (3-3): Total antioxidant levels ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean ($\mu\text{mol/l}$)	SD	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	1.6043	0.60404	0.06040	1.4845	1.7242	1	2	0.001*
							3	0.001*
G2	1.0609	0.41804	0.04180	0.9779	1.1438	2	1	0.001*
							3	0.544
G3	1.0188	0.42378	0.04238	0.9347	1.1029	3	1	0.001*
							2	0.544

*. The mean difference is significant at the 0.05 level.

This study observed a significant reduction in TAO levels within T2DM patients and those with nephropathy, which have also been documented by other researchers in their studies (Jamuina and Mythili et al ., 2014). Other research showed that diabetic individuals with T2D patients with overt nephropathy had lower overall antioxidant potential than healthy men (Lotfi et al ., 2020).

May be Increasing oxidative stress may be to blame for the decrease in TAO levels , this is demonstrated by elevated lipid peroxidation as well as an overuse of antioxidants to prevent oxidative stress , these reductions in levels of TAO may be attributable to its increased use to scavenge the high concentrations of "free radicals" created by increased oxidative stress .

Despite the fact that everyone agrees that diabetic patients have more free radicals, antioxidant levels have been found to be significantly lower (Kharroubi et al ., 2015; Coyne et al ., 2005) , increasing (Srivatsan et al ., 2009), or stay the same (Giugliano et al ., 1996) . The impact of diabetes on overall antioxidant levels appears to be complicated by the impact of diabetes on antioxidant systems, according to a study that found a rise in "extracellular superoxide dismutase" levels (Kimura et al ., 2003), whereas a simultaneous decrease of plasma Gpx and an increase of plasma SOD was observed (Palanduz et al ., 2001). Furthermore, antioxidant levels in diabetics have been shown to be lower in other studies , therefore , catalase, SOD, and GPx activity are observed to decrease in diabetics with a rise in free radicals in T2D , implying that plasma/serum antioxidant status is decreased together with specific "antioxidants" involve vitamin E and ascorbic acid together (Nishikawa et al ., 2000). Previous research has shown that total antioxidant potential in adults is inversely related to the risk of abdominal obesity, and metabolic syndrome (Bahadoran et al ., 2012) . A linear inverse relationship between total antioxidant potential and glucose and lipid biochemical markers, as well as central adiposity

assessments, was also discovered in healthy males and females (Hermsdorff et al ., 2011).

In addition, curcuminoids are powerful antioxidants that can prevent lipid peroxidation as well as scavenge " hydroxyl and superoxide radicals" (Sahebkar et al., 2015),curcuminoids have been shown to boost the activity of enzymatic antioxidants including catalase, Gpx, and SOD (Panahi et al ., 2017), Glutathione-S-transferase and other detoxification enzymes (Piper et al., 1998). However, previous research into the antioxidant properties of curcuminoids in patients with diabetes has been restricted, with no proof of the impact of curcuminoids with improved bioavailability (Panahi et al ., 2017).

3.4. Antioxidant enzymes activities in blood of diabetic patients and controls

Catalase is a dual-function enzyme that stimulates the breakdown of H_2O_2 into H_2O and O_2 , as well as the per oxidative oxidation of hydrogen donors , which are a per oxidative function (Ezeiruaku et al ., 2016) ,which is a portion of cellular metabolism and occurs frequently in the existence of LPO and reducing antioxidant enzyme levels (Turk et al., 2002 ; Veal and Day , 2011) . Several theories have been offered to explain why OS is higher in diabetic patients, due to , reactive oxygen species generation is up, and antioxidant defenses are low (West, 2000) as found in a variety of diseases. Therefore , reduced catalase activity can lead to a rise in hydrogen peroxide levels, which can contribute to the development of age-related diseases such as DM (Góth and Nagy , 2013) . Other studies , The highest concentrations of catalase are found in human tissues such as "erythrocytes and liver", and erythrocytes account for more than 99 percent of blood catalase (Góth et al ., 2016) . Table (3-4) shows levels of the Catalase activity in the present study. Catalase activity was shown to be considerably

lower in type 2 diabetics and those with nephropathy in this study when compared to control groups .

Table (3-4): Catalase activity (Katal/l) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean (Katal/l)	SD	SE	95% C.I.		Compare d groups		Sign.
				Lower Bound	Upper Bound			
G1	164.427	57.73622	5.77362	152.9709	175.8831	1	2	0.001*
							3	0.001*
G2	103.237	45.78657	4.57866	94.1519	112.3220	2	1	0.001*
							3	0.788
G3	101.197	56.44806	5.64481	89.9970	112.3981	3	1	0.001*
							2	0.788

*. The mean difference is significant at the 0.05 level.

In this study, the result showed that assessment of serum catalase behavior in diabetic patients represents the powerful predictor to assessment of the oxidative stress. However, when T2D patients and diabetese with nephropathy were compared to control groups, serum catalase activity levels were found to be significantly lower.

The previously studies suggest that decreased serum catalase activity in diabetic patients might well be due to "hyperglycemia", which causes "peroxisome" degradation (Hristosozova et al., 2000) or ,inhibition of the enzyme catalase may occur as a result of glycation triggered by the current glucose levels. As a result, elevated glycation in diabetics and subsequent protein reactions can affect amino acids near to the enzyme's active site or disrupt the stereo chemical structure, resulting in biological changes in the molecules (Rahbani et al., 1999). Furthermore, decreased serum catalase activity in diabetics is a reliable predictor of elevated H₂O₂ levels, which increases oxidative stress and diabetes consequences.

Previous study has shown that a reduction in SOD and catalase activity in a hyperglycemic condition causes a rise in ROS, which leads to oxidation-induced liver injury (Han *et al.*, 2006; Sarkar *et al.*, 2019). methemoglobinaemia and hemolytic anemia can be caused by low catalase activity, which can be caused by a "glucose-6-phosphate dehydrogenase" deficiency (Góth and Bigler, 2007). also, when low catalase activity is combined with redox active metal ions, it can damage heme proteins, induce cell death (Góth *et al.*, 2005).

3.5. Vitamin E levels in sera of diabetic patients and control .

"Vitamin E is a fat-soluble antioxidants", it functions to defend cells against free radicals, that are potentially harmful results of the metabolism (Pavithra *et al.*, 2018). Antioxidant substances, notably vitamin E, have the ability to prevent diseases such as DM (Mayer-Davis *et al.*, 2002). Vitamin E may increase insulin action and secretion by helping to protect peripheral tissue and " β -cells" from damage produced by free radicals, according to in vitro research, This has led to the suggestion that this vitamin could help prevent the onset of T2D (Tajiri and Grill, 1999).

Vitamin E is an antioxidant that can help to reduce oxidative stress and inflammation, two variables linked to the development of diabetes complications (Khatami *et al.*, 2016), of "vitamin E are tocopherol and tocotrienol". When compared to tocopherol, tocotrienol have been shown to be approximately 50 times more effective antioxidant, with higher "antiglycemic, anticholesterolemic, antiinflammatory, neuroprotective, and cardioprotective" characteristics (Peh *et al.*, 2016). The fact that - tocopherol has antioxidant properties by contributing "hydrogen radical" to eliminate free radicals interacting with it and forming non-radical products (Choi, 2008; Ekeanyanwu *et al.*, 2016). Table (3-5) shows in this investigation, that type 2 diabetics and those with nephropathy were

compared to control groups, vitamin E levels were found to be considerably lower.

Table (3-5): Vitamin E (mg/dl) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean (mg/dl)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	1.7459	0.50065	0.05007	1.6466	1.8453	1	2	0.01*
							3	0.01*
G2	1.1841	0.55979	0.05598	1.0730	1.2952	2	1	0.01*
							3	0.801
G3	1.1615	0.80253	0.08025	1.0023	1.3207	3	1	0.01*
							2	0.801

*. The mean difference is significant at the 0.05 level.

Vitamin E levels in diabetic patients were found to be lower compared with control . In this study, diabetes patients had considerably decreased mean serum vitamin E levels, therefore vitamin E can be used to scavenge free radicals, according to previously studies. Vitamin E has antiplatelet effects and inhibits vascular permeability, in addition to its antioxidant properties.

Other study , Vitamin E is an important component of the antioxidant system that traps peroxy radicals, (Peroxy and superoxide radicals), as well as "singlet oxygen ", interact directly with it ,Vitamin E levels were marginally lower in hypertensive and diabetic patients than in individuals with normal blood pressure (Bisht and Sosadia , 2010; Ekeanyanwu et al ., 2016) . A study by Bernaedo Rodreiguez-Iturbe et al , found which an antioxidant-rich diet rich in" vitamin E, vitamin C, selenium, and zinc "reduces renal interstitial inflammation, lowers MDA levels in renal tissue, and improves hypertension (Bernardo et al ., 2003) . also, the cardio

protective possibility of vitamin E have been attributed to its active antioxidant .

In diabetic patients, vitamin E concentrations were slightly lower, was compare to controls . These findings are consistent with those of a previous article (Quilliot et al ., 2005; Eljaoudi et al ., 2017) , others, on the other hand, discovered slightly lower levels of both "vitamin E" and "vitamin C" in DM patients (Armstrong et al ., 1996) . "Vitamin E" and "vitamin C" are essential in diabetes, and good "vitamin E and C status" or supplementation tends to lower the levels of OS associated with hyperglycemia and a incidence of diabetes complications, such as cardiovascular disease (Rafighi et al ., 2013) . Other study , Other study ,to see whether high-dose vitamin E supplementation has any impact on markers of cardiovascular disease and OS in diabetes nephropathy patients (Aghadavod et al ., 2018) . The action of these vitamins as scavengers which are oxidized instead of physiological molecules including "proteins", "DNA", and "lipids" is their most important function in the OS (Gaede et al ., 2001; Eljaoudi et al ., 2017).

In addition , vitamin E reduces the oxidation sensitivity of LDL and inhibit the generation of "pro-inflammatory" "cytokines", as well as improving action of "insulin in patients with insulin resistance" (Upritchard et al ., 2008) . Reactive free radicals have been shown to cause diabetes in animal models, and vitamin E may act as a scavenger of these ROS, preventing diabetes from developing (Laight et al ., 1999) . Some epidemiologic research have discovered an inverse relationship between levels of "vitamin E" and the risk of T2D (Mayer-Davis et al ., 2002).

3.6 . Afamin activity in sera of diabetic patients and controls

The "human vitamin E-binding glycoprotein" afamin is predominantly expressed in the liver and has been linked to metabolic syndrome, both prevalent and incident (Kollerits et al ., 2017). Afamin appears to have a

variety of effects depending on the region of action. Afamin has been demonstrated to exhibit binding characteristics for two important forms of "anti-oxidative vitamin E, γ -tocopherol and α -tocopherol" (Voegele et al ., 2002; Kollerits et al ., 2017) , vitamin E transport in bodily fluids may be mediated by human afamin, which has been proven to be a particular binding protein for the vitamin (Juhász et al ., 2019). The causality of afamin's link to T2D, as well as potential underlying mechanisms, are yet unknown. Thus observations of a hyperglycemic phenotype in mice transgenic for the human afamin gene imply a causative role for afamin in T2D development (Kronenberg et al ., 2014) . Shen et al, have demonstrated a direct involvement for afamin in glucose metabolism in a "thyroid cancer cell" line transfected with human afamin (Shen et al ., 2016).

In addition , The discovery that afamin up regulates multiple critical enzymes and metabolites involved in glucose metabolism sheds new light on the molecular functions of the afamin (Kollerits et al ., 2017). Afamin is linked to "insulin resistance" and other symptoms of metabolic diseases. It plays a role in anti-apoptotic cellular processes connected to OS (Köninger et al ., 2018).

As a result, afamin's hypothesized vitamin E binding activity may be functionally relevant only in extravascular fluids or tissues for disorders like T2D and metabolic syndrome .Table (3-6) shows in this investigation, the Afamin activity was examined. In diabetic individuals, Afamin activity was substantially higher than in control groups.

Table (3-6): Afamin ($\mu\text{g/ml}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean ($\mu\text{g/ml}$)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	80.0825	19.06175	3.48018	72.9648	87.2003	1	2	0.01*
							3	0.01*
G2	102.984	21.9635	4.0099	94.7828	111.185	2	1	0.01*
							3	0.799
G3	104.61	31.03156	5.66556	93.0158	116.1905	3	1	0.01*
							2	0.799

*. The mean difference is significant at the 0.05 level.

The current study found which afamin is strongly linked to type 2 diabetes and the development of T2D, even when other key metabolic risk variables or characteristics were controlled for. Elevated plasma afamin levels may so signal the onset of T2D and nephropathy at an early stage, and global diabetes prevalence have doubled since 1980, according to the WHO, finding critical markers that participate to the development of (T2D) is critical for accurate and timely identification of influenced or high-risk patients, as well as clarification of the disease's pathophysiology. In line with these findings, higher afamin levels were recently discovered in patients with polycystic ovarian syndrome, that is linked to insulin resistance, supporting afamin's participation in a similar metabolic condition in "polycystic ovary syndrome patients" (Koninger et al., 2014; Dieplinger and Dieplinger, 2015).

Previously, discovered that afamin concentrations assessed at baseline were strongly connected to all components of the metabolic syndrome, with higher waist circumference being one of the strongest connections seen at both the baseline and follow-up research (Kronenberg et al., 2014). Increased waist circumference and body mass index are well-established

risk factors for T2D and metabolic syndrome (NCD , 2015) . Furthermore, This increase in body fat raises the risk of both T2D and insulin resistance .The findings on incident T2D, taken combined, clearly imply that afamin could be a useful marker for predicting a high risk of acquiring T2D. Most crucially, independent of key metabolic determinants or indicators, afamin was linked to , insulin resistance, and the prevalence and incidence of type 2 diabetes in our big study. In addition ,The metabolic syndrome is linked to the early onset of life-threatening complications such as type 2 diabetes , Cardiovascular, and stroke (Gami et al ., 2007). In recent decades, metabolic syndrome has become more common in the general population. hyperglycemia, abdominal obesity, are all risk factors for T2D and cardiovascular disease . Afamin has been found in high concentrations in plasma and various bodily fluids such as follicular and cerebrospinal fluids (Eckel et al ., 2010; Seeber et al ., 2014).

3.7. Peroxiredoxin activity in sera of diabetic patients and controls

Peroxiredoxins are a widespread family of cysteine dependent "antioxidant peroxidases" They are useful oxidative stress biomarkers because they play a vital role in controlling peroxide levels (Poynton and Hampton 2014). They serve as a powerful defensive mechanisms which keeps the redox balance in both natural and oxidative stress situations. They also have the ability to serve as a chaperone (Jang et al ., 2004) . Six isoforms have been described, and they are expressed in the "cytosol, mitochondria, and peroxisomes", among other places where ROS are produced (Rhee et al ., 2005) . Peroxiredoxins have become increasingly common as antioxidative proteins in biomedical research , Peroxynitrate and hydro peroxide can be reduced by peroxiredoxins (Wood et al ., 2003). As a result, a large amount of H₂O₂ causes radical damage. Prxs which have been exposed to a high volume of free radicals are thought to over oxidize, according to the literature.

Although there is a connection between peroxiredoxin expression and DM, the link between this enzyme family and cardiovascular disease is still unknown. Other studies , baseline levels of circulating peroxiredoxin isoforms (1, 2, 4, and 6) as well as their relationships with typical cardiovascular disease were determined , including "blood pressure (BP)", "hyperlipidemia", and "C-reactive protein (CRP)" in patients with T2DM (El Eter and Al-Masri , 2015). Table (3-7) and Table (3-8) shows that diabetic patients with and without nephropathy have significantly higher overall peroxiredoxin activity than control groups.

Table (3-7): Peroxiredoxin enzyme activity (U/L) was measured when using H₂O₂ as the Prx substrate in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean (U/L)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	80.57	30.535	3.053	74.512	86.629	1	2	0.001*
							3	0.001*
G2	133.64	47.646	4.764	124.188	143.097	2	1	0.001*
							3	0.171
G3	124.86	54.122	5.4122	114.127	135.605	3	1	0.001*
							2	0.171

*. The mean difference is significant at the 0.05 level.

Table (3-8): Peroxiredoxin enzyme activity (U/L) was measured when using t-BOOH as the Prx substrate in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean (U/L)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	75.831	32.9843	3.2984	69.286	82.3763	1	2	0.001*
							3	0.001*
G2	127.569	45.2353	4.5235	118.593	136.545	2	1	0.001*
							3	0.888
G3	128.414	43.544	4.3544	119.774	137.055	3	1	0.001*
							2	0.888

*. The mean difference is significant at the 0.05 level.

In the present study, patients with type 2 diabetes had significantly higher levels of peroxiredoxins than control subjects. In this analysis, we believed that the levels in the stable control subjects were not pathological. Previous research has found a decrease in antioxidant defense system and a rise in oxidative damage markers in patients with "T2D", particularly in those with diabetes-related complications (Kasznicki et al ., 2012) . Furthermore , the patients with diabetic have higher plasma levels of Prx 1, Prx 2, Prx 4, and Prx 6 than safe "control subjects" (El Eteer and Al-Masrii 2015; Heecker et al ., 2018). Peroxiredoxin 1 activity in red blood cells was found to be higher in T2D patients than in non-diabetic control, that was similar to our findings (Brinkmann et al ., 2011) .

Moreover, even after controlling for common diabetes risk factors, the population-based Prevention of Renal and Cardiovascular end stage Disease cohort study found that increased circulating Prx4 levels are linked to a higher risk of Diabetes (Abbasi et al 2014; Hecker et al ., 2018). Prx 6 has been shown to be a primary mediator of hyperglycemia in T2D-related glucose metabolism. (Pacifiici et al 2014). Other studies , Prx 6 knockout

mice evolve a phenotype that resembles early-stage T2D, with decreased "glucose-dependent" insulin secretion and increased insulin resistance", resulting in a decrease in muscle glucose uptake (Pacifici et al 2014). In addition, In human pulmonary artery smooth muscle cells, Prx 6 was found to be carbonylated in response to a short-term treatment with cell proliferation-inducing platelet-derived growth factor (Wong et al 2013). Interestingly, Prx 6 is abundant in beta -cells (Tiedge et al ., 1999), and muscle, and have been previously reported to have a strong antioxidant function (Fisher et al ., 2011) . Other studies, Prx 6 has been shown to impair metabolic homeostasis in mice, suggesting that it could be a determinant of DM "susceptibility" in humans (Fisher et al ., 2011) .

Other research also during the WHO endurance step test on a bicycle ergometer, a rise in the contents of overoxidized peroxiredoxins was only seen in the red blood cells of patients with "T2D" men (Brinkmann et al ., 2012) .In addition , Exercise-induced improvements in antioxidant defence ability can be particularly beneficial for patients with diseases like diabetes mellitus, which are linked to high levels of free radicals (Brinkmann et al ., 2012) . Evidently, exercise increases the expression of Prx isoforms in skeletal and heart muscle cells, especially those found in the mitochondria (Richters et al ., 2011) . This indicates that during exercise, many free radicals (and thus H₂O₂) are produced in the mitochondria, and antioxidative defense is especially beneficial in this case (Sahlin et al ., 2010) .

3.8 . Peroxiredoxin 4 activity in sera of diabetic patients and controls

Prx4, a thiol-dependent "antioxidant" family member, and play a vital role in oxidant scavenger and signaling pathways that protect against oxidative damage, according to mounting data (Nabeshima et al ., 2013) . Endothelial damage is also a product of oxidative stress, that could lead to more Prx4 leakage from endothelial tissue, resulting in even greater blood

peroxiredoxin 4 levels (Gerrits et al ., 2014). Prx4's ability to remove H₂O₂ from within the cell, as well as the enzyme's secretion, is hypothesized be boosted in response to "oxidative stress" (Tavender and Bulleid , 2010) .

peroxiredoxin 4 is the only isoform encoded by the "X chromosome" (the human Prx4 gene is found on "Xp22.11") and the only one released into circulation among the six members of the family (Nabeshima et al ., 2013) . In the other results, Prx4 supplementation has a diabetes-protective impact in vivo and it can ameliorate insulin resistance (Hivert et al ., 2011) . In both humans and animal models of T2D , elevated ROS generation is a crucial alteration in the development of "insulin resistance" and early stage beta cell dysfunction. Prx4 expression changes govern cellular redox status, implying that oxidative metabolism is boosted in the islets of animals fed high fatty or high carbohydrate diet , Prx4 may also help to protect cells from oxidative stress by inhibiting apoptosis and increasing progenitor cell proliferation in vivo (Ding et al ., 2010) . Moreover, Prx4 is the sole member of the Prx family that has been discovered to be secreted (Ding et al ., 2010; Gateva et al ., 2016) .

Peroxiredoxin 4 levels in diabetic patients are greater than in controls, according to studies, and are linked to cardiovascular disease in people with T2D (Gateva et al ., 2016). Table (3-9) demonstrates the study individuals' Prx 4 activity. When diabetes individuals were compared to a healthy subject group, their peroxiredoxin 4 activity were considerably higher .

Table (3-9): Peroxiredoxin 4 ($\mu\text{g/ml}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean ($\mu\text{g/ml}$)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	1407.67	238.757	43.591	1318.5213	1496.8283	1	2	0.014*
							3	0.013*
G2	1519.94	137.830	25.164	1468.4735	1571.4070	2	1	0.014*
							3	0.973
G3	1521.45	118.151	21.571	1477.3404	1565.5773	3	1	0.013*
							2	0.973

*. The mean difference is significant at the 0.05 level.

In the present study, Prx4 level was found to be significantly higher in T2D patients and those with nephropathy when compared to a healthy control group. Prx4 levels were also found to be higher in T2D patients in cross-sectional in vivo studies (Nabeshima et al ., 2013; Abbasi et al ., 2012). In other studies , El Eter et al found that T2D patients with peripheral atherosclerosis had considerably greater Prx4 levels than healthy controls (Eter et al ., 2014) . In addition, Nabeeshima et al colleagues discovered which male patients with T2D had greater serum peroxiredoxin 4 levels than healthy males (Nabeshima et al ., 2013) . In a recent examination of clinical data, it was discovered that septic patients had higher serum Prx4 levels than healthy people (Schulte et al ., 2011) . In other researches , Prx4 levels in the blood were found to be higher in septic patients than in healthy people, and it was linked to well-known inflammatory indicators such "procalcitonin, C-reactive protein (CRP), and "interleukins 6 levels (IL-6)" (Schulte et al ., 2010). In keeping with the findings of the latter study, peroxiredoxin 4 levels have previously been linked to inflammatory markers , adiposity measurements (such as BMI), blood pressure, and glucose levels (Abbasi et al ., 2012) . These factors

underlie the central biological pathways of metabolic syndrome and T2D. Up regulated intracellular Prx4 production and increased extracellular Prx4 levels may protect against the metabolic anomalies that contribute to T2D by suppressing oxidative stress and improving local such as islet cells or hepatic and "systemic inflammatory signaling and insulin sensitivity" (Wilson et al ., 2007; Abbasi et al ., 2012) . The patients with greater Prx4 levels of other research revealed various variables which might have influenced Prx4 or OS in general, including older age, a higher incidence of albuminuria, and a greater prevalence of oxidative stress in general (Tavender and Bulleid , 2010).

Recently , Prx4 expression or oxidation status in pancreatic islet cells has been observed in animal models of DM (Ding et al ., 2010) .Thus , Prx4 was tested in a generic Dutch sample, which included people with micro albuminuria and was collected from the general community. (Abbasi et al ., 2012) . Other research has linked raised serum Prx4 levels to an elevated risk of heart disease , cardiovascular mortality, and all-cause mortality (Chang et al ., 2009; Schulte et al ., 2011).

Prx4 also enhances antioxidant activity through a variety of pathways, including "nuclear factor-B , p53 , the thromboxane A2 receptor , "NF-E2 related factor 2 (Nrf2)" (Paynter et al ., 2011; Abbasi et al ., 2012) . Thus treatment with "Nrf2 antagonist", a particular antioxidants that inhibits the peroxiredoxin 4 pathway, has been shown to be an effective intervention against kidney function loss in patients with renal impairment and diabetes (Abbasi et al ., 2012).

3.9. Lipid peroxidation levels in sera of diabetic patients with and without nephropathy

Lipid peroxidation is a damaging process involving autocatalytic free radicals in which "polyunsaturated fatty acids" in cell membranes are degraded to create lipid hydro peroxides (SHENOY et al ., 2018) . However

during process of lipid peroxidation, by-products including conjugated dienes and malondialdehyde are observed to be higher in patients with type - 2 diabetes , These compounds attack "carbohydrates, lipids, protein, and DNA", causing cell damage. (Valko et al ., 2007 ; SHENOY et al ., 2018). "TBA reactive species" (TBARS) are commonly use as biomarkers of LPO, and multiple investigations have indicated higher amounts of LPO products in the plasma of T2D patients (Matkovics et al ., 1982) . Other studies , in diabetics, Rbc membrane "lipid peroxidation" had also been observed to be higher and linked with the quantity of glycated hemoglobin (Jain et al ., 1989). The specific method by which high blood glucose causes lipid peroxidation in diabetic patients' plasma, "erythrocyte, and leukocyte" membranes is unknown . However, under physiological conditions, glucose can enolize and thereby decrease molecular oxygen, resulting in -keto aldehydes, H₂O₂, and free radical intermediates, according to in vitro studies (Mashino and Fridovich , 1987) . Therefore , It also emphasizes the presence of lipid peroxidation and systemic inflammation, which contributing to metabolic imbalance even in well-controlled type 2 diabetes, as other authors have observed (de Souza et al ., 2016). In addition , Through "non-enzymatic and autoglycation" mechanisms, hyperglycemia enhances ROS-mediated LPO (Gunawardena et al ., 2019). Table (3-10) shows levels of the Lipid peroxidation in the present study. Lipid peroxidation levels were considerably higher in diabetic individuals with and without nephropathy when compared to control groups.

Table (3-10): Malondialdehyde concentrations ($\mu\text{mol/l}$) in sera of healthy subject, diabetic patients and diabetic patients with nephropathy.

	Mean ($\mu\text{mol/l}$)	S D	SE	95% C.I.		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	1.6741	.86215	0.08621	1.5030	1.8452	1	2	0.001*
							3	0.001*
G2	2.5765	1.16235	0.11623	2.3459	2.8072	2	1	0.001*
							3	0.713
G3	2.5183	1.28133	0.12813	2.2641	2.7726	3	1	0.001*
							2	0.713

*. The mean difference is significant at the 0.05 level.

Type 2 diabetes patients' hyperlipidemia may be one of the causes of increased lipid peroxide generation (Kesavulu et al ., 2000 ; Gunawardena et al ., 2019) . By promoting the generation of RNS and ROS , chronic hyperglycemia, dyslipidemia, and increased FFAs cause oxidative stress (Kuroki et al 2003), which causes peroxidation by attacking lipids in plasma membranes, "mitochondrial membranes, and endoplasmic reticulum membranes" (Chen and Tseng , 2013). After , lipid peroxidation begins and propagate chain reactions occur until termination products, include lipid hydro peroxide, are formed , which breakdown further to aldehydes like " 4-hydroxy-2 nonenal (4-HNE) and MDA " ,it's also possible for cyclic "endoperoxides, isoprostanes, and hydrocarbons" to create (Kharroubi et al ., 2015) .

The current study's reported increase in malondialdehyde levels in type 2 diabetes patients is likely to be related to increased lipid peroxide generation and release into circulation, which would be consistent with earlier findings (Jamuna et al ., 2014). In addition , similar to our results, an increase in plasma malondialdehyde has been previously recorded in T2D

patients (Gunawardena et al ., 2019). Despite the fact that previous studies have shown elevated MDA levels in T2D patients with impaired glycaemic regulation (Manohar et al ., 2013) . It also emphasizes the presence of LPO and systemic inflammation, which lead to metabolic imbalance even in well controlled type 2 diabetes , as previous authors have suggested (de Souza et al ., 2016) . As a result, OS in "T2D" is indicated by decreased plasma TAC and increased malondialdehyde levels. Because of auto oxidation of glucose, which produces free radicals, an raise in malondialdehyde levels can be linked to hyperglycemia in these patients . The continual, raise in blood glucose in DM results in the production of rise levels of reactive oxygen species / malondialdehyde, same finding is present in our study. Therefore "micro vascular complications" of DM may be the result of this increase level of oxidative stress (elevated in the level of malondialdehyde).

3.10. Genetic Study

3.10.1. Amplification of afamin and peroxiredoxin 4 genes primers using temperature gradient of PCR reaction

PCR temperature gradient was used to detection optimum annealing T_m for afamin primer as shown in (Figure 3-1).

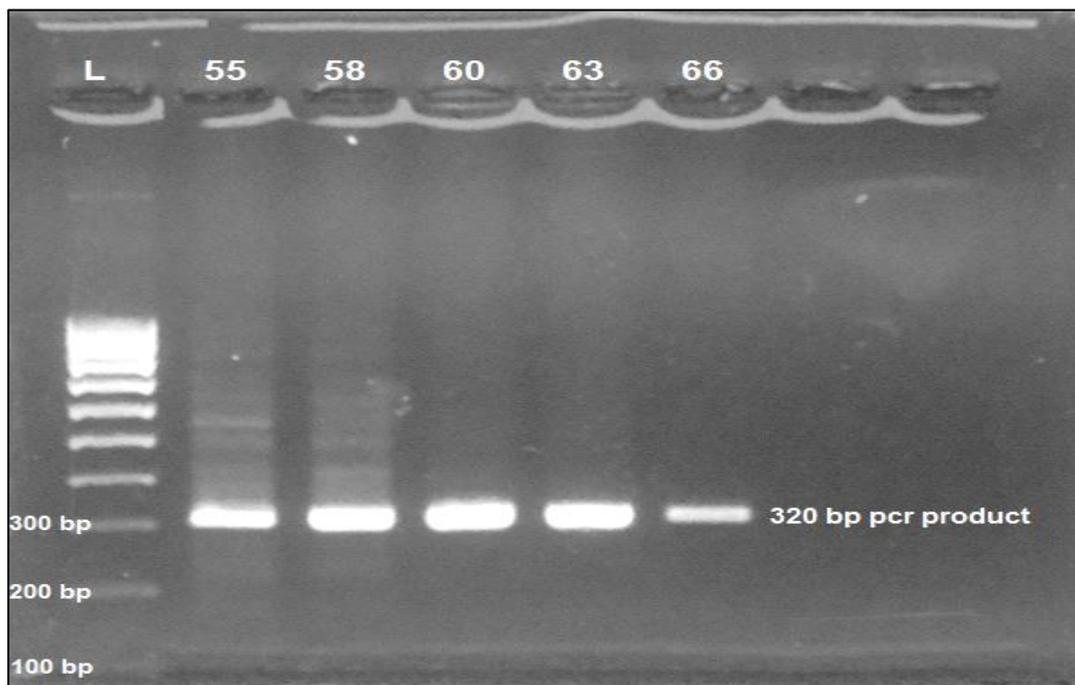


Figure (3-1): PCR temperature gradient was used to detection optimum annealing T_m for afamin primer.

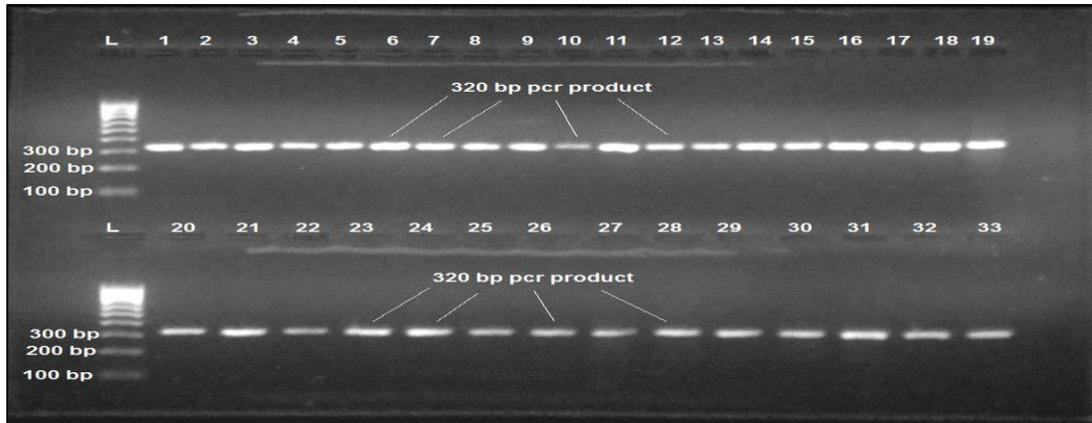


Figure (3-2): The electrophoresis pattern of PCR product for afamin gene .

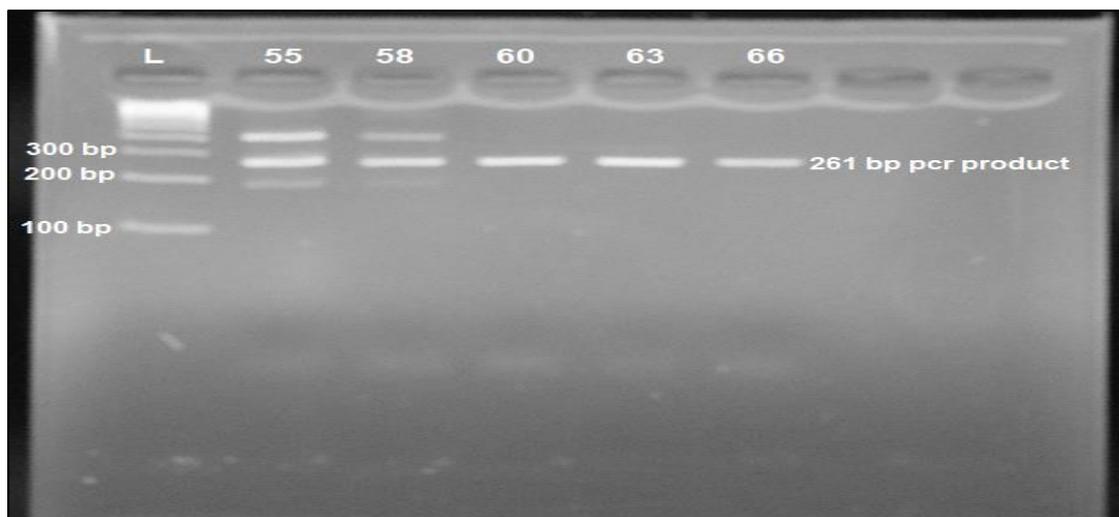


Figure (3-3): PCR temperature gradient was used to detection optimum annealing T_m for peroxiredoxin 4 primer.

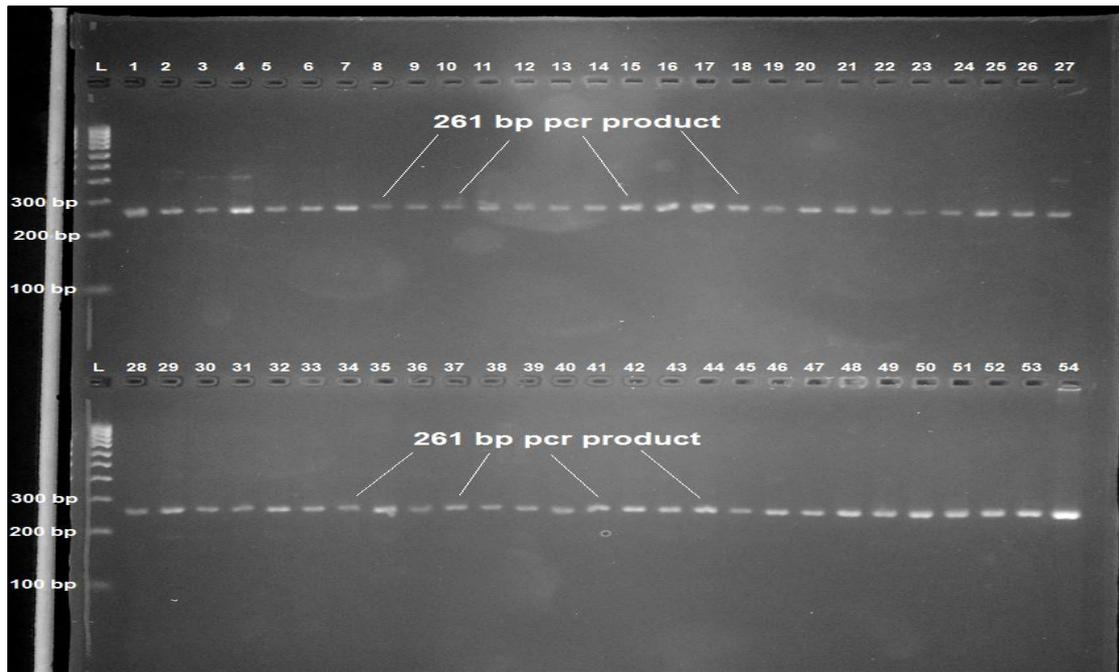


Figure (3-4): The electrophoresis pattern of PCR product for peroxiredoxin 4 genes .

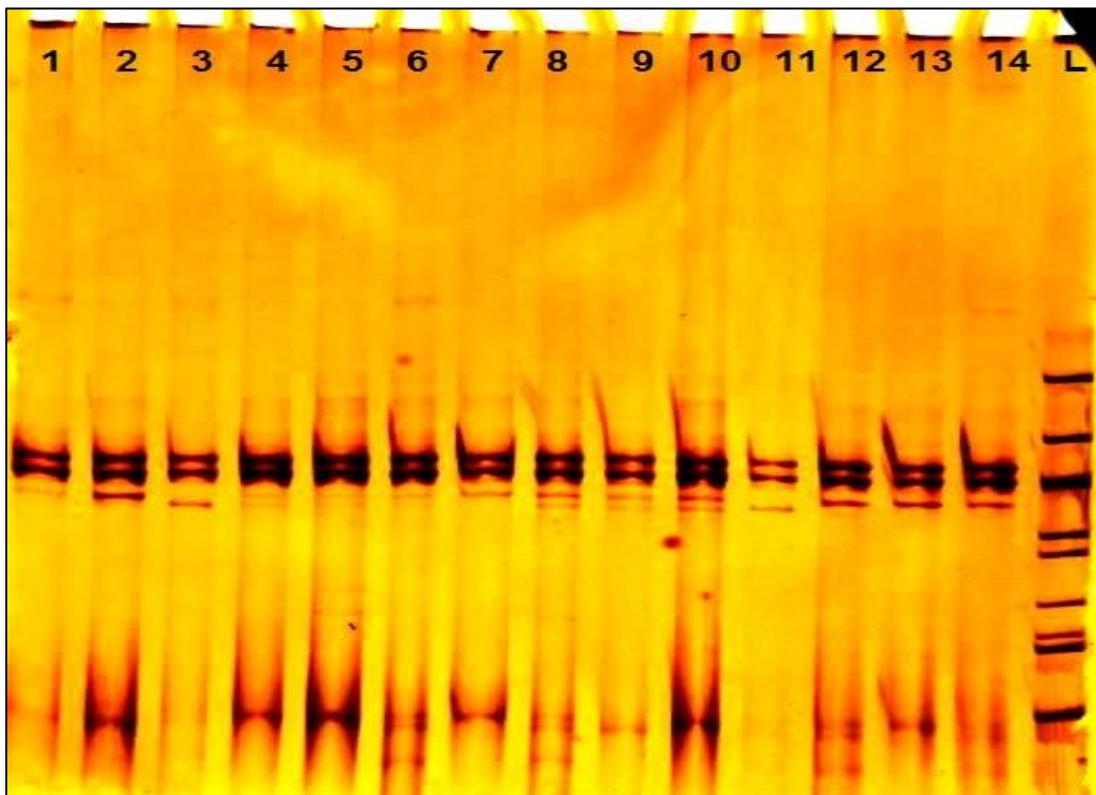


Figure (3-5) : Silver stained SSCP electrophoresis for afamin gene genotyping , lane L DNA ladder lanes 1,2,7,12,13,and 14 A pattern ; lanes 3and 11 B pattern ; other lanes C pattern .

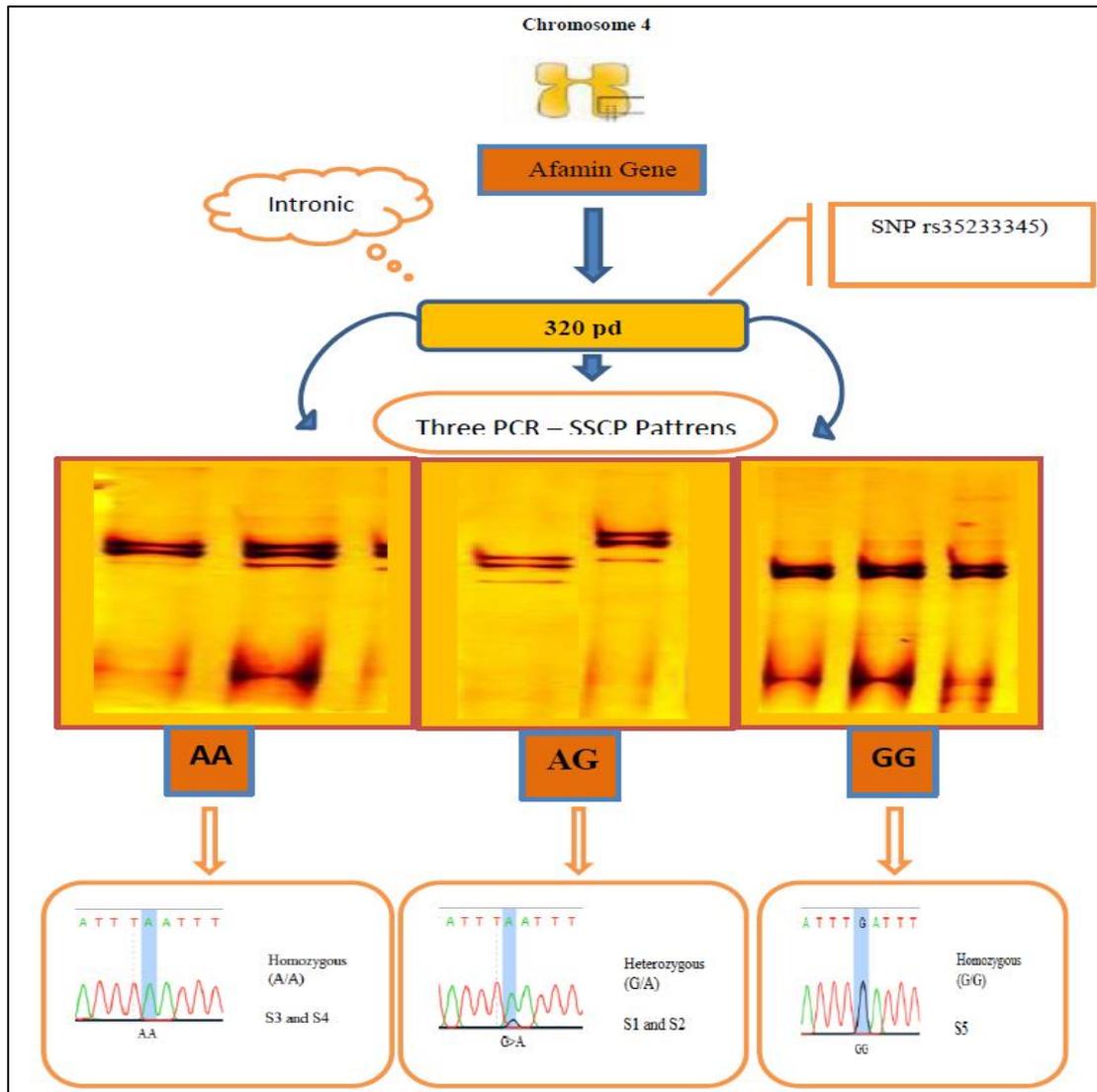


Figure (3-6) : The sequencing of amplified 320 bp gene in ((SNP rs35233345) after using specific PCR primer to detection the single nucleotide polymorphism of afamin gene

3.10.2. Sequencing of the 320 bp region within the afamin gene polymorphisms

The current study includes five samples from this locus. These samples were examined for afamin genetic sequences on chromosome 4 to see if they could be amplified. The latter gene codes for alpha-albumin, a protein that acts as a transporter for hydrophobic compounds in bodily fluids (Probable).

It is necessary for the solubility and action of "lipidated Wnt family members, links vitamin E, and transports vitamin E in body fluids" when

the lipoprotein system fails, as well as across the blood-brain barrier (<https://www.uniprot.org/uniprot/P43652>). The sequencing processes indicated their exact identification after NCBI blastn for these PCR amplicons (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The NCBI BLASTn engine found 99 percent sequence similarity between the sequenced samples and the planned reference target sequences for the alleged 320 bp amplicons. The observed DNA sequences of these studied samples were compared to the recovered DNA sequences (GenBank acc. U51243.1), the precise locations and other features of the obtained PCR fragments were identified as demonstrated in, (Figure 3-7).

Human alpha-albumin gene, complete cds

GenBank: U51243.1

[GenBank](#) [FASTA](#)

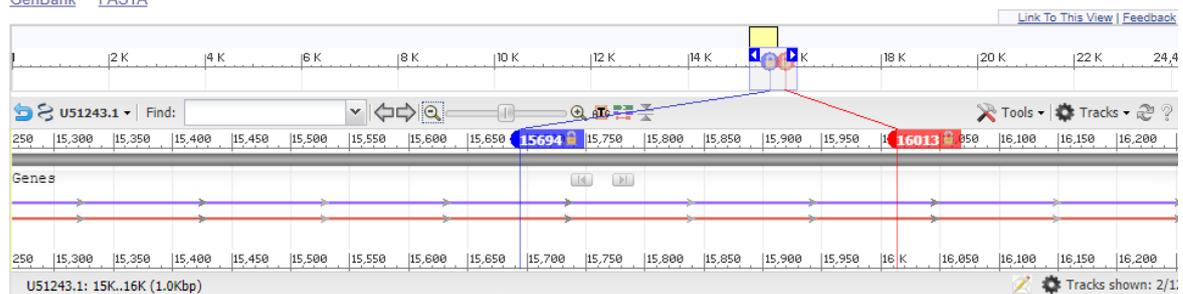


Figure (3-7): The precise location on chromosome 4 of the 320 bp amplicon that partially covered a section of the afamin gene (GenBank acc no. U51243.1). The amplicon's beginning point is indicated by the cyan arrow, while its end point is indicated by the red arrow.

Following the placement of the 320 bp amplicons' sequences on chromosome no. 4, the sequences of the 320 bp amplified amplicon's forward and reverse primers were highlighted as shown in Table (3-11).

Table (3-11): The location and length of the 320 bp PCR amplicons utilized to amplify a segment of the "afamin gene on chromosome no. 4" (GenBank

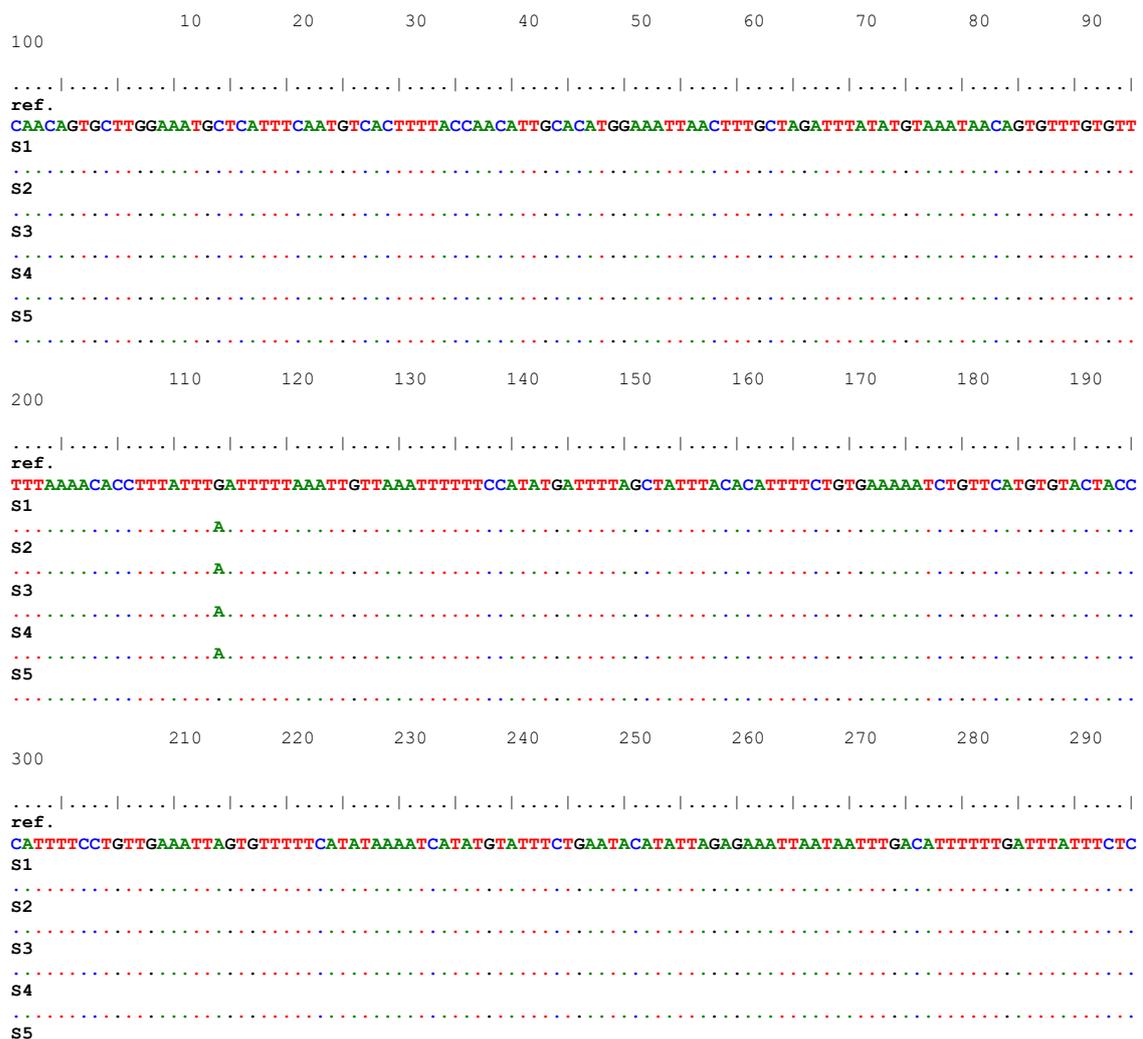
acc. no. U51243.1). The reverse and forward primer positions were indicated by the gray colored sequences.

Amplicon	Referring locus sequences (5' - 3')	length
DNA sequences within the afamin gene	*CAACAGTGCTTGGAAATGCTCATTTC AATGTCAC TTTTACCAACAT TGCACATGGAAATTAAC TTTGCTAGATTTATATGTAAATAACAGTGT TTGTGTTTTTAAAACACCTTTATTTGATTTTTTAAATTGTTAAATTTT TTCCATATGATTTTAGCTATTTACACATTTTCTGTGAAAAATCTGTT CATGTGTACTACCCATTTTCTGTTGAAATTAGTGT TTTTCATATAA AATCATATGTATTTCTGAATACATATTAGAGAAATTAATAATTTGAC ATTTTTTGATTTATTTCTCCTTCCTTTGATATGTGCTAC**	320 bp

* refers to the forward primer sequences (placed in a forward direction)

** refers to the reverse primer sequences (placed in a reverse direction)

In comparison to the referencing reference DNA sequences, the alignment results of the 320 bp samples revealed the existence of only one SNP in the majority of the tested samples, as shown in (Figure 3-8).



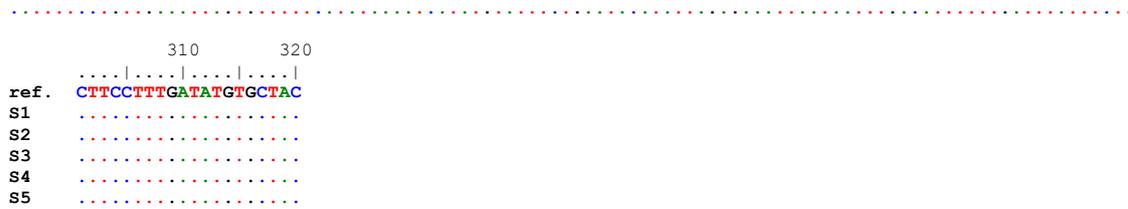


Figure (3-8): The 320 bp amplicons of the afamin genetic DNA sequences were aligned with the appropriate reference sequences of 5 samples. The letters "ref" stand for the NCBI referencing sequence, whereas "S1-S5" stand for samples 1 through 5.

In this investigation, an extremely interesting nucleic acid substitution SNP was discovered in the tissues examined. However, no further SNPs were found in the current investigation when compared to the other sequences. The detected variation region's sequencing chromatogram, as well as their extensive annotations, were noted, and the chromatogram for this sequencing was displayed according to its location in the PCR amplicon . However, Only the "homozygous and heterozygous" forms of G119A were found. as it was as shown in (Figure 3-9).

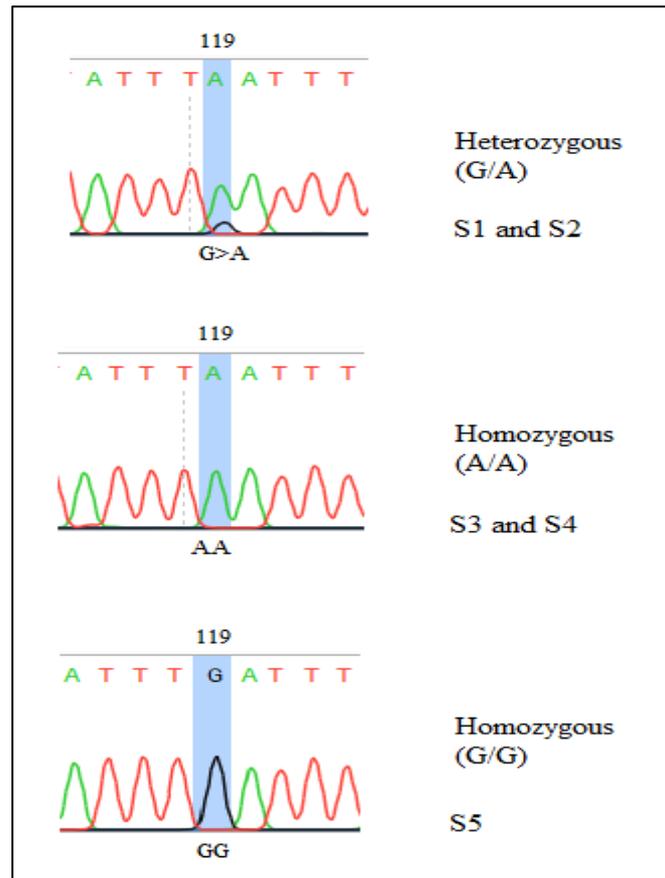


Figure (3-9): The pattern of the G119A SNP inside the DNA chromatogram of the AMF gene's targeted 320 bp amplicons. The position of the discovered SNP in the PCR amplicons was marked. The symbol “>” refers to “substitution” mutation

The AMF gene's matching position was downloaded from the dbSNP service to elucidate the locations of the targeted SNP in relation to their deposited SNP database of the sequenced 320 bp segment (<https://www.ncbi.nlm.nih.gov/projects/SNP/>). A graphical representation of the AMF dbSNP database inside chromosome no. 4 (GenBank Acc. No. NC 000002.12) was used to determine the nature of this SNP. This identified SNP was found to be previously known, rs35233345, which is suited at an intronic position, after analyzing the dbSNP engine as shown in (Figure 3-10). This SNP, however, has yet to be registered in the ClinVar database (<https://www.ncbi.nlm.nih.gov/snp/rs35233345>).

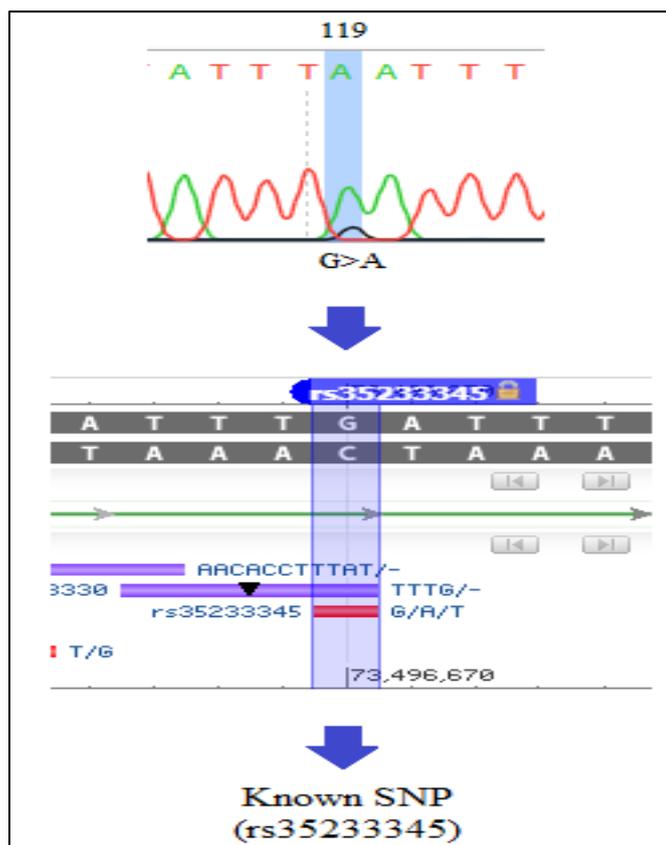


Figure (3-10): The dbSNP server was used to check the novelty of afamin genetic single nucleotide polymorphisms. The detected SNP was highlighted in red. The highlighted substitution SNP was positioned using the GenBank accession number NC 000002.12. The targeted sequences were discovered in the negative strand.

The exact sites of the detected differences in the NCBI reference sequences were given to summarize all of the results acquired from the sequenced 320 bp fragments as shown in (Table 3- 12) .

Table (3- 12): In comparison to the NCBI reference sequences (GenBank acc. no. NC 000004.12), the detected SNP pattern in the 320 bp amplicons of the afamin gene. The symbol “S” refers to the sample number.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	SNP type	Variant summary
S1,S2,S3,S4	G	A	119	73496670	Intronic	NC_000004.12;73496670G>A (Known SNP rs35233345)

The genotype distribution and allele frequencies of the afamin gene (rs35233345 G>A) polymorphisms in all patients, and in the healthy controls, are shown in Table (3- 13) for diabetes Mellitus in patients with Diabetic patients and healthy controls . The genotype frequencies were in HWE in both patients and healthy controls for afamin (rs35233345 G>A) ; No statistical differences were observed for afamin (rs35233345 G>A) polymorphisms (Table 3-15), for diabetes Mellitus in patients with Diabetic patients and healthy controls.

Table (3-13): Allelic frequency of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 allele frequencies (n=200)						
Allele	All subjects		V1=1Control		V1=2Diabetes	
	Count	Proportion	Count	Proportion	Count	Proportion
A	224	0.56	95	0.48	129	0.64
G	176	0.44	105	0.52	71	0.36

Table (3-14): genotype frequency of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 genotype frequencies (n=200)						
Genotype	All subjects		V1=1Control		V1=2Diabetes	
	Count	Proportion	Count	Proportion	Count	Proportion
A/A	58	0.29	21	0.21	37	0.37
A/G	108	0.54	53	0.53	55	0.55
G/G	34	0.17	26	0.26	8	0.08

Table (3-15): Hardy-Weinberg equilibrium test of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 exact test for Hardy-Weinberg equilibrium (n=200)						
	A/A	A/G	G/G	A	G	P-value*
All subjects	58	108	34	224	176	0.2
V1=1Control	21	53	26	95	105	0.69
V1=2Diabetes	37	55	8	129	71	0.079

* two tailed p value of chi-square

Table (3-16): Genotypic association of afamin polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 association with response V1 (n=200, crude analysis)							
Model	Genotype	V1=1Control	V1=2Diabetes	OR (95% CI)	P- value*	AIC	BIC
Codominant	A/A	21 (21%)	37 (37%)	1.00	7e-04	268.7	278.6
	A/G	53 (53%)	55 (55%)	0.59 (0.31-1.13)			
	G/G	26 (26%)	8 (8%)	0.17 (0.07-0.45)			
Dominant	A/A	21 (21%)	37 (37%)	1.00	0.012	275	281.6
	A/G-G/G	79 (79%)	63 (63%)	0.45 (0.24-0.85)			
Recessive	A/A-A/G	74 (74%)	92 (92%)	1.00	5e-04	269.3	275.9
	G/G	26 (26%)	8 (8%)	0.25 (0.11-0.58)			
Overdominant	A/A-G/G	47 (47%)	45 (45%)	1.00	0.78	281.2	287.8
	A/G	53 (53%)	55 (55%)	1.08 (0.62-1.89)			

* two tailed p value of chi-square

The genotype distribution and allele frequencies of the afamin gene (rs35233345 G>A) polymorphisms in all patients, and in the healthy controls, are shown in Table (3- 17), for diabetic nephropathy in patients with Diabetic patients and healthy controls. The genotype frequencies were in HWE in both patients and healthy controls for afamin (rs35233345 G>A); No statistical differences were observed for afamin (rs35233345 G>A) polymorphisms (Table 3-19) (Table 3-20) , for diabetic nephropathy in patients with Diabetic patients and healthy controls.

Table (3-17): Allelic frequency of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 allele frequencies (n=200)						
	All subjects		V1=1Diabetes		V1=2DN	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	250	0.62	129	0.64	121	0.6
G	150	0.38	71	0.36	79	0.4

Table (3-18): genotype frequency of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 genotype frequencies (n=200)						
	All subjects		V1=1Diabetes		V1=2DN	
Genoty	Count	Proportion	Count	Proportion	Count	Proportion

pe						
A/A	64	0.32	37	0.37	27	0.27
A/G	122	0.61	55	0.55	67	0.67
G/G	14	0.07	8	0.08	6	0.06

Table (3-19): Hardy-Weinberg equilibrium test of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 exact test for Hardy-Weinberg equilibrium (n=200)						
	A/A	A/G	G/G	A	G	P-value*
All subjects	64	122	14	250	150	<0.0001
V1=1Diabetes	37	55	8	129	71	0.079
V1=2DN	27	67	6	121	79	<0.0001

* two tailed p value of chi-square

Table (3-20): Genotypic association of afamin polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 association with response V1 (n=200, crude analysis)							
Model	Genotype	V1=1Diabetes	V1=2DN	OR (95% CI)	P-value*	AIC	BIC
Codominant	A/A	37 (37%)	27 (27%)	1.00	0.22	280.2	290.1
	A/G	55 (55%)	67 (67%)	1.67 (0.91-3.08)			
	G/G	8 (8%)	6 (6%)	1.03 (0.32-3.31)			
Dominant	A/A	37 (37%)	27 (27%)	1.00	0.13	279	285.6
	A/G-G/G	63 (63%)	73 (73%)	1.59 (0.87-2.89)			
Recessive	A/A-A/G	92 (92%)	94 (94%)	1.00	0.58	281	287.5
	G/G	8 (8%)	6 (6%)	0.73 (0.25-2.20)			
Overdominant	A/A-G/G	45 (45%)	33 (33%)	1.00	0.081	278.2	284.8
	A/G	55 (55%)	67 (67%)	1.66 (0.94-2.95)			

* two tailed p value of chi-square

Additionally, the biochemical characteristics of subject studied according to the afamin gene SNP (rs35233345 G>A) genotype are shown in Table (3-21). The results revealed that there are significant and no significant associations between AA, AG and GG polymorphisms for diabetic patients, diabetic patients with nephropathy and control group are shown in Table (3-22).

Table (3-21): Biochemical characteristics of subject studied according to the afamin gene rs 35233345 genotype.

biochemical characteristics	genotype	Healthy subjects	diabetic patients	diabetic with nephropathy
Total antioxidant levels ($\mu\text{mol/l}$)	AA	1.723 \pm 0.491	0.909 \pm 0.466	0.858 \pm 0.376
	AG	1.603 \pm 0.734	1.150 \pm 0.390	1.021 \pm 0.423
	GG	1.338 \pm 0.229	0.923 \pm 0.135	1.290 \pm 0.415
Total oxidant levels ($\mu\text{mol/l}$)	AA	1.479 \pm 0.218	1.553 \pm 0.392	1.737 \pm 0.773
	AG	1.343 \pm 0.240	1.565 \pm 0.599	1.664 \pm 0.764
	GG	1.365 \pm 0.258	1.452 \pm 0.523	1.512 \pm 0.446
malondialdehyde concentrations ($\mu\text{mol/l}$)	AA	1.827 \pm 1.034	2.418 \pm 1.321	2.268 \pm 1.399
	AG	1.591 \pm 0.782	2.622 \pm 1.052	2.568 \pm 1.278
	GG	1.617 \pm 0.884	2.176 \pm 1.159	2.649 \pm 1.115
Vitamin E (mg/dl)	AA	5.984 \pm 0.829	5.076 \pm 1.315	5.519 \pm 2.529
	AG	5.639 \pm 1.357	4.897 \pm 1.807	4.496 \pm 2.706
	GG	5.195 \pm 1.309	4.658 \pm 1.044	2.555 \pm 1.154
Catalase activity (Katal/l)	AA	140.283 \pm 57.87	126.26 \pm 57.827	126.55 \pm 71.863
	AG	166.620 \pm 64.471	85.170 \pm 22.356	89.378 \pm 46.715
	GG	167.168 \pm 37.576	103.613 \pm 56.561	63.016 \pm 21.543
Afamin ($\mu\text{g/ml}$)	AA	73.586 \pm 16.755	100.42 \pm 21.598	100.494 \pm 34.293
	AG	76.839 \pm 17.643	101.41 \pm 21.886	104.804 \pm 28.589
	GG	84.779 \pm 23.118	94.998 \pm 23.80	103.058 \pm 32.636
Prx activity (U/L) using t-BOOH as the Prx substrate	AA	72.187 \pm 34.914	97.780 \pm 50.60	100.576 \pm 44.47
	AG	73.569 \pm 33.625	143.85 \pm 32.805	136.883 \pm 40.322
	GG	75.527 \pm 31.183	130.982 \pm 15.193	126.891 \pm 20.566
Prx activity (U/L) using H ₂ O ₂ as the Prx substrate	AA	66.452 \pm 25.313	95.312 \pm 50.603	90.364 \pm 54.216
	AG	80.343 \pm 31.727	153.773 \pm 30.065	136.328 \pm 50.72
	GG	87.160 \pm 82.892	143.485 \pm 5.807	128.640 \pm 18.633
Prx 4 ($\mu\text{g/ml}$)	AA	1303.36 \pm 174.962	1481.55 \pm 130.512	1431.86 \pm 121.532
	AG	1392.299 \pm 166.06	1497.06 \pm 139.650	1496.571 \pm 112.11
	GG	1344.578 \pm 163.74	1363.74 \pm 124.273	1292.17 \pm 68.477

Table (3-22): Genotypic association of afamin polymorphisms for healthy controls ,Diabetic patients and Diabetic patients with nephropathy.

biochemical characteristics	genotype	Healthy subjects			diabetic patients			Diabetic patients with nephropathy		
		Compared groups		Sign.	Compared groups		Sign.	Compared groups		Sign.
Total antioxidant levels (µmol/l)	AA	1	2	0.289	1	2	0.013 *	1	2	0.129
			3	0.024 *		3	0.422		3	0.006 *
	AG	2	1	0.289	2	1	0.013 *	2	1	0.129
			3	0.100		3	0.557		3	0.032 *
	GG	3	1	0.024 *	3	1	0.422	3	1	0.006 *
			2	0.100		2	0.557		2	0.032 *
Total oxidant levels (µmol/l)	AA	1	2	0.006*	1	2	0.969	1	2	0.641
			3	0.061		3	0.884		3	0.819
	AG	2	1	0.006*	2	1	0.969	2	1	0.641
			3	0.492		3	0.898		3	0.994
	GG	3	1	0.061	3	1	0.884	3	1	0.819
			2	0.492		2	0.898		2	0.994
malondialdehyde concentrations (µmol/l)	AA	1	2	0.369	1	2	0.288	1	2	0.366
			3	0.591		3	0.975		3	0.255
	AG	2	1	0.369	2	1	0.288	2	1	0.366
			3	0.757		3	0.571		3	0.470
	GG	3	1	0.591	3	1	0.975	3	1	0.255
			2	0.757		2	0.571		2	0.470
Vitamin E (mg/dl)	AA	1	2	0.109	1	2	0.533	1	2	0.069
			3	0.016*		3	0.738		3	0.017 *
	AG	2	1	0.109	2	1	0.533	2	1	0.069
			3	0.213		3	0.487		3	0.115
	GG	3	1	0.016*	3	1	0.738	3	1	0.017 *
			2	0.213		2	0.487		2	0.115
Catalase activity (Katal/l)	AA	1	2	0.104	1	2	0.000 *	1	2	0.002 *
			3	0.085		3	0.122		3	0.013 *
	AG	2	1	0.104	2	1	0.000 *	2	1	0.002 *
			3	0.714		3	0.319		3	0.312
	GG	3	1	0.085	3	1	0.122	3	1	0.013 *
			2	0.714		2	0.319		2	0.312
Afamin (µg/ml)	AA	1	2	0.814	1	2	0.883	1	2	0.483
			3	0.066		3	0.561		3	0.597
	AG	2	1	0.814	2	1	0.883	2	1	0.483
			3	0.046*		3	0.496		3	0.854
	GG	3	1	0.066	3	1	0.561	3	1	0.597
			2	0.046*		2	0.496		2	0.854
Prx activity (U/L) using t-BOOH as the Prx substrate	AA	1	2	0.995	1	2	0.000 *	1	2	0.000 *
			3	0.725		3	0.001 *		3	0.007 *
	AG	2	1	0.995	2	1	0.000 *	2	1	0.000 *
			3	0.672		3	0.709		3	0.367
	GG	3	1	0.725	3	1	0.001 *	3	1	0.007 *
			2	0.672		2	0.709		2	0.367
Prx activity (U/L) using H ₂ O ₂ as the Prx substrate	AA	1	2	0.088	1	2	0.000 *	1	2	0.000 *
			3	0.009*		3	0.000 *		3	0.008 *
	AG	2	1	0.088	2	1	0.000 *	2	1	0.000 *
			3	0.163		3	0.567		3	0.417
	GG	3	1	0.009*	3	1	0.000 *	3	1	0.008 *
			2	0.163		2	0.567		2	0.417
Prx 4 (µg/ml)	AA	1	2	0.169	1	2	0.995	1	2	0.203
			3	0.446		3	0.753		3	0.200
	AG	2	1	0.169	2	1	0.995	2	1	0.203
			3	0.581		3	0.748		3	0.498
	GG	3	1	0.446	3	1	0.753	3	1	0.200
			2	0.581		2	0.748		2	0.498

*. The mean difference is significant at the 0.05 level.

3.10.3. Distribution of genotype and allele frequencies of peroxiredoxin 4 gene polymorphisms

The results showed that the present of three different haplotypes (CC,GC,GG) as revealed in Figure (3-11).

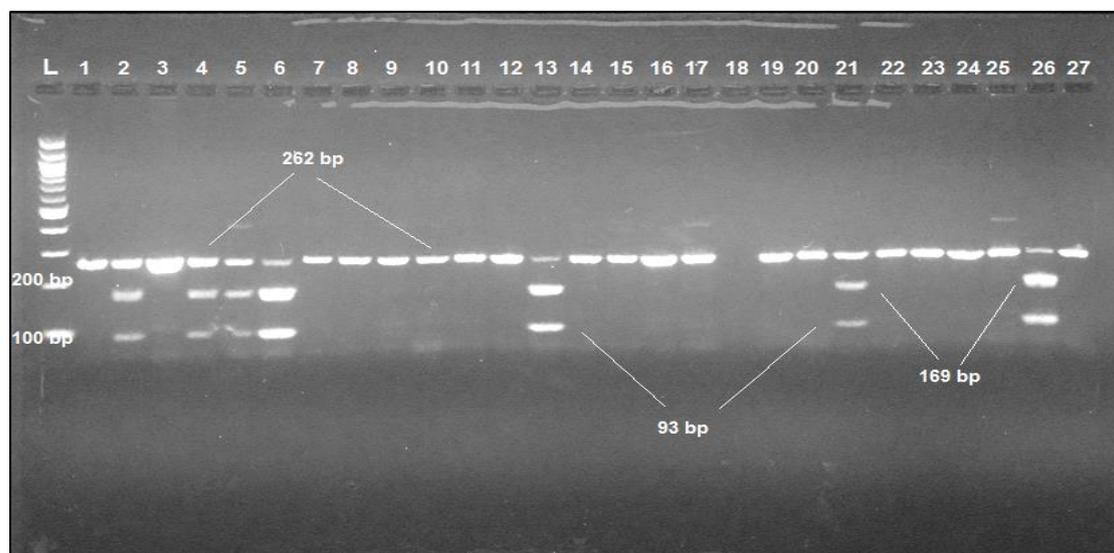


Figure (3-11): Polymerase Chain Reaction-Restriction fragment Length Polymorphism (PCR-RFLP) for peroxiredoxin 4 genes .

The genotype distribution and allele frequencies of the peroxiredoxin 4 gene (rs 35233345 G>C) polymorphisms in all patients, and in the healthy controls, are shown in Table (3- 24) , for diabetes Mellitus in patients with Diabetic patients and healthy controls .The genotype frequencies were in HWE in both patients and healthy controls for peroxiredoxin 4 (rs 564561 G> C); No statistical differences were observed for peroxiredoxin 4 (rs 564561 G> C) polymorphisms (Table 3-26) (Table 3-27), for diabetes Mellitus in patients with diabetic patients and healthy controls.

Table (3-24): Allelic frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 allele frequencies (n=200)						
Allele	All subjects		V1=1Control		V1=2Diabetes	
	Count	Proportion	Count	Proportion	Count	Proportion
C	336	0.84	168	0.84	168	0.84
G	64	0.16	32	0.16	32	0.16

Table (3-25): genotype frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 genotype frequencies (n=200)						
Genotype	All subjects		V1=1Control		V1=2Diabetes	
	Count	Proportion	Count	Proportion	Count	Proportion
C/C	164	0.82	82	0.82	82	0.82
C/G	8	0.04	4	0.04	4	0.04
G/G	28	0.14	14	0.14	14	0.14

Table (3-26): Hardy-Weinberg equilibrium test of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 exact test for Hardy-Weinberg equilibrium (n=200)						
	C/C	C/G	G/G	C	G	P-value*
All subjects	164	8	28	336	64	<0.0001
V1=1Control	82	4	14	168	32	<0.0001
V1=2Diabetes	82	4	14	168	32	<0.0001

* two tailed p value of chi-square

Table (3-27): Genotypic association of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetes Mellitus in patients with Diabetic patients and healthy controls.

V2 association with response V1 (n=200, crude analysis)							
Model	Genotype	V1=1Control	V1=2Diabetes	OR (95% CI)	P-value*	AIC	BIC
Codominant	C/C	82 (82%)	82 (82%)	1.00	1	283.3	293.2
	G/C	4 (4%)	4 (4%)	1.00 (0.24-4.13)			
	G/G	14 (14%)	14 (14%)	1.00 (0.45-2.23)			
Dominant	C/C	82 (82%)	82 (82%)	1.00	1	281.3	287.9
	G/C-G/G	18 (18%)	18 (18%)	1.00 (0.49-2.06)			
Recessive	C/C-G/C	86 (86%)	86 (86%)	1.00	1	281.3	287.9
	G/G	14 (14%)	14 (14%)	1.00 (0.45-2.22)			
Overdominant	C/C-G/G	96 (96%)	96 (96%)	1.00	1	281.3	287.9
	G/C	4 (4%)	4 (4%)	1.00 (0.24-4.11)			

* two tailed p value of chi-square

The genotype distribution and allele frequencies of the peroxiredoxin 4 gene (rs 564561 G>C) polymorphisms in all patients, and in the healthy controls, are shown in Table (3- 28), for diabetic nephropathy in patients with Diabetic patients and healthy controls. The genotype frequencies were in HWE in both patients and healthy controls for peroxiredoxin 4 (rs564561

G>C); No statistical differences were observed for peroxiredoxin 4 (rs 564561 G>C) polymorphisms (Table 3-29) (Table 3-30) , for diabetic nephropathy in patients with Diabetic patients and healthy controls.

Table (3-28): Allelic frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 allele frequencies (n=200)						
Allele	All subjects		V1=1Diabetes		V1=2DN	
	Count	Proportion	Count	Proportion	Count	Proportion
C	336	0.84	168	0.84	168	0.84
G	64	0.16	32	0.16	32	0.16

Table (3-29): genotype frequency of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 genotype frequencies (n=200)						
Genotype	All subjects		V1=1Diabetes		V1=2DN	
	Count	Proportion	Count	Proportion	Count	Proportion
C/C	161	0.8	82	0.82	79	0.79
C/G	14	0.07	4	0.04	10	0.1
G/G	25	0.12	14	0.14	11	0.11

Table (3-30): Hardy-Weinberg equilibrium test of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 exact test for Hardy-Weinberg equilibrium (n=200)						
	C/C	C/G	G/G	C	G	P-value*
All subjects	161	14	25	336	64	<0.0001
V1=1Diabetes	82	4	14	168	32	<0.0001
V1=2DN	79	10	11	168	32	<0.0001

* two tailed p value of chi-square

Table (3-31): Genotypic association of peroxiredoxin 4 polymorphisms and their associations with risks of Diabetic nephropathy in patients with Diabetic patients.

V2 association with response V1 (n=200, crude analysis)							
Model	Genotype	V1=1Diabetes	V1=2DN	OR (95% CI)	P-value*	AIC	BIC
Codominant	C/C	82 (82%)	79 (79%)	1.00	0.22	280.2	290.1
	G/C	4 (4%)	10 (10%)	2.59 (0.78-8.62)			
	G/G	14 (14%)	11 (11%)	0.82 (0.35-1.90)			
Dominant	C/C	82 (82%)	79 (79%)	1.00	0.59	281	287.6
	G/C-G/G	18 (18%)	21 (21%)	1.21 (0.60-2.44)			
Recessive	C/C-G/C	86 (86%)	89 (89%)	1.00	0.52	280.8	287.4

	G/G	14 (14%)	11 (11%)	0.76 (0.33-1.76)			
Overdominant	C/C-G/G	96 (96%)	90 (90%)	1.00	0.091	278.4	285
	G/C	4 (4%)	10 (10%)	2.67 (0.81-8.81)			

* two tailed p value of chi-square

Additionally, the biochemical characteristics of subject studied according to the peroxiredoxin 4 gene SNP (rs 564561 G>C) genotype are shown in Table (3- 32). The results revealed that there are significant and no significant associations between CC, GC and GG polymorphisms for diabetic patients, diabetic patients with nephropathy and control group are shown in Table (3- 33) .

Table (3-32) Biochemical characteristics of subject studied according to peroxiredoxin 4 genes Rs 564561 genotype.

biochemical characteristics	genotype	Healthy subjects	diabetic patients	diabetic with nephropathy
Total antioxidant levels (µmol/l)	CC	1.645 ± 0.646	1.023 ± 0.436	0.958 ± 0.411
	GC	1.034 ± 0.238	0.938 ± 0.142	1.151 ± 0.453
	GG	1.270 ± 0.210	1.133 ± 0.328	1.130 ± 0.424
Total oxidant levels (µmol/l)	CC	1.376 ± 0.245	1.554 ± 0.502	1.706 ± 0.791
	GC	1.137 ± 0.261	1.409 ± 0.386	1.298 ± 0.278
	GG	1.423 ± 0.241	1.571 ± 0.667	1.786 ± 0.691
malondialdehyde concentrations (µmol/l)	CC	1.724 ± 0.875	2.559 ± 1.163	2.484 ± 1.322
	GC	0.591 ± 0.229	2.633 ± 1.703	2.611 ± 1.114
	GG	1.641 ± 0.787	2.390 ± 1.065	2.354 ± 1.214
Vitamin E (mg/dl)	CC	5.855 ± 1.249	5.196 ± 1.569	4.978 ± 2.682
	GC	4.122 ± 0.518	3.002 ± 0.359	3.444 ± 3.177
	GG	4.422 ± 1.242	4.281 ± 1.670	2.852 ± 1.068
Catalase activity (Katal/l)	CC	161.133 ± 57.28	105.87 ± 48.153	106.242 ± 60.90
	GC	135.847 ± 64.48	73.083 ± 20.529	78.475 ± 23.00
	GG	165.860 ± 61.44	80.44 ± 31.30	69.145 ± 21.40
Afamin (µg/ml)	CC	79.217 ± 19.43	101.99 ± 21.673	106.324 ± 31.08
	GC	67.668 ± 18.292	71.24 ± 25.946	100.391 ± 24.86
	GG	74.889 ± 20.260	97.459 ± 22.532	91.693 ± 28.623
Prx activity (U/L) using t-BOOH as the Prx substrate	CC	73.186 ± 33.930	123.799 ± 48.621	122.55 ± 46.65
	GC	72.988 ± 34.632	119.27 ± 21.772	135.49 ± 19.801
	GG	80.698 ± 26.279	153.54 ± 17.557	139.709 ± 18.97
Prx activity (U/L) using H ₂ O ₂ as the Prx substrate	CC	78.204 ± 30.139	126.851 ± 50.204	116.781 ± 57.97
	GC	63.812 ± 33.276	118.446 ± 16.915	136.612 ± 17.69
	GG	87.234 ± 31.779	153.04 ± 6.811	139.844 ± 17.78
Prx 4 (µg/ml)	CC	1389.1 ± 165.36	1503.34 ± 137.53	1495.08 ± 115.75
	GC	1078.06 ± 179.23	1208.91 ± 160.85	1365.576 ± 118.60
	GG	1325.9 ± 168.59	1414.71 ± 112.42	1363.801 ± 97.160

Table (3-33): Genotypic association of peroxiredoxin 4 polymorphisms for healthy controls ,Diabetic patients and Diabetic patients with nephropathy.

biochemical characteristics	genotype	Healthy subjects			diabetic patients			Diabetic patients with nephropathy		
		Compared groups		Sign.	Compared groups		Sign.	Compared groups		Sign.
Total antioxidant levels (µmol/l)	CC	1	2	0.181	1	2	0.500	1	2	0.091
			3	0.093		3	0.182		3	0.087
	GC	2	1	0.181	2	1	0.500	2	1	0.091
			3	0.725		3	0.941		3	0.969
	GG	3	1	0.093	3	1	0.182	3	1	0.087
			2	0.725		2	0.941		2	0.969
Total oxidant levels (µmol/l)	CC	1	2	0.510	1	2	0.517	1	2	0.316
			3	0.067		3	0.575		3	0.530
	GC	2	1	0.510	2	1	0.517	2	1	0.316
			3	0.728		3	0.667		3	0.218
	GG	3	1	0.067	3	1	0.757	3	1	0.530
			2	0.728		2	0.669		2	0.318
malondialdehyde concentrations (µmol/l)	CC	1	2	0.041 *	1	2	0.516	1	2	0.638
			3	0.703		3	0.912		3	0.957
	GC	2	1	0.041 *	2	1	0.516	2	1	0.638
			3	0.097		3	0.520		3	0.747
	GG	3	1	0.703	3	1	0.912	3	1	0.957
			2	0.97		2	0.520		2	0.747
Vitamin E (mg/dl)	CC	1	2	0.468	1	2	0.087	1	2	0.465
			3	0.003 *		3	0.129		3	0.019 *
	GC	2	1	0.468	2	1	0.087	2	1	0.465
			3	0.373		3	0.437		3	0.237
	GG	3	1	0.003 *	3	1	0.129	3	1	0.019 *
			2	0.373		2	0.437		2	0.237
Catalase activity (Katal/l)	CC	1	2	0.809	1	2	0.456	1	2	0.175
			3	0.384		3	0.128		3	0.045 *
	GC	2	1	0.809	2	1	0.456	2	1	0.175
			3	0.821		3	0.915		3	0.654
	GG	3	1	0.384	3	1	0.128	3	1	0.045 *
			2	0.821		2	0.915		2	0.645
Aflamin (µg/ml)	CC	1	2	0.912	1	2	0.567	1	2	0.952
			3	0.902		3	0.840		3	0.242
	GC	2	1	0.912	2	1	0.576	2	1	0.952
			3	0.970		3	0.543		3	0.414
	GG	3	1	0.902	3	1	0.840	3	1	0.242
			2	0.970		2	0.543		2	0.414
Prx activity (U/L) using t-BOOH as the Prx substrate	CC	1	2	0.332	1	2	0.394	1	2	0.099
			3	0.342		3	0.137		3	0.045 *
	GC	2	1	0.332	2	1	0.394	2	1	0.099
			3	0.695		3	0.993		3	0.875
	GG	3	1	0.342	3	1	0.137	3	1	0.045 *
			2	0.695		2	0.993		2	0.875
Prx activity (U/L) using H ₂ O ₂ as the Prx substrate	CC	1	2	0.489	1	2	0.193	1	2	0.050 *
			3	0.167		3	0.006 *		3	0.029*
	GC	2	1	0.489	2	1	0.193	2	1	0.050
			3	0.935		3	0.806		3	0.916 *
	GG	3	1	0.167	3	1	0.006 *	3	1	0.029 *
			2	0.935		2	0.806		2	0.916
Prx 4 (µg/ml)	CC	1	2	0.146	1	2	0.768	1	2	0.933
			3	0.636		3	0.863		3	0.718
	GC	2	1	0.146	2	1	0.768	2	1	0.933
			3	0.121		3	0.723		3	0.741
	GG	3	1	0.636	3	1	0.863	3	1	0.718
			2	0.121		2	0.723		2	0.741

*. The mean difference is significant at the 0.05 level.

3.11. Assessment Catalase Activity

The current method utilized the reaction between a hydroquinone/anilinium sulfate/ammonium molybdate reagent and unreacted hydrogen peroxide, resulting in the formation of a purple, disubstituted quinone compound with a maximum absorbance at 550 nm (Hadwan and Abid, 2015). The method takes place in a neutral pH, and this is an additional positive feature. In the present method, the hydroquinone/ anilinium sulfate/ammonium molybdate reagent acts as a catalase enzymatic reaction stop bath. Ammonium molybdate reagent was used to halt the catalase reaction *via* consuming all the hydrogen peroxide molecules. Ammonium molybdate reacts with H₂O₂ molecule that is not dissociated by the catalase enzyme to form singlet oxygen (1O₂). Subsequently, 1O₂ molecules react with reduced hydroquinone to form the oxidized form in the presence of aniline and molybdate to form a purple, disubstituted quinone compound (**Figure. 3-12 A**). A reduction in the intensity of the purple disubstituted quinone compound is associated with increased catalase activity (**Figure. 3-12 B**). The spectrum of the resulting disubstituted quinone compound was scanned from 330 nm to 700 nm, and the maximum absorbance was observed at 550 nm (**Figure. 3-12 C**); therefore, this peak was used to measure catalase activity.

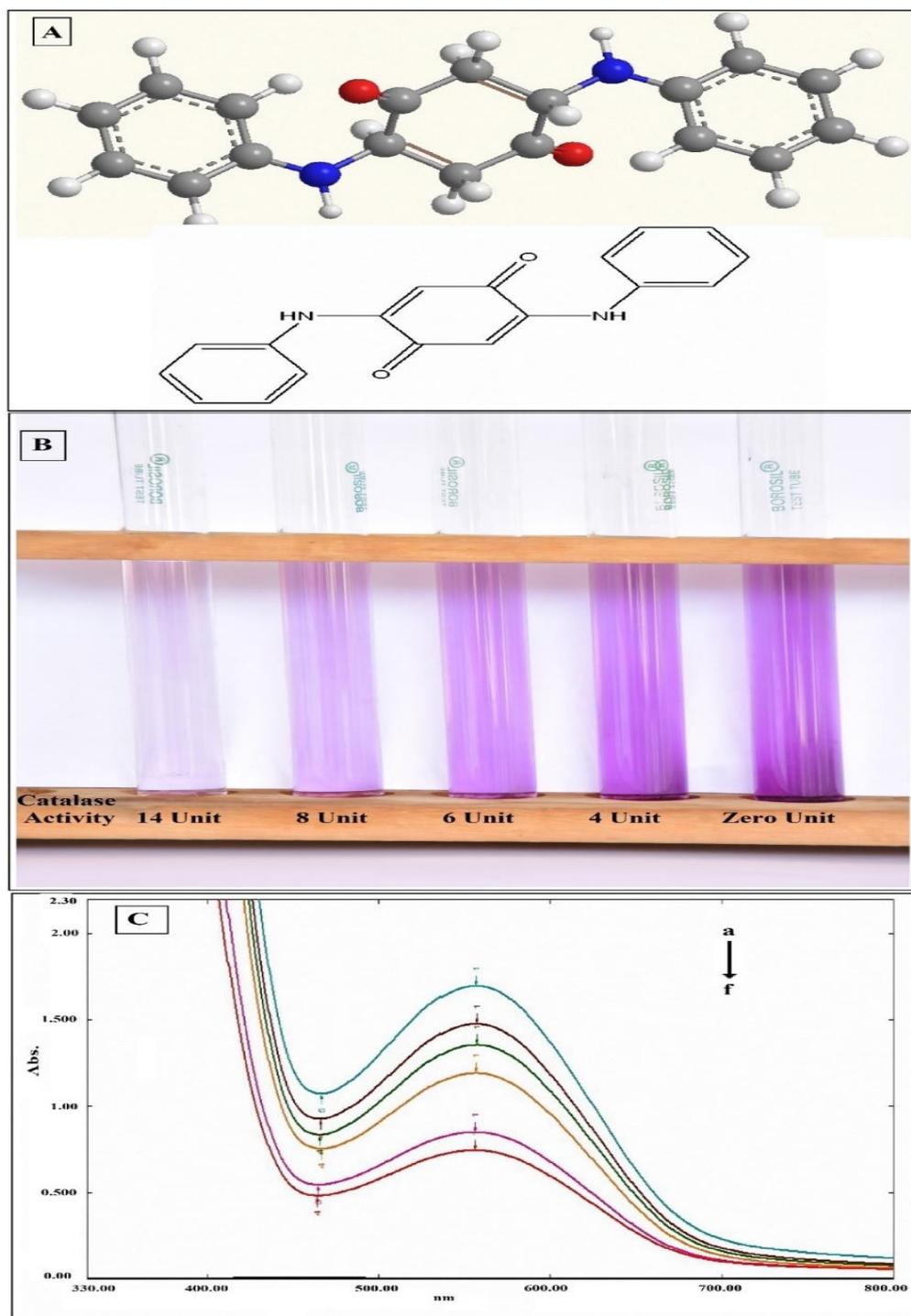


Figure (3-12) : Catalase enzyme activity correlated with the spectrophotometric properties of the disubstituted quinone. **A** The disubstituted quinone compound. **B** Catalase enzyme activity was linked to reductions in color intensity of the resulting disubstituted quinone compound. **C** Absorption spectra achieved for the resulting disubstituted quinone compound: (a) 16 mM H_2O_2 ; (b) 15 mM H_2O_2 ; (c) 14 mM H_2O_2 ;

(d) 12 Mm H₂O₂; (e) 8 mM H₂O₂; and (f) 6 mM H₂O₂. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

The optimal incubation time for the measurement of catalase enzyme activity was assessed by determining the activity of 5 units of catalase activity (HiMedia company; the code product: TC037, India) using the current method. In the current study, the catalase enzyme solution was prepared in cold phosphate buffer (pH 7; 50 mM). The resulting activity was standardized using the peroxovanadate protocol, as described by Hadwan and Ali (Hadwan and Ali , 2018) . (Table 3-34) shows that the optimal incubation time was determined to be 120 sec.

Table 3-34 : Correlation between incubation time and catalase activity.

Prepared catalase enzyme activity	5	5	5	5	5	5
Incubation	60	120 ^b	180	240	300	360
Obtained catalase enzyme activity ^a	3.0 ± 0.5	5.1 ± 0.1	5.0 ± 0.35	4.8 ± 0.3	4,8 ± 0.5	4,8 ± 0.3

^a mean of triplicate determinations.

^b optimal incubation time.

The protocol described by Hadwan and Abid (Hadwan and Abid , 2015) was used to determine the potential interference of the chemicals used during the current method for the assessment of catalase activity. In brief, 9 ml solutions of potentially interfering chemicals (Table 3-35), were prepared in phosphate buffer solution (pH 7.4; 50 mM) and mixed with 1 ml catalase enzyme solutions with previously determined activity levels (30 U/mL).

Table 3-35: The probable interference of selected chemicals on the assessment of catalase enzyme activity using the disubstituted quinone method.

Chemicals	Concentration of chemical	Added catalase Unit	Found catalase Unit	Relative error (%)
Glucose	120 mg dl ⁻¹	3	3.02	0.66
Cellulose	120 mg dl ⁻¹	3	3.02	0.66
Glycine	50 μM	3	3.04	1.3
Aspartic acid	50 μM	3	3.01	0.33
Lysine	50 μM	3	3.04	1.3
Methionine	50 μM	3	3.01	0.33
Albumin	50 mg/ml	3	3.02	0.66
Ascorbic acid	50 μM	3	3.07	2.3
Uric Acid	50 μM	3	3.05	1.6
Triton x 100	0.5 % v/v	3	2.9	3.3
EDTA	20.0 μM	3	3	0.0
Heparin	78.4 USP/10 mL	3	3	0.0

The peroxovanadate method was used to calibrate the observed catalase activity. The final enzyme activity was determined to be 3 U·mL⁻¹. (Table 3-35) lists the effects of all examined potentially interfering chemicals on the assessment of catalase activity. The reliability of the disubstituted quinone method was also examined, using homogenized red blood cells as a source of catalase activity. The disubstituted quinone method was used to evaluate catalase enzyme activity, and the results were compared with those using the peroxovanadate protocol (Hadwan and Ali , 2018). Similar buffers, samples, and reagents were utilized during both methods. (Table 3-36) demonstrates that the disubstituted quinone assay has good precision.

Table 3-36 : Precision of the disubstituted quinone method.

	n.	Mean (±SD): U.mL ⁻¹	95% Confidence Interval	CV%
Within-run	20	7.5 ± 0.2	(7.41 to 7.59)	2.6%
Between-run	20	7.38 ± 0.35	(7.15 to 7.45)	4.7%

In addition, the data obtained from the disubstituted quinone method were significantly associated with the data obtained from the peroxovanadate assay, as shown in (Table 3-37).

Table 3-37 : The statistical correlation between the catalase activity assessment that obtained by applied the disubstituted quinone method and the peroxovanadium method.

The numbers of measurements	20
Mean of catalase activity that assessed by the present method U.mL ⁻¹ .	5.74
Mean of catalase activity that assessed by the peroxovanadate method U.mL ⁻¹ .	5.67
Mean of catalase activity that assessed by both methods U.mL ⁻¹ .	5.7
The regression coefficient B	0.9863
The regression coefficient A	-0.101
The correlation coefficient	1.006

The recovery of added known catalase enzyme activities (HiMedia company; the code product: TC037, India) was used to determine the accuracy of the current method. In these experiments, the catalase enzyme solution was prepared in cold phosphate buffer (pH 7; 50 mM), and the resulting activity was standardized using the peroxovanadate method. The recovery of catalase enzyme activity was equal to 92% in the presence of 10 U·mL⁻¹ of enzyme and increased to 97.15% when the activity of the enzyme was varied between 0.5 to 6.5 U·mL⁻¹ (Table 3-38).

Table 3-38 : The recovery percentage of catalase enzyme activity after addition a suitable activities of catalase to enzymatic reaction solutions.

Contents of catalase enzyme	Catalase enzyme activity added U mL ⁻¹	Catalase enzyme calculated activity U mL ⁻¹	Catalase enzyme observed activity ^a U mL ⁻¹	Recovery %
Enzymatic sample	-----	-----	3.0	-----
Catalase enzyme added + enzymatic sample	0.5	3.5	3.6	-2.85
Catalase enzyme added + enzymatic sample	1.0	4.0	4.1	-2.5
Catalase enzyme added + enzymatic sample	2.0	5.0	4.9	2
Catalase enzyme added + enzymatic sample	3.0	6.0	6.1	-1.66
Catalase enzyme added + enzymatic sample	4.0	7.0	7.05	-0.7
Catalase enzyme added + enzymatic sample	5.0	8.0	7.85	1.87
Catalase enzyme added + enzymatic sample	6.0	9.0	8.9	1.11
Catalase enzyme added + enzymatic sample	6.5	9.5	9.1	4.2
Catalase enzyme added + enzymatic sample	7.0	10	9.2	8
^a mean of triplicate determinations				

Various dilutions of red blood cell homogenates were used to evaluate the sensitivity of the disubstituted quinone method. The sensitivity was determined by comparing the measured catalase activity against the expected catalase activity, as shown in **Figure (3-13)**. The present activity was adjusted using the peroxovanadate method, whereas the expected activity was measured using the disubstituted quinone method. The correlation between the measured catalase activity and the expected catalase activity was highly linear ($r = 0.9982$), and the best linear curve passed through the origin.

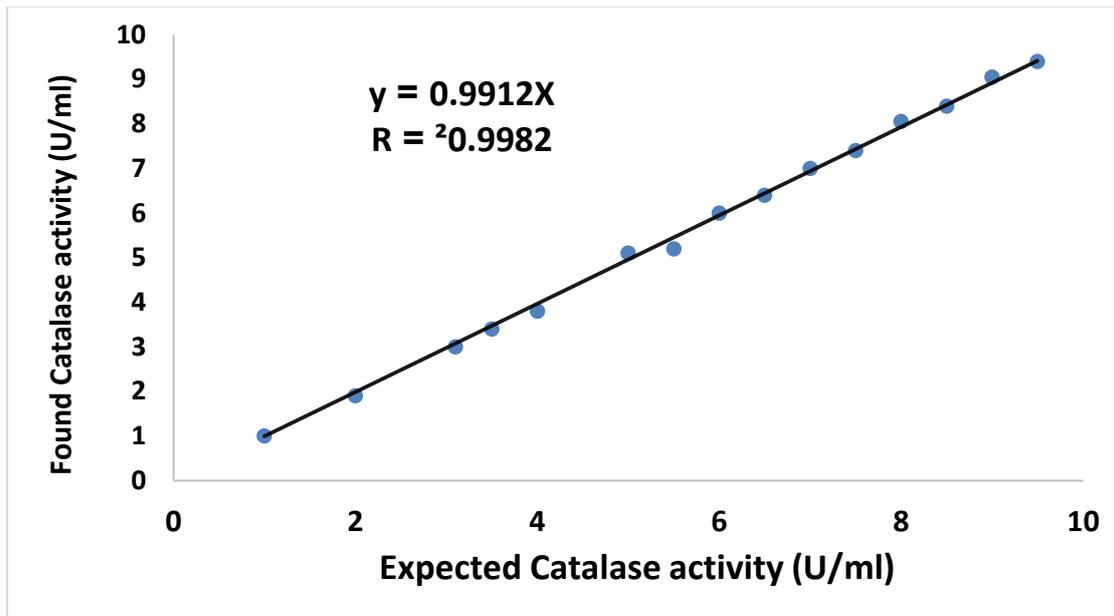


Figure (3-13) :The comparison between catalase enzyme activity of RBC homogenates that assessed by utilize the disubstituted quinone method and peroxovanadate method.

Catalase activity assays were then performed using lysates from five different bacterial laboratory strains to clarify the further potential applications of the disubstituted quinone method. The results of these experiments indicated that similar catalase enzyme activities were obtained using the disubstituted quinone method as were obtained using the peroxovanadate method in bacterial strains (Hadwan and Ali , 2018) . In agreement with their findings, our results (Table 3-42) showed that *Staphylococcus aureus* has higher catalase enzyme activity than other types of bacteria.

Table 3-39: Comparison between the disubstituted quinone and peroxyvanadate methods for the assessment of catalase activities (KU) in different types of bacteria.

Name of Bacteria	Peroxyvanadate Method	Disubstituted Quinone Method
Staphylococcus aureus	15.5	15.2
Pseudomonas aeruginosa	12.0	12.3
Escherichia coli	7.7	7.5
Klebsiella pneumonia	12.0	12.7
Enterococcus faecalis	0	0

The disubstituted quinone method was utilized to assess catalase enzyme activities in liver tissue homogenates from male albino mice, male albino rats, and broiler chickens. Liver tissue homogenates exhibited high catalase enzyme activities **Figure (3- 14)**.

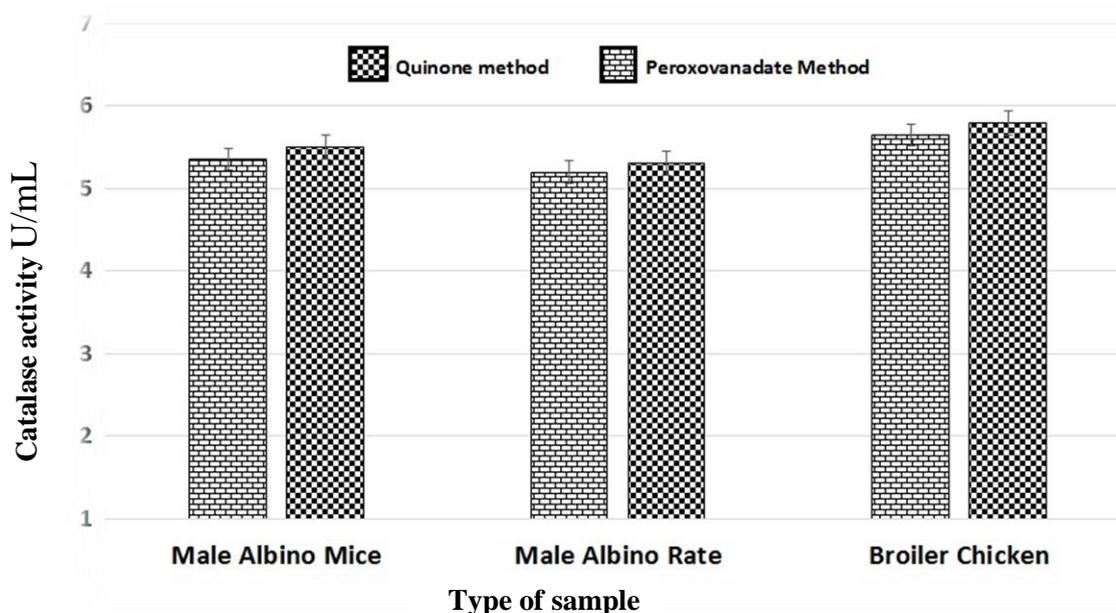


Figure (3-14) :The comparison of the disubstituted quinone method and peroxyvanadate methods for assessment of catalase activities in (1-500) dilution of homogenate tissues.

The assessment of catalase activity represents a good method for determining the ability of the liver to resist oxidative stress (Shin et al ., 2019) , and many scientific reports have investigated catalase enzyme activities in the livers of albino rats and mice (Adisa et al ., 2019 ;

Aldulaimi and Husain , 2019) . In parallel, the evaluation of oxidative stress in broiler chickens has been used to assess catalase activities in the liver (Qadri et al ., 2019 ; Baradaran et al ., 2019) . Moreover, utilized catalase activity measurements from the livers of broiler chickens to study the effects of *Lactobacillus plantarum* 299v consumption on growth and aflatoxin-related oxidative damage in the liver (Khanian et al ., 2019) . In addition, utilized catalase measurements of broiler chicken livers to clarify the effects of rutin on growth performance, antioxidant capacity, lipidrelated transcript expression, and the economics of broiler chickens (Hassan et al ., 2018) .

The disubstituted quinone assay presents several improvements over other protocols used to assess catalase enzyme activities in biological tissues. First, this method is free from the negative characteristics associated with the UV spectrophotometric method. The disubstituted quinone method requires a hydrogen peroxide concentration equal to 10 mM, in contrast with the UV spectrophotometric method, which requires a hydrogen peroxide concentration equal to 30 mM, which is a high enough concentration to inhibit catalase by modifying its active site structure (Mueller et al ., 1997) . Additionally, proteins and DNA molecules can absorb UV light during the UV spectrophotometric method, whereas the disubstituted quinone assay is based upon the decreased absorbance of the characteristic disubstituted quinone band at 550 nm, which is not in the UV spectrum. The disubstituted quinone protocol could be made accessible as assay kits, does not require the use of cumbersome techniques, and is inexpensive. Additionally, the method is simple, shows high precision, and can be applied at low H₂O₂ concentrations and in the presence of significant quantities of several types of biochemicals without interference.

3.12. Assessment Peroxiredoxin Activity

Prx containing samples were incubated with suitable concentrations of peroxide and DTT that dissolved in HEPES buffer (pH7.0; 25mM). A working reagent containing of ammonium ferrous sulfate (FAS) and amino salicylic acid (ASA) was used to stop prx enzymatic reaction. Unreacted residual peroxide acts to oxidize Fe^{+2} to Fe^{+3} , which will be complexed with aminosalicilyc acid (ASA) to form a brown -colored aminosalicylate complex Figure (3-15), with a λ_{max} at 425 nm.

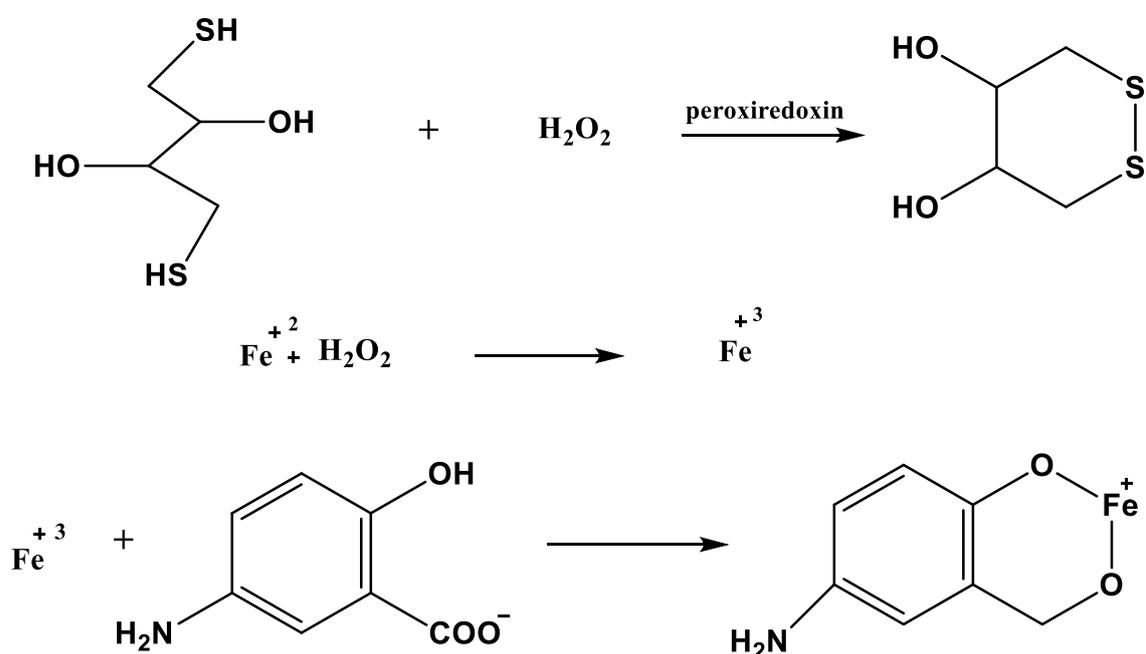


Figure (3-15). Peroxiredoxin estimated reaction. The final product includes the production of the brown ferriaminosalicylate complex.

The ferriaminosalicylate complex has a single peak at 425 nm. The residual peroxide concentrations were directly proportional to absorbance of the formed complex as shown in **Figure (3-16)**.

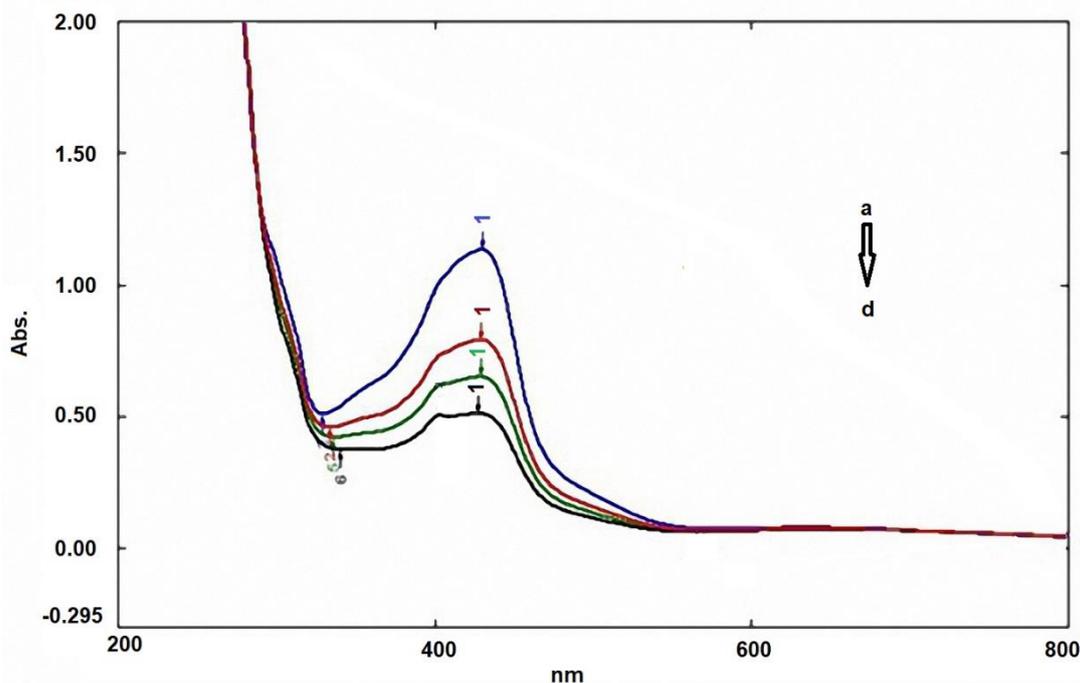


Figure (3-16). A spectrophotometric spectrum of the ferriaminosalicylate complex. The residual peroxide concentrations were directly proportional to absorbance of the formed complex: (a) 200 μM H_2O_2 ; (b) 125 μM H_2O_2 ; (c) 100 μM H_2O_2 ; (d) 75 μM H_2O_2 .

This assay is appropriate for peroxiredoxin enzyme and it can be used to estimate peroxide concentrations as low as 1 to 1000 μM . The colored end product is very stable and its absorbance at 425 nm does not change for more than three hours.

Peroxiredoxin activities of liver tissue homogenate were determined using the aminosalicylate method. Liver peroxiredoxin activity was previously used to estimate oxidative stress in Rats (Freitas et al., 2016). Moreover, peroxiredoxin protects against alcohol-induced oxidative injury in mouse liver (Bae et al., 2011). Previous studies demonstrated that consumption of chronic ethanol in rat results in the hyper-oxidation of peroxiredoxin I, but not other peroxiredoxin (Bae et al., 2011), specific inactivation of peroxiredoxin I was colocalization of a large ratio of CYP2E1 with peroxiredoxin I of the ER membrane at the cytosolic side (Bae et al., 2013)

. Moreover, the accumulation of the acidic inactive form of peroxiredoxin III in rat's liver might decreased the protease activities that involved in the degradation of oxidized proteins (Musicco et al ., 2009) . In addition, peroxiredoxins II were identified on the basis of its capacity to protect proteins from oxidative damage that induced by reactive oxygen species, which generated in the presence of DTT (Rhee et al ., 2012) . The current ferriaminosalicylate method was compared with the ferrithiocyanate method to measure peroxiredoxin activity in animal tissues, as shown in Table (3-40) . The comparison produces compatible results.

Table (3-40): The activity of peroxiredoxin enzyme measured using the ferrithiocyanate and ferriaminosalicylate protocols for tissues of a male albino rat.

Samples	Type of substrate	Prx activity [(U l ⁻¹ protein) for serum and (U mg ⁻¹ protein) for tissues]	
		Aminosalicylate method ^a	Ferrithiocyanate method ^a
		Mean ± SD	Mean ± SD
Serum	H ₂ O ₂	155±3	151±5
	t-BOOH	172±5	180±4
Erythrocytes	H ₂ O ₂	31±1	33±2
	t-BOOH	19±0.5	18±1
Kidney	H ₂ O ₂	12±0.7	11.5±1
	t-BOOH	9±0.5	9.0±0.7
Liver	H ₂ O ₂	15±0.9	15.5±1
	t-BOOH	14±0.5	15±0.7

^a mean of triplicate determinations

The precision of the current peroxiredoxin protocol was estimated in the presence of the four types of biochemical interfering molecules. The first contained of 25 mM HEPES buffer (pH 7.0); the second contained 5 mM of three types of mono-saccharides: mannose, lactose and glucose that dissolved in 25 mM HEPES buffer (pH 7.0); the third contained 5 mM of three types of amino acids: aspartic acid, methionine, leucine, and histidine dissolved in 25 mM HEPES buffer (pH 7.0); and the forth contained 3% casein and 3% bovine serum albumin that dissolved in 25 mM HEPES buffer (pH 7.0). An enzymatic reaction, consists of 1 ml 1000 (U/l) Prx

mixed with 9 ml aliquots of the solutions containing biochemical interfering molecules. Total peroxiredoxin activity was adjusted to 100 (U/l), using the thiocyanate method (Netto et al ., 1996) . The results in Table (3-41) demonstrate the correlation between relative percentage errors and interfering biological contaminants.

Table (3-41): Effect of interfering biomolecules on relative percentage errors during the measurement of prx activity, using the aminosalicylate protocol.

	Added peroxiredoxin U/l	Found peroxiredoxin U/l	Relative error (%)
# Solution 1	100	100	0.00
# Solution 2	100	98	2.0
# Solution 3	100	103	3.0
# Solution 4	100	99	1.0

The Bland-Altman plot was applied to confirm the developed protocol (Bahar et al ., 2017) . The sensitivity and linearity of the ferriaminosalicylate protocol were evaluated for several prx activities (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 U. L⁻¹). The linearity of the proposed assay was evaluated by compare the results of the current method with that obtained from ferrithiocyanate method (Netto et al ., 1996) . Figure (3-17) shows the result of analysis of the Bland–Altman plot. The analysis elucidates the mean relative bias and the relative difference between the ferriaminosalicylate method and the ferrithiocyanate method.

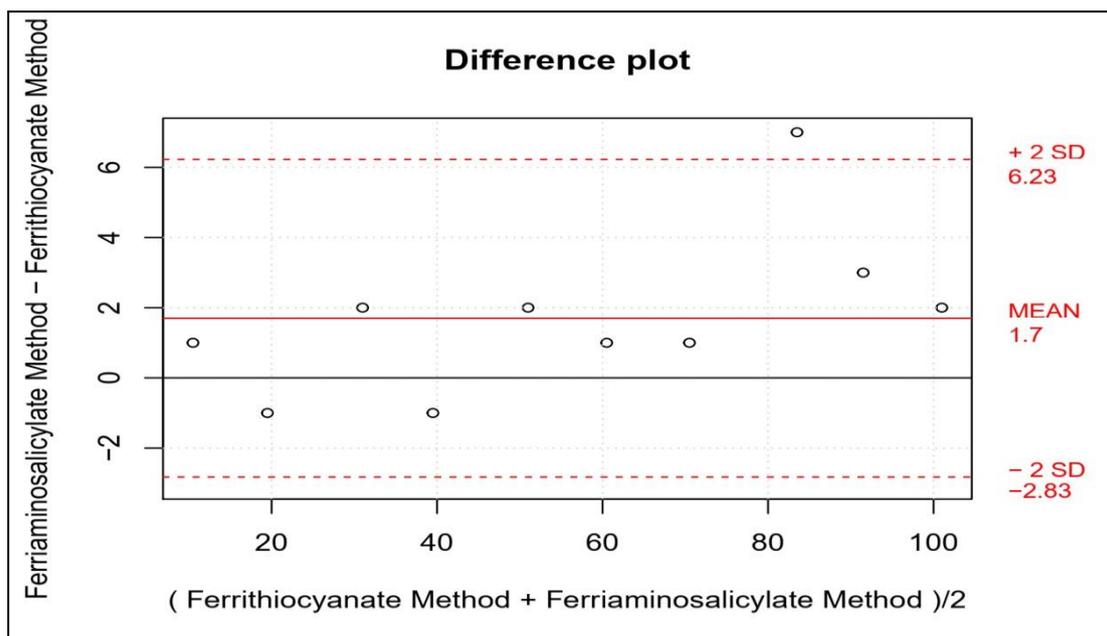


Figure (3-17). Bland-Altman plot indicating the mean relative bias the relative difference between the ferrithiocyanate and ferriaminosalicylate methods.

Additionally, the Passing–Bablok analysis correlation of the ferriaminosalicylate method and the ferrithiocyanate method showed good agreement Figure (3-18). The Pearson correlation confirmed the visually impressive correlation for the different samples, with a Pearson $r > 0.98$.

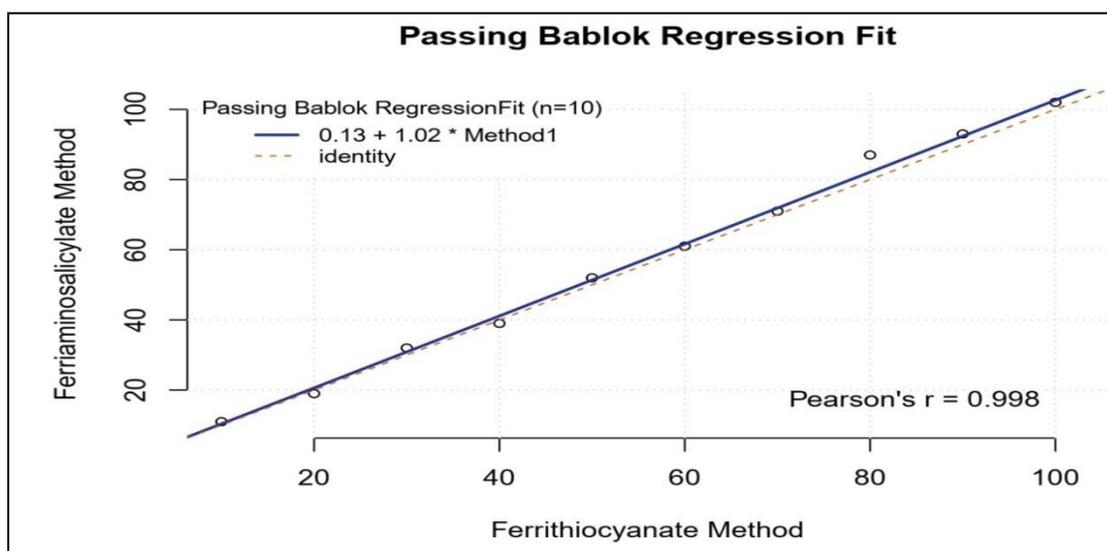


Figure (3-18). The Passing–Bablok analysis correlation of the ferriaminosalicylate method and the ferrithiocyanate method.

The current protocol is compatible with the previous method that described by Khalifa and Hadwan (Khalifa and Hadwan , 2020) . The two protocol were used salicylic acid derivatives to form colored complex with unreacted residual peroxide. The disadvantages of the thiocyanate method include the high toxicity of thiocyanate and associated environmental risks. The substitution of thiocyanate with SSA is consistent with the rules and principles of green chemistry (Koel and Kaljurand , 2006; Keith et al 2007) . According of the above results, the present protocol is free of interference, and gives alternative probe to assess peroxide with high sensitivity. The results showed that this assay can be used for clinical pathology laboratories because it is simple, rapid, low-cost. The systematic reagent consists of ammonium ferrous sulfate (FAS)/ aminosalicylic acid (ASA) acts as a sensitive probe for assessment peroxide.

3.13. Conclusion

1- The described method for assessments of catalase activity that show high precision and accuracy in the presence of high concentrations of several types of biomolecules, and at low H₂O₂ concentrations. These data indicate that hydroquinone/ anilinium sulfate/ammonium molybdate reagent is a sensitive reagent for measurements of hydrogen peroxide concentrations, and allows assessments of catalase activity at low substrate concentrations.

2- The described method for assessments of peroxiredoxin activity that show high precision and accuracy in the presence of high concentrations of several types of interference, and at low H₂O₂ concentrations

3- Diabetic nephropathy associated with increment Afamin activity and increment peroxiredoxin 4 activity.

4- Diabetic nephropathy associated with increment reactive oxygen species concentration and decrement total antioxidant levels.

5- Diabetic nephropathy associated with decrement catalase and Vitamin E and increment Malondialdehyde (MDA)

6- Diabetic nephropathy associated with the genotyping (Deletion) of Afamin activity and genotyping (G) of peroxiredoxin 4 activity

3.14. Recommendations

1- Afamin and peroxiredoxin 4 they consider of the important biochemical markers altered to type 2 diabetic and diabetic nephropathy, thus this marker requires more study, especially its associations with total antioxidant and total oxidant.

2- Determination DNA sequencing of afamin and peroxiredoxin 4 gene polymorphisms to detected precise order of nucleotides in mutated DNA piece

3- Analysis Genotypic of afamin and peroxiredoxin 4 gene and their recognized of associations with risks of Diabetic patients and Diabetic patients with nephropathy.

Supervisor Certification

I certify that this thesis entitled (**The association of afamin and peroxiredoxin 4 genes polymorphisms with antioxidant status in diabetic patients with nephropathy**) was prepared under my supervision at the Department of chemistry/ College of Science / University of Babylon, in partial requirements for the Degree of Doctor of Science in chemistry and this work has never been published anywhere.

Signature:

Name: **Dr. Mahmoud Hussein Hadwan**

Title: Professor

Address: Department of chemistry -College of Sciences - University of Babylon

Date: / / 2021

In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

Signature:

Name: **Dr. Saadon Abdulla Aowda**

Title: Professor

Address: Head of Department of Chemistry-College of Sciences- University of Babylon

Date: / /2021

References

Abbasi A, Corpeleijn E, Gansevoort RT et al Circulating peroxiredoxin 4 and type 2 diabetes risk: the prevention of renal and vascular endstage disease (PREVEND) study. *Diabetologia* (2014) ;57:1842–1849.

Abbasi A, Corpeleijn E, Postmus D, Gansevoort RT, de Jong PE, et al. Peroxiredoxin 4, a novel circulating biomarker for oxidative stress and the risk of incident cardiovascular disease and all-cause mortality. *J Am Heart Assoc* (2012) ;1: 002956.

Abdalla, A.E., The role of antioxidant (Vitamin E) in the control of lead pollution and enhancement of growth within Nile tilapia (*Oreochromis niloticus*). *Intern. J. Appl. Res. Vet. Med.* 2009, 3(7): 97-101.

Abiru N, Kawasaki E, Eguchi K. Current knowledge of Japanese type 1 diabetic syndrome. *Diabetes Metab Res Rev* 2002;18:357–66.

ADA (American Diabetes Association), 2018. The cost of diabetes. <http://www.diabetes.org/advocacy/newsevents/cost-of-diabetes.html>. (Accessed 31 December 2018).

ADA (American Diabetes Association), Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2009;32(Suppl. 1): S62–7.

Adisa, R.A.; Kolawole, N.; Sulaimon, L.A.; Brai, B.; Ijaola, A. Alterations of Antioxidant Status and Mitochondrial Succinate Dehydrogenase Activity in the Liver of Wistar Strain Albino Rats Treated with by Ethanol Extracts of *Annona senegalensis* Pers (Annonaceae) Stem Bark. *Toxicol. Res.*, **2019**, 35(1), 13-24. <http://dx.doi.org/10.5487/TR.2019.35.1.013> PMID: 30766654.

Adriano, S., D. Bartolomeo, X. Cristos and M. Andrea, 2004. Effects of different irradiance levels on some antioxidant enzymes and on

References

malondialdehyde content during rewatering in olive tree. *Plant Sci.*, 166: 293-302.

Aghadavod E, Soleimani A, Hamidi G, Keneshlou F, Heidari A, Asemi Z. Effects of high-dose vitamin E supplementation on markers of cardiometabolic risk and oxidative stress in patients with diabetic nephropathy: A randomized double-blinded controlled trial. *Iranian journal of kidney diseases.* 2018 May 1;12(3):156.

Aldulaimi, A.M.; Husain, F.F. Effect of Aqueous Extract *Cyperus rotundus* Tubers as Antioxidant on Liver and Kidney Functions in Albino Males Rats Exposed to Cadmium Chloride Toxic. *Baghdad Science Journal*, 2019, 16(2), 315-322. <http://dx.doi.org/10.21123/bsj.16.2.0315>

Aly DG, Shahin RS (2010). "Oxidative stress in lichen planus". *Acta Dermatovenerol Alp Pannonica Adriat.* **19** (1): 3–11. [PMID 20372767](https://pubmed.ncbi.nlm.nih.gov/20372767/).

Amer J, Ghoti H, Rachmilewitz E, Koren A, Levin C, Fibach E (January 2006). "Red blood cells, platelets and polymorphonuclear neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants". *Br. J. Haematol.* **132** (1): 108–13. [doi:10.1111/j.1365-2141.2005.05834.x](https://doi.org/10.1111/j.1365-2141.2005.05834.x). [PMID 16371026](https://pubmed.ncbi.nlm.nih.gov/16371026/).

American Diabetes Association. Classification and diagnosis of diabetes, *Diabetes Care.*(2010), 2015, 38(Suppl. 1):S8-S16.

American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2014;37(Suppl 1):S81–90.

American Diabetes Association. Strategies for improving care. Sec. 1. In *Standards of Medical Care in Diabetes—2015*, *Diabetes Care*, 2015, 38(Suppl. 1):S5–S7.

Aon MA, Tocchetti CG, Bhatt N, Paolucci N, Cortassa S. Protective mechanisms of mitochondria and heart function in diabetes. *Antioxidants & redox signaling.* 2015 Jun 10;22(17):1563-86.

References

Arican O, Kurutas EB. "Oxidative stress in the blood of patients with active localized vitiligo". *Acta Dermatovenerol Alp Pannonica Adriat.* (March 2008),**17** (1): 12–6. [PMID 18454264](#).

Armstrong A, Chesnutt JE, Gormley MJ, Young IS. The effect of dietary treatment on lipid peroxidation and antioxidant status in newly diagnosed noninsulin dependent diabetes. *Free Radic Biol Med* 1996; 21: 719-726.

Ashok, A. and A. Sushil,. Oxidative stress and antioxidants in male infertility: A difficult balance. *Iranian J. Reprod. Med.* 2005, 3(1): 1-8.

Ashton T, Rowlands CC, Jones E, Young IS, Jackson SK, Davies B, Peters JR (1998) Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *Eur J Appl Physiol Occup Physiol* 77: 498–502 *J Physiol Sci* (2013) 63:1–5 5 123.

Atip, L., P. Natchai, P. Thavatchai and S. Charn,. Lipid peroxidation and antioxidant enzyme activities in erythrocytes of type 2 diabetic patients. *J. Med. Associ. Thailand.*2010, 93(6):

Aune D, Keum N, Giovannucci E, Fadnes LT, Boffetta P, Greenwood DC, Tonstad S, Vatten LJ, Riboli E, Norat T. Dietary intake and blood concentrations of antioxidants and the risk of cardiovascular disease, total cancer, and all-cause mortality: a systematic review and dose-response meta-analysis of prospective studies. *The American journal of clinical nutrition.* 2018 Nov 1;108(5):1069-91.

Auten, R.L., Davis, J.M. Oxygen toxicity and reactive oxygen species: the devil is in the details. *Pediatr. Res* .2009. 66 (2), 121–127.

Bae SH, Sung SH, Cho EJ, Lee SK, Lee HE, Woo HA, Yu D, Kil IS, and Rhee SG. Concerted action of sulfiredoxin and peroxiredoxin I

References

protects against alcohol-induced oxidative injury in mouse liver. *Hepatology*. 2011; 53: 945–953.

Bae SH, Sung SH, Lee HE, Kang HT, Lee SK, Oh SY, Woo HA, Kil IS, Rhee SG. Peroxiredoxin III and sulfiredoxin together protect mice from pyrazole-induced oxidative liver injury. *Antioxidants & redox signaling*. 2012 Nov 15;17(10):1351-61.

Bahadoran Z, Golzarand M, Mirmiran P, Shiva N, Azizi F .Dietary total antioxidant capacity and the occurrence of metabolic syndrome and its components after a 3-year follow-up in adults: Tehran Lipid and Glucose Study. *Nutr Metab (Lond)* (2012) , 9:70

Bahar B, Tuncel AF, Holmes EW, Holmes DT. An interactive website for analytical method comparison and bias estimation. *Clinical biochemistry*. 2017 Dec 1;50(18):1025-9.

Bajaj S, Khan A (2012) Antioxidants and diabetes. *Indian J Endocrinol Metab* 16:S267–S271. doi:10.4103/2230-8210. 104057.

Bakris GL. Microalbuminuria marker of kidney and cardiovascular disease. London, UK: Current Medicine Group Ltd.; 2007.

Baradaran, A.; Samadi, F.; Ramezani, S.S.; Yousefdoust, S. Hepatoprotective effects of silymarin on CCl₄-induced hepatic damage in broiler chickens model. *Toxicol. Rep.*, **2019**, *6*, 788-794. <http://dx.doi.org/10.1016/j.toxrep.2019.07.011> PMID: 31440455

Bashir, M.R., M.H. Guido, J.F.V. Wim and B. Aalt, 2004. The extraordinary antioxidant activity of vitamin E phosphate. *Bioch. Biophys. Acta*, 1683: 16-21.

References

Battelino, T. et al. Clinical targets for continuous glucose monitoring data interpretation: Recommendations from the international consensus on time in range. *Diabetes Care* **42**, 1593–1603 (2019).

Bell CM, Chapman RH, Stone PW, et al. An off-the-shelf help list: A comprehensive catalog of preference scores from published cost-utility analyses. *MedDecis Making* 2001;21:288–94.

Bernardo Rodriguez-Iturbe, Chang-De Zhan, Yasmir Quiroz. Antioxidant-Rich Diet Relieves Hypertension and Reduces Renal Immune Infiltration in Spontaneously Hypertensive Rats. *Hypertension* 2003; 41: 341. *Biol.* 2007;39:44–84.
<https://doi.org/10.1016/j.biocel.2006.07.001>.

Biró A, Markovics A, Fazekas MÉ, Fidler G, Szalóki G, Paholcsek M, Lukács J, Stündl L, Remenyik J. Allithiamine alleviates hyperglycaemia-induced endothelial dysfunction. *Nutrients*. 2020 Jun;12(6):1690.

Bisht S, Sosodia SS. Diabetes, Dyslipidaemia, Antioxidant and status of oxidative stress. *International Journal of Research in Ayurveda* 2010; 1: 33-42.

Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of botany*. 2003;91(2):179-94.

Bonadonna RC. Alterations of glucose metabolism in type 2 diabetes mellitus. An overview. *Rev Endocr Metab Disord* 2004;5:89-97.

Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R. "Atherosclerosis and oxidative stress". *Histol. Histopathol.* (March 2008), **23** (3): 381–90.

References

Breitenbach M, Eckl P. Introduction to oxidative stress in biomedical and biological research. Multidisciplinary Digital Publishing Institute; 2015.

Brinkmann C, Blossfeld J, Pesch M, Krone B, Wiesiollek K, Capin D, Montiel G, Hellmich M, Bloch W, Brixius K . Lipid-peroxidation and peroxiredoxin-overoxidation in the erythrocytes of non-insulin-dependent type 2 diabetic men during acute exercise. *Eur J Appl Physiol*(2012); 112: 2277–2787.

Brinkmann C, Chung N, Schmidt U, Kreutz T, Lenzen E, Schiffer T, Geisler S, Graf C, Montiel-Garcia G, Renner R, Bloch W, Brixius K. Training alters the skeletal muscle antioxidative capacity in non-insulin-dependent type 2 diabetic men. *Scand J Med Sci Sports*(2012) ; 22:462–470

Brinkmann C, Neumann E, Blossfeld J, Frese S, Orthmann P, Montiel G, et al. Influence of glycemic status and physical fitness on oxidative stress and the peroxiredoxin system in the erythrocytes of non-insulin-dependent type 2 diabetic men. *Exp Clin Endocrinol Diabetes* 2011; 119: 559-564, doi: 10.1055/s-0031-1279712.

Buchan IE .Arcus QuickStat Biomedical version. Cambridge: Addison Wesley Longman Ltd (1997) .

Butler AE, Janson J, Bonner-Weir S, et al. b-cell deficit and increased b-Cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003;52(1):102–10. [http:// dx.doi.org/10.2337/diabetes.52.1.102](http://dx.doi.org/10.2337/diabetes.52.1.102).

Byun SO, Fang Q, Zhou H, Hickford JG. An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical biochemistry*. 2009 Feb 1;385(1):174-5.

References

Cardoso MLV, Ferreira PB, Wanderley AM, Torres RA, Gomes MT, Teixeira RH, et al. a cost-effective method for rapid identification of the southern muriqui (*brachyteles arachnoides*): a contribution for the control of illegal bushmeat trade. 2018.

Carmelli D, Cardon LR, Fabsitz R. Clustering of hypertension, diabetes, and obesity in adult male twins: same genes or same environments? *Am J Hum Genet* 1994;55:566-73.

Chang X, Cui Y, Zong M, Zhao Y, Yan X, et al. Identification of proteins with increased expression in rheumatoid arthritis synovial tissues. *J Rheumatol*(2009) ; 36: 872–880.

Cheeseman KH, Slater TF. An introduction to free radical biochemistry. *Br Med Bull.* 1993;49(3):481–493.

Chen M, Yang ZD, Smith KM, Carter JD, Nadler JL. Activation of 12-lipoxygenase in proinflammatory cytokine-mediated cell toxicity. *Diabetologia.* 2005;48:486 – 495.

Chen S, Tseng C. Dyslipidaemia, kidney disease, and cardiovascular disease in diabetic patients. *Rev Diabet Stud.* 2013;10:80–100.

Cheng HT, Xu X, Lim PS, Hung KY. Worldwide Epidemiology of Diabetes-Related End-Stage Renal Disease, 2000–2015. *Diabetes Care.* 2021 Jan 1;44(1):89-97.

Chin SO, Hwang JK, Rhee SY, Chon S, Hwang YC, et al. (2013) Risk factors for the progression of intima-media thickness of carotid arteries: a 2-year follow-up study in patients with newly diagnosed type 2 diabetes. *Diabetes Metab J* 37: 365-374.

Choi H. Mechanism of angiotensin induced superoxide production in cells reconstituted with angiotensin type 1 receptor and the components of NADPH oxidase. *Journal of Biological Chemistry* 2008; 283: 255-267.

References

Christian P, Stewart CP. "Maternal micronutrient deficiency, fetal development, and the risk of chronic disease". *The Journal of Nutrition*. (March 2010).**140** (3): 437–45. doi:10.3945/jn.109.116327. PMID 20071652.

Coppieters KT ,van Belle TL, von HerrathMG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev* 2011;91:79–118.

Coyne, T. T. Ibiebele, I. P. Baade, D. et al., “Diabetes mellitus and serum carotenoids: findings of a population-based study in Queensland, Australia,” *American Journal of Clinical Nutrition*, vol. 82, no. 3, pp. 685–693, 2005.

Cuschieri S. The genetic side of type 2 diabetes—a review. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*. 2019 Jul 1;13(4):2503-6.

de Boer IH, Sibley SD, Kestenbaum B, et al. Central obesity, incident **De Duve C, Bauduhin P.** peroxisomes (microbodies and related particles) *Physiol Rev*. 1966;46:323–357.

De Rosa S, Arcidiacono B, Chiefari E, Brunetti A, Indolfi C, Foti DP. Type 2 diabetes mellitus and cardiovascular disease: genetic and epigenetic links. *Frontiers in endocrinology*. 2018 Jan 17;9:2.

de Souza Bastos ,A., Graves, D. T., de Melo Loureiro ,A. P. et al.“Diabetes and increased lipid peroxidation are associated with systemic inflammation even in well-controlled patients,” *Journal of Diabetes and its Complications*, vol. 30, no. 8, pp. 1593– 1599, 2016.

De Vrieze J, Ijaz UZ, Saunders AM, Theuerl S. Terminal restriction fragment length polymorphism is an “old school” reliable technique for swift microbial community screening in anaerobic digestion. *Scientific Reports*. 2018;8(1):16818.

References

Dean OM, van den Buuse M, Berk M, Copolov DL, Mavros C, Bush AI (July 2011). "N-acetyl cysteine restores brain glutathione loss in combined 2-cyclohexene-1-one and D-amphetamine-treated rats: relevance to schizophrenia and bipolar disorder". *Neurosci. Lett.* **499** (3): 149–53. [doi:10.1016/j.neulet.2011.05.027](https://doi.org/10.1016/j.neulet.2011.05.027). [PMID 21621586](https://pubmed.ncbi.nlm.nih.gov/21621586/).

Del Prato S. Role of glucotoxicity and lipotoxicity in the pathophysiology of Type 2 diabetes mellitus and emerging treatment strategies. *Diabet Med* 2009; 26(12):1185–92.

Devasagayam, T.P.A., J.C. Tilak, K.K. Boloor, S.S. Ketaki, S.G. Saroj and R.D. Lele, 2004. Free radicals and antioxidants in human health: Current status and future prospects. *JAPI.*, 52: 804-794.

Dieplinger H, Dieplinger B. Afamin—a pleiotropic glycoprotein involved in various disease states. *Clinica Chimica Acta.* 2015 Jun 15;446:105-10.

Ding Y, Yamada S, Wang KY, Shimajiri S, Guo X, et al. Overexpression of peroxiredoxin 4 protects against high-dose streptozotocin-induced diabetes by suppressing oxidative stress and cytokines in transgenic mice. *Antioxid Redox Signal* (2010) ; 13: 1477–1490.

Doria A, Patti M-E, Kahn CR. The emerging genetic architecture of type 2 diabetes. *Cell Metabol* 2008;8:186-200.

Eckel RH, Alberti KG, Grundy SM, Zimmet PZ: The metabolic syndrome. *Lancet* 2010, 375:181–183.

Egan AM, Dinneen SF. What is diabetes?. *Medicine.* 2019 Jan 1;47(1):1-4.

References

Egan AM, Laurenti MC, Hurtado Andrade MD, Dalla Man C, Cobelli C, Bailey KR, Vella A. Limitations of the fasting proinsulin to insulin ratio as a measure of β -cell health in people with and without impaired glucose tolerance. *European Journal of Clinical Investigation*. 2020 Dec 8:e13469.

Eizirik DL, Darville MI. beta-Cell apoptosis and defense mechanisms: lessons from type 1 diabetes. *Diabetes* 2001;50(Suppl. 1):S64–9.

Ekeanyanwu, R.C., Ejiogu, R.N. and Egbogu, M.C.,. Lipid peroxidation and non-enzymatic antioxidants status in hypertension in diabetic and non-diabetic patients in Nigeria: a comparative study 2016.

El Eter E, Al-Masri AA. Peroxiredoxin isoforms are associated with cardiovascular risk factors in type 2 diabetes mellitus. *Brazilian Journal of Medical and Biological Research*. 2015 May;48(5):465-9.

Eljaoudi R, Elomri N, Laamarti M, Cherrah Y, Amezyane T, Ghafir D, Ibrahimi A. Antioxidants status in type 2 diabetic patients in Morocco. *Turkish journal of medical sciences*. 2017 Jun 12;47(3):782-8.

Elnemma, E.M. Spectrophotometric determination of hydrogen peroxide by a hydroquinone-aniline system catalyzed by molybdate. *Bull. Korean Chem. Soc.*, 2004, 25(1), 127-129.

Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, ELISA: III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *The Journal of Immunology*. 1972;109(1):129-35.

Epstein FH, Ho'ppener J, Ahre'n B. Islet amyloid and type 2 diabetes mellitus. *N Engl J Med* 2000.

References

Erel O. A new automated colorimetric method for measuring total oxidant status. *Clinical biochemistry*. 2005;38(12):1103-11.

Eter .El E, Al Masri .A, Habib S et al . Novel links among peroxiredoxins, endothelial dysfunction, and severity of atherosclerosis in type 2 diabetic patients with peripheral atherosclerotic disease. *Cell Stress Chaperones* (2014) ;19:173–181.

Ezeiruaku FC, Tonkey EO, Ojoye NB. Plasma catalase evaluation in type 1 and type 2 chronic diabetic patients in Yenegoa, Bayelsa State, Nigeria. *Int. J. Med. Clin. Sci.* 2016;3(2):36-42.

F. Kronenberg, B. Kollerits, S. Kiechl, et al. Plasma concentrations of afamin are associated with the prevalence and development of metabolic syndrome *Circ Cardiovasc Genet*, 7 (2014), pp. 822-829

F. Prattichizzo, V. De Nigris, E. Mancuso, R. Spiga, A. Giuliani, G. Maticchione, R. Lazzarini, F. Marcheselli, R. Recchioni, R. Testa, L. La Sala, Short-term sustained hyperglycaemia fosters an archetypal senescence-associated secretory phenotype in endothelial cells and macrophages, *Redox Biol.* (May (15)) (2018) 170 181.

Filomeni G, Rotilio G, Ciriolo MR. Cell signalling and the glutathione redox system. *Biochem Pharmacol.* 2002;64:1057–1064.

Fisher AB. Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A₂ activities. *Antioxid Redox Signal* 2011;15:831–844.

Flohé L. The impact of thiol peroxidases on redox regulation. *Free radical research.* 2016 Feb 1;50(2):126-42.

References

Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998;32:S112–19.

Freitas I, Boncompagni E, Tarantola E, Gruppi C, Bertone V, Ferrigno A, Milanesi G, Vaccarone R, Tira ME, Vairetti M. In situ evaluation of oxidative stress in rat fatty liver induced by a methionine- and choline-deficient diet. *Oxidative medicine and cellular longevity*. 2016;2016.

Fuchsberger, Christian; Flannick, Jason; Teslovich, Tanya M.; Mahajan, Anubha; Agarwala, Vineeta; Gaulton, Kyle J.; Ma, Clement; Fontanillas, Pierre; Moutsianas, Loukas; McCarthy, Davis J.; Rivas, Manuel A. (4 August 2016). "The genetic architecture of type 2 diabetes". *Nature*. **536** (7614): 41–47.

Funk CD, Cyrus T. 12/15-lipoxygenase, oxidative modification of LDL and atherogenesis. *Trends Cardiovasc Med*. 2001;11:116.

Fanali ,G., Fasano ,M., Ascenzi P., Zingg , A. Azzi Alpha-tocopherol binding to human serum albumin *Biofactors*, 39 (2013), pp. 294-303.

Gaede P, Poulsen HE, Parving HH, Pedersen O. Double-blind, randomized study of the effect of combined treatment with vitamin C and E on albuminuria in type 2 diabetic patients. *Diabetes Med* 2001; 18: 756-760.

Gambara V, Mecca G, Remuzzi G, et al. Heterogeneous nature of renal lesions in type II diabetes. *J Am Soc Nephrol* 1993;3:1458–66.

Gami AS, Witt BJ, Howard DE, Erwin PJ, Gami LA, Somers VK, Montori VM: Metabolic syndrome and risk of incident cardiovascular

References

events and death: a systematic review and meta-analysis of longitudinal studies. *J Am Coll Cardiol* 2007, 49:403–414.

Garg, N. & Manchanda. G, " ROS generation in plants: boon or bane" *Plant Biosys.* 143 (2009) 8e96.

Gateva A, Assyov Y, Velikova T, Kamenov Z. Increased peroxiredoxin 4 levels in patients with prediabetes compared to normal glucose tolerance subjects. *Clinical endocrinology.* 2016 Oct;85(4):551-5.

Gems D, Partridge L (March 2008). "Stress-response hormesis and aging: "that which does not kill us makes us stronger"". *Cell Metab.* **7** (3): 200–3.

Gerrits EG, Alkhalaf A, Landman GW, van Hateren KJ, Groenier KH, Struck J, Schulte J, Gans RO, Bakker SJ, Kleefstra N, Bilo HJ. Serum peroxiredoxin 4: a marker of oxidative stress associated with mortality in type 2 diabetes (ZODIAC-28). *PLoS One.* 2014 Feb 25;9(2):e89719.

Gezginci-Oktayoglu S, Basaraner H, Yanardag R, Bolkent S. The effects of combined treatment of antioxidants on the liver injury in STZ diabetic rats. *Dig Dis Sci.* 2009;54(3):538-46.

Giugliano ,D. Ceriello ,A.,and Paolisso , G. "Oxidative stress and diabetic vascular complications," *Diabetes Care*, vol. 19, no. 3, pp. 257–267, 1996.

Glovaci D, Fan W, Wong ND. Epidemiology of diabetes mellitus and cardiovascular disease. *Current cardiology reports.* 2019 Apr;21(4):1-8.

Görlach A, Dimova EY, Petry A, Martínez-Ruiz A, Hernansanz-Agustín P, Rolo A.P, Kietzmann T. Reactive oxygen species, nutrition, hypoxia and diseases: Problems solved? *Redox Biol.* 2015; 6: 372–385.

References

Góth L, Bigler NW. Catalase deficiency may complicate urate oxidase (rasburicase) therapy. *Free radical research.* 2007;41(9):953-5.

Góth L, Nagy T (2013). Inherited catalase deficiency: is it benign or a factor in various age related disorders? *Mut. Res./ Rev. Mut. Res* 753: 147-154.

Góth L, Nagy T, Paragh G, Káplár M. Blood Catalase Activities, Catalase Gene Polymorphisms and Acatlasemia Mutations in Hungarian Patients with Diabetes Mellitus. *Glob J Obes Diabetes Metab Syndr* 3 (1): 001 2016;2.

Góth L, Tóth Z, Tarnai I, Bérces M, Török P, Bigler WN. Blood catalase activity in gestational diabetes is decreased but not associated with pregnancy complications. *Clinical chemistry.* 2005;51(12):2401-4.

Gross E, Sevier CS, Heldman N, Vitu E, Bentzur M, Kaiser CA, et al. Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p. *Proc Nat Acad Sci USA.* 2006;103(2):299–304.

Gross JL, Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care.* 2005; 28(1):164-76.

Gunawardena HP, Silva R, Sivakanesan R, Ranasinghe P, Katulanda P. Poor glycaemic control is associated with increased lipid peroxidation and glutathione peroxidase activity in type 2 diabetes patients. *Oxidative medicine and cellular longevity.* 2019 Aug 5;2019.

Gutteridge JMC, Maitt L, Poyer L. Superoxide dismutase and Fenton chemistry. *Biochem J* 1990;269:169–74.

References

Hadwan, M.H.; Abed, H.N. Data supporting the spectrophotometric method for the estimation of catalase activity. *Data Brief*, **2015**, *6*, 194-199.

Hadwan, M.H.; **Ali, S.K.** New spectrophotometric assay for assessments of catalase activity in biological samples. *Anal. Biochem.*, **2018**, *542*, 29-33.

Halliwell ,B. and Gutteridge ,J. M. *Free Radicals in Biology and Medicine*, Oxford University Press, New York, NY, USA, 4th edition, 2007.

Halliwell, Barry . "Oxidative stress and cancer: have we moved forward?". *Biochem. J.* (2007). **401** (1): 1–11.

Han D, Hanawa N, Saberi B, Kaplowitz N. Mechanisms of liver injury: III. Role of glutathione redox status in liver injury. *Am J Physiol Gastrointest Liver Physiol.* 2006;291(1):G1-G7.

Hanafusa T, Imagawa A. Fulminant type 1 diabetes: a novel clinical entity requiring special attention by all medical practitioners. *Nat Clin Pract Endocrinol Metab* 2007;3:36–45 .

Handan, M., K. Suleyman, M. Nihat and D. Yeter, 2007. Vitamin status in yearling rams with growth failure. *Turk. J. Vet. Anim. Sci.*, *31*(6): 407-409.

Hansberg W, Salas-Lizana R, Domínguez L (September 2012). "Fungal catalases: function, phylogenetic origin and structure". *Archives of Biochemistry and Biophysics.* **525** (2): 170–80.

Haridas V, Ni J, Meager A, Su J, Yu GL, Zhai Y, Kyaw H, Akama KT, Hu J, Van Eldik LJ & Aggarwal BB (1998) TRANK, a novel

References

cytokine that activates NF-kappa B and c-Jun Nterminal kinase. *J Immunol* 161: 1-6.

Hasan MS, Mashkur MS, Siadat SO, Ali HA. Assessment of Serum Afamin and Preptin Levels as a Potential Diagnosis Markers for Cardiovascular Patients Undergoing Catheterization. *Medico Legal Update.* 2020 May 22;20(2):479-84.

Hassan, F.A.M.; Roushdy, E.M.; Kishawy, A.T.Y.; Zagloul, A.W.; Tukur, H.A.; Saadeldin, I.M. Growth Performance, Antioxidant Capacity, Lipid-Related Transcript Expression and the Economics of Broiler Chickens Fed Different Levels of Rutin. *Animals (Basel)*, **2018**, 9(1), 7. <http://dx.doi.org/10.3390/ani9010007> PMID: 30583506.

He L, He T, Farrar S, Ji L, Liu T, Ma X. Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cell Physiol Biochem.* 2017; 44(2):532-553.

Hecker M, Wagner AH. Role of protein carbonylation in diabetes. *Journal of inherited metabolic disease.* 2018 Jan;41(1):29-38.

Hegazy HG. Ameliorative effects of ginger and-lipoic acid on oxidative stress and inflammation in senile female rats. *African Journal of Pharmacy and Pharmacology.* 2011;5(8):1096-105.

Heit C, Marshall S, Singh S, Yu X, Charkoftaki G, Zhao H, Orlicky DJ, Fritz KS, Thompson DC, Vasiliou V (2017). "Catalase deletion promotes prediabetic phenotype in mice". *Free Radical Biology & Medicine.* **103**: 48–56.

References

Herder C, Roden M (June 2011). "Genetics of type 2 diabetes: pathophysiologic and clinical relevance". *European Journal of Clinical Investigation*. **41** (6): 679–92.

Hermsdorff HH, Puchau B, Volp AC et al (2011) Dietary total antioxidant capacity is inversely related to central adiposity as well as to metabolic and oxidative stress markers in healthy young adults. *Nutr Metab (Lond)* 8:59

Herold KC, Vignali DA, Cooke A, Bluestone JA. Type 1 diabetes: translating mechanistic observations into effective clinical outcomes. *Nat Rev Immunol* 2013;13:243–56.

Hivert MF, Sullivan LM, Shrader P et al .Insulin resistance influences the association of adiponectin levels with diabetes incidence in two population-based cohorts: the Cooperative Health Research in the Region of Augsburg (KORA) S4/F4 study and the Framingham Offspring Study. *Diabetologia* (2011) ;54:1019–1024.

Hristozova, T.; Rasher, T.; Nedeva, T., and Kujumdzieva, A. Superoxide Dismutase During Glucose Repression of *Hansenula Polymorpha*. CBS4732. *Z. Naturforsch.*, 2000; 57: 313-318.

Hwang O. "Role of oxidative stress in Parkinson's disease". *Exp Neurobiol*. (March 2013). **22** (1): 11–7.

I. Kratzer, E. Bernhart, A. Wintersperger, et al . Afamin is synthesized by cerebrovascular endothelial cells and mediates alpha-tocopherol transport across an in vitro model of the blood–brain barrier *J Neurochem*, 108 (2009), pp. 707-718.

References

Ighodaro OM. Molecular pathways associated with oxidative stress in diabetes mellitus. *Biomedicine & Pharmacotherapy*. 2018 Dec 1;108:656-62.

Imagawa A, Hanafusa T, Miyagawa JI, Matsuzawa Y, for the Osaka IDDM Study Group. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. *N Engl J Med* 2000;342: 301–7.

Irshaid FI, Birmingham DJ. Cq1 Exon Polymorphisms in Caucasian and African American Systemic Lupus Erythematosus patients. *Pakistan journal of biological sciences: PJBS*. 2018;21(3):119-26.

Ishii T, Miyazawa M, Hartman PS, Ishii N. Mitochondrial superoxide anion (O₂⁻) inducible. *BMB reports*. 2011;44(5):298-305.

Issaka A, Paradies Y, Stevenson C. Modifiable and emerging risk factors for type 2 diabetes in Africa: a systematic review and meta-analysis protocol. *Systematic reviews*. 2018 Dec;7(1):1-0.

J. Styskal, H. Van Remmen, A. Richardson, A.B. Salmon, Oxidative stress and diabetes: what can we learn about insulin resistance from antioxidant mutant mouse models? *Free Radic. Biol. Med.* 52 (January (1)) (2012) 46–58.

J.L. Rains, S.K. Jain, Oxidative stress, insulin signaling, and diabetes, *Free Radic. Biol. Med.* 50 (March (5)) (2011) 567–575.

Jaber HT, Hailu A, Pralong F, Lami P, Bastien P, Jaffe CL. Analysis of genetic polymorphisms and tropism in East African *Leishmania donovani* by Amplified Fragment Length Polymorphism and kDNA minicircle sequencing. *Infection, Genetics and Evolution*. 2018;65:80-90.

References

Jain SK, McVie R, Duett J, Herbst JJ. Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. *Diabetes M. Brownlee*, “The pathobiology of diabetic complications: a unifying mechanism,” *Diabetes*, vol. 54, no. 6, pp. 1615– 1625, 2005.

Jakubczyk K, Kalduńska JU, Dec K, Kawczuga DO, Janda KA. Antioxidant properties of small-molecule non-enzymatic compounds. *Pol. Merkur. Lekarski*. 2020 Apr 22;48:128-32.

James SJ, Cutler P, Melnyk S, Jernigan S, Janak L, Gaylor DW, Neubrandner JA (December 2004). "Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism". *Am. J. Clin. Nutr.* **80** (6): 1611–7. [doi:10.1093/ajcn/80.6.1611](https://doi.org/10.1093/ajcn/80.6.1611). [PMID 15585776](https://pubmed.ncbi.nlm.nih.gov/15585776/).

Jamuna Rani A, and Mythili SV. Study on total antioxidant status in relation to oxidative stress in type 2 diabetes mellitus. *J Clin Diagn Res*. 2014;8 (3):108–10.

Jang HH, Lee KO, Chi YH, Jung BG, Park SK, Park JH, et al. Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 2004; 117: 625-635, doi: 10.1016/j.cell.2004.05.002.

Jayedi A, Rashidy-Pour A, Parohan M, Zargar MS, Shab-Bidar S. Dietary antioxidants, circulating antioxidant concentrations, total antioxidant capacity, and risk of all-cause mortality: a systematic review and dose-response meta-analysis of prospective observational studies. *Advances in Nutrition*. 2018 Nov 1;9(6):701-16.

Jelinek, H. F., Stranieri, A., Yatsko, A., & Venkatraman, S. (2016). Data analytics identify glycated haemoglobin co-markers for type 2

References

diabetes mellitus diagnosis. *Computers in biology and medicine*, 75, 90-97.

Jeong W, Bae SH, Toledano MB, Rhee SG. Role of sulfiredoxin as a regulator of peroxiredoxin function and regulation of its expression. *Free Radic Biol Med*. 2012 Aug 1;53(3):447-56.

Jerkovic L, Voegele AF, Chwatal S, Kronenberg F, Radcliffe CM, Wormald MR, Lobentanz EM, Ezeh B, Eller P, Dejori N, Dieplinger B, Lottspeich F, Sattler W, Uhr M, Mechtler K, Dwek RA, Rudd PM, Baier Dieplinger .G, Afamin .H: is a novel human vitamin E-binding glycoprotein characterization and in vitro expression. *J Proteome Res* 2005, 4:889–899.

Jia W, Chen P, Cheng Y. PRDX4 and its roles in various cancers. *Technology in cancer research & treatment*. 2019 Jul 19;18:1533033819864313.

Jiang L, Yang KH, Tian JH, Guan QL, Yao N, Cao N, Mi DH, Wu J, Ma B, Yang SH (2010). "Efficacy of antioxidant vitamins and selenium supplement in prostate cancer prevention: a meta-analysis of randomized controlled trials". *Nutrition and Cancer*. **62** (6): 719–27.

Jiang S, Fang J, Yu T, Liu L, Zou G, Gao H, Zhuo L, Li W. Novel Model predicts diabetic nephropathy in type 2 diabetes. *American journal of nephrology*. 2020;51(2):130-8.

Jiménez S, Gurpegui M, Díaz-Atienza F, Pérez-Costillas L, Gerstenberg M, Correll CU (December 2015). "Oxidative stress and antioxidant parameters in patients with major depressive disorder compared to healthy controls before and after antidepressant treatment:

References

results from a meta-analysis". *J Clin Psychiatry*. **76** (12): 1658–67. doi:10.4088/JCP.14r09179. PMID 26579881.

Jin D.Y., Chae H.Z., Rhee S.G., Jeang K.T. 1997. Regulatory role for a novel human thioredoxin peroxidase in NF- κ B activation. *J. Biol. Chem.* **272**, 30952–30961.

Jin DY, Chae HZ, Rhee SG, Jeang KT (Jan 1998). "Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation". *J. Biol. Chem.* **272** (49): 30952–61.

Jin L, Long L, Green MA, Spear BT. The alpha-fetoprotein enhancer region activates the albumin and alpha-fetoprotein promoters during liver development. *Dev Biol* 2009;336:294–300.

Jo C, Ahn D. Fluorometric analysis of 2-thiobarbituric acid reactive substances in turkey. *Poultry Science*. 1998;77(3):475-80.

Joseph N, Zhang-James Y, Perl A, Faraone SV . "Oxidative Stress and ADHD: A Meta-Analysis". *J Atten Disord* (November 2015). **19** (11): 915–24.

Joy SM, Little E, Maruthur NM, Purnell TS, Bridges JF (2013) Patient preferences for the treatment of type 2 diabetes: a scoping review. *Pharmacoeconomics* 31: 877-892.

Juhász I, Ujfalusi S, Lőrincz H, Somodi S, Harangi M, Seres I, Paragh G: Circulating Afamin Level Correlates With Lipoprotein Subpopulations In Non-Diabetic Obese Patients. *Atherosclerosis*. 2019 Aug 1;287:e165.

K.S. Yang, S.W. Kang, H.A.Woo, S.C. Hwang, H.Z. Chae, K. Kim, S.G. Rhee, Inactivation of human peroxiredoxin I during catalysis as the

References

result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid, *J. Biol. Chem.* 277 (2002) 38029–38036.

Karakus YY. Typical Catalases: Function and Structure. *Glutathione System and Oxidative Stress in Health and Disease.* 2020 Feb 6:111.

Kasznicki J, Kosmalski M, Sliwinska A, Mrowicka M, Stanczyk M, Majsterek I, et al. Evaluation of oxidative stress markers in pathogenesis of diabetic neuropathy. *Mol Biol Rep* 2012; 39: 8669-8678, doi: 10.1007/s11033-012-1722-9.

Kawabata Y, Ikegami H. Genetics of fulminant type 1 diabetes. *Diabetology international.* 2020 Sep 21:1-8.

Kei S. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. *Clinica chimica acta.* 1978;90(1):37-43.

Keith LH, Gron LU, Young JL. Green analytical methodologies. *Chemical reviews.* 2007 Jun 13;107(6):2695-708.

Kennedy G, Spence VA, McLaren M, Hill A, Underwood C, Belch JJ (September 2005). "Oxidative stress levels are raised in chronic fatigue syndrome and are associated with clinical symptoms". *Free Radic. Biol. Med.* 39 (5): 584–9. doi:[10.1016/j.freeradbiomed.2005.04.020](https://doi.org/10.1016/j.freeradbiomed.2005.04.020). PMID [16085177](https://pubmed.ncbi.nlm.nih.gov/16085177/).

Kesavulu ,M. M., Giri ,R., B. “Lipid peroxidation and antioxidant enzyme levels in type 2 diabetics with microvascular complications,” *Diabetes & Metabolism*, vol. 26, no. 5, pp. 387–392, 2000.

Kettunen JL, Tuomi T. Human physiology of genetic defects causing beta-cell dysfunction. *Journal of molecular biology.* 2020 Mar 6;432(5):1579-98.

References

Khalifa HH, Hadwan MH. Simple Method for the Assessment of Peroxiredoxin Activity in Biological Samples. *Chemical Data Collections*. 2020 May 11:100376.

Khanian, M.; Karimi-Torshizi, M.A.; Allameh, A. Alleviation of aflatoxin-related oxidative damage to liver and improvement of growth performance in broiler chickens consumed *Lactobacillus plantarum* 299v for entire growth period. *Toxicon*, **2019**, *158*, 57- 62. <http://dx.doi.org/10.1016/j.toxicon.2018.11.431> PMID: 30529382

Kharroubi AT, Darwish HM, Akkawi MA, Ashareef AA, Almasri ZA, Bader KA, Khammash UM. Total antioxidant status in type 2 diabetic patients in Palestine. *Journal of Diabetes Research*. 2015 May 27;2015.

Khatami, P.G.; Soleimani, A.; Sharifi, N.; Aghadavod, E.; Asemi, Z. The effects of high-dose vitamin E supplementation on biomarkers of kidney injury, inflammation, and oxidative stress in patients with diabetic

Kim Y, Jang HH. The role of peroxiredoxin family in cancer signaling. *Journal of cancer prevention*. 2019 Jun;24(2):65.

Kimura, F. Hasegawa, G. Obayashi ,H. et al., “Serum extracellular superoxide dismutase in patients with type 2 diabetes: relationship to the development of micro- and macrovascular complications,” *Diabetes Care*, vol. 26, no. 4, pp. 1246–1250, 2003.

Kiran BSR, Lakshmi TM, Srikumar R, Reddy EP. Total antioxidant status and oxidative stress in diabetes mellitus and metabolic syndrome. *Int. J. Pharm. Sci. Rev. Res.* 2016; 40(1): 271-277.

References

Koel M, Kaljurand M. Application of the principles of green chemistry in analytical chemistry. *Pure and applied chemistry*. 2006 Jan 1;78(11):1993-2002.

Kollerits B, Lamina C, Huth C, Marques-Vidal P, Kiechl S, Seppälä I, Cooper J, Hunt SC, Meisinger C, Herder C, Kedenko L. Plasma concentrations of afamin are associated with prevalent and incident type 2 diabetes: a pooled analysis in more than 20,000 individuals. *Diabetes Care*. 2017 Oct 1;40(10):1386-93.

Koltover VK, Skipa TA. Free-radical redox timer, sirtuins and aging: from chemistry of free radicals to systems theory of reliability. In *Sirtuin Biology in Medicine* 2021 Jan 1 (pp. 213-223). Academic Press.

Koninger A, Edimiris P, Koch L, et al. Serum concentrations of afamin are elevated in patients with polycystic ovary syndrome. *Endocr Connect* 2014;3:120–6.

Königer A, Mathan A, Mach P, Frank M, Schmidt B, Schleussner E, Kimmig R, Gellhaus A, Dieplinger H. Is afamin a novel biomarker for gestational diabetes mellitus? A pilot study. *Reproductive Biology and Endocrinology*. 2018 Dec;16(1):1-1.

Kronenberg F, Kollerits B, Kiechl S, Lamina C, Kedenko L, Meisinger C, Willeit J, Huth C, Wietzorrek G, Altmann ME, Thorand B, Melmer A, Dähnhardt D, Santer P, Rathmann W, Paulweber B, Koenig W, Peters A, Adham IM, Dieplinger H: Plasma concentrations of afamin are associated with the prevalence and development of metabolic syndrome. *Circ Cardiovasc Genet* 7:822-829, 2014

References

Kuroki T, Isshiki K, King GL. Oxidative stress: the lead or supporting actor in the pathogenesis of diabetic complications. *J Am Soc Nephrol.* 2003;14:S216–20. <https://doi.org/10.1097/01>.

L. Jerkovic, A.F. Voegelé, S. Chwatal, et al . Afamin is a novel human vitamin E-binding glycoprotein characterization and in vitro expression *J Proteome Res*, 4 (2005), pp. 889-899.

L. Jin, L. Long, M.A. Green, B.T. Spear The alpha-fetoprotein enhancer region activates the albumin and alpha-fetoprotein promoters during liver development *Dev Biol*, 336 (2009), pp. 294-300.

Laight DW, Desai KM, Gopaul NK, Anggard EE, Carrier MJ: Pro-oxidant challenge in vivo provokes the onset of NIDDM in the insulin resistant obese Zucker rat. *Br J Pharmacol* 128:269–271, 1999.

Lapolla A, Mosca A, Fedele D (2011) The general use of glycosylated haemoglobin for the diagnosis of diabetes and other categories of glucose intolerance: still a long way to go. *Nutr Metab Cardiovasc Dis* 21: 467-475.

Lazzarini PA, Hurn SE, Fernando ME, Jen SD, Kuys SS, Kamp MC, et al. Prevalence of foot disease and risk factors in general inpatient populations: a systematic review and meta-analysis. *BMJ Open.* 2015;5:e008544.

Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). *Clinical chemistry.* 2005;51(12):2415-8.

Lim, Y.S., Cha, M.K., Yun., C.H, Kim., H.K., Kim, K.W., Kim, I.H. Purification and characterization of thiol-specific antioxidant protein from human red blood cell: a new type of antioxidant protein,

References

Biochemical and biophysical research communications. 1994; 199 : 199-206.

Liu H, Ren H, Spear BT. Themouse alpha-albumin (afamin) promoter is differentially regulated by hepatocyte nuclear factor 1alpha and hepatocyte nuclear factor 1beta. *DNA Cell Biol* 2011;30:137–47.

Lotfi A, Shapourabadi MA, Kachuei A, Saneei P, Naeini AA. Assessment and comparison of the antioxidant defense system in patients with type 2 diabetes, diabetic nephropathy and healthy people: A case–control study. *Clinical nutrition ESPEN*. 2020 Jun 1;37:173-7.

Lowe LP, Metzger BE, Dyer AR, et al. Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study Associations of maternal A1C and glucose with pregnancy outcomes. *Diabetes* 2012;35(3):574–80.

Lugrin J, Rosenblatt-Velin N, Parapanov R, Liaudet L. The role of oxidative stress [15]during inflammatory processes. *Biol Chem*. 2014;395:203-30.

M. Alipour, I. Salehi, F.G. Soufi, Effect of exercise on diabetes-induced oxidative stress in the rat hippocampus, Iran. *Red Crescent Med. J.* 14 (April (4)) (2012) 222.

M. Brownlee, The pathobiology of diabetic complications, *Diabetes* 54 (June (6)) (2005) 1615–1625.

MacIsaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. *Am J Kidney Dis* 2014;63:S39– 62.

References

Mailloux RJ. Protein S-glutathionylation reactions as a global inhibitor of cell metabolism for the desensitization of hydrogen peroxide signals. *Redox biology*. 2020 May 1;32:101472.

Makino ,A., Scott ,B,T., Dillmann ,W,H. Mitochondrial fragmentation and superoxide anion production in coronary endothelial cells from a mouse model of type 1 diabetes, *Diabetologia* 53 (August (8)) (2010) 1783–1794.

Manafa PO, Okafor CC, Okeke CO, Chukwuma GO, Ibeh NC, Ogenyi SI, Nwene EK, Aneke JC. Assessment of Superoxide dismutase activity and total antioxidant capacity in adult male cigarette smokers in Nnewi metropolis, Nigeria. *J. Med. Res.* 2017; 3(1):23-26.

Maneesh, M. and H. Jayalekshmi, 2006. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. *Indian J. Clini. Bioch.*, (21)2: 80-89.

Manohar ,S. M., Vaikasuvu S. R., Deepthi, K., A. Sachan, and S. R. Narasimha, “An association of hyperglycemia with plasma malondialdehyde and atherogenic lipid risk factors in newly diagnosed type 2 diabetic patients,” *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences*, vol. 18, no. 2, pp. 89–93, 2013.

Maritim AC, Sanders RA, Watkins JB. Diabetes, oxidative stress, and antioxidants: [14]A review. *J Biochem Mol Toxicol.* 2003;17:24-38.

Matkovics B, Varga SI, Szabo L, Witas H. The effect of diabetes on the activities of the peroxide metabolism enzymes. *Hor Metab Res* 1982;14:77-79.

References

Mauvais-Jarvis F, Sobngwi E, Porcher R, et al. Ketosis-prone type 2 diabetes in patients of Sub-Saharan African origin: clinical pathophysiology and natural history of β -cell dysfunction and insulin resistance. *Diabetes* 2004;53(3): 645–53.

Mayer-Davis EJ, Costacou T, King I, Zaccaro DJ, Bell RA. Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS). *Diabetes care*. 2002 Dec 1;25(12):2172-7.

Mazzucco G, Bertani T, Fortunato M, et al. Different patterns of renal damage in type 2 diabetes mellitus: A multicentric study on 393 biopsies. *Am J Kidney Dis* 2002;39:713–20.

McFarlane, Philip, et al. "Chronic kidney disease in diabetes." *Canadian journal of diabetes* 42 (2018): S201-S209.

Meigs JB, Muller DC, Nathan DM, et al. The natural history of progression from normal glucose tolerance to type 2 diabetes in the Baltimore Longitudinal Study of Aging. *Diabetes* 2003;52(6):1475–84.

Melmed, Shlomo; Polonsky, Kenneth S.; Larsen, P. Reed; Kronenberg, Henry M. *Williams textbook of endocrinology* (12th ed.). Philadelphia: Elsevier/Saunders. (2011). 1371–1435. ISBN 978-1-4377-0324-5.

Melton DA. Using stem cells to study and possibly treat type 1 diabetes. *Philos Trans R Soc Lond B Biol Sci* 2011;366:2307–11.

Mishra M, Chawsheen HA, Wu L, Jiang H, Wei Q. PRDX4 (peroxiredoxin 4). *Atlas of Genetics and Cytogenetics in Oncology and Haematology*. 2013.

References

Misra K, Dhillon GS, Brar SK, Verma M. Antioxidants. In *Biotransformation of Waste Biomass into High Value Biochemicals* 2014 (pp. 117-138). Springer, New York, NY.

Mitsui R, Fukushima M, Nishi Y, et al. Factors responsible for deteriorating glucose tolerance in newly diagnosed type 2 diabetes in Japanese men. *Metabolism* 2006;55(1):53–8.

Mogensen CE Microalbuminuria, blood pressure and diabetic renal disease: origin and development of ideas. *Diabetologia*. (1999) . 42:263-285.

Mohieldein A. H., Hasan M., K. al-Harbi ,K., S. Alodailah S., R. Azahrani ,M., and al-Mushawwah, S. A. “Dyslipidemia and reduced total antioxidant status in young adult Saudis with prediabetes,” *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, vol. 9, no. 4, pp. 287–291, 2015.

Molitch ME, Adler AI, Flyvbjerg A, Nelson RG, So WY, Wanner C, Kasiske BL, Wheeler DC, De Zeeuw D, Mogensen CE. Diabetic kidney disease: a clinical update from *Kidney Disease: Improving Global Outcomes*. *Kidney international*. 2015 Jan 1;87(1):20-30.

Morinaka, A., Funato, Y., Uesugi, K., & Miki, H. (2011). Oligomeric peroxiredoxin-I is an essential intermediate for p53 to activate MST1 kinase and apoptosis. *Oncogene*, 30(40), 4208–4218.

Moussa, S.A. Oxidative stress in diabetes mellitus. *Romanian J. Biophys.* 2008,18 (3), 225–236.

References

Mueller, S.; Riedel, H.D.; Stremmel, W. Determination of catalase activity at physiological hydrogen peroxide concentrations. *Anal. Biochem.*, **1997**, *245*(1), 55-60.

Muralidharan C, Conteh AM, Marasco MR, Crowder JJ, Kuipers J, de Boer P, Linnemann AK. Pancreatic beta cell autophagy is impaired in type 1 diabetes. *Diabetologia*. 2021 Jan 30:1-3.

Musicco C, Capelli V, Pesce V, Timperio AM, Calvani M, Mosconi L, Zolla L, Cantatore P, Gadaleta MN. Accumulation of overoxidized Peroxiredoxin III in aged rat liver mitochondria. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*. 2009 Jul 1;1787(7):890-6.

Nabeshima A, Yamada S, Guo X et al .Peroxiredoxin 4 protects against nonalcoholic steatohepatitis and type 2 diabetes in a nongenetic mouse model. *Antioxid Redox Signal* (2013) .19:1983–1998.

Nachiappan, Vasanthi; Muthukumar, Kannan (December 2010). "Cadmium-induced oxidative stress in *Saccharomyces cerevisiae*". *Indian Journal of Biochemistry and Biophysics*. **47** (6). ISSN 0975-0959.

Naidu S, PetersonML, Spear BT. Alpha-fetoprotein related gene (ARG): a new member of the albumin gene family that is no longer functional in primates. *Gene* 2010; 449:95–102.

Nardello, V.; Bouttemy, S.; Aubry, J.M. Olefin oxidation by the system H₂O₂MoO₄²⁻: competition between epoxidation and peroxidation. *J. Mol. Catal. Chem.*, **1997**, *117*(1-3), 439-447.

NCD Risk Factor Collaboration (NCD-RisC): Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698

References

population-based measurement studies with 19.2 million participants. *Lancet* 387:1377-1396, 2016.

Nesma, E., A.A. Hany, H.A. Mohammed and M.Y. Mohammed, 2010. Red palm olein: Characterization and utilization in formulating novel functional biscuits. *J. Am. Oil. Chem. Soc.*, 87(3): 295-304. DOI: 10.1007/s11746-009-1497-x.

Netto L.E., Chae ,H.Z., Kang ,S.W., Rhee, E.R. Stadtman, Removal of hydrogen peroxide by thiol-specific antioxidant enzyme (TSA) is involved with its antioxidant properties TSA possesses thiol peroxidase activity, *J. Biol. Chem.* 271 (1996) 15315–15321.

Niedworok, E. and A. Bielaszka, 2007. Comparison of the effect of vitamins A and E on aging processes of edible vegetable oils. *Polish. J. Environ. Stud.*, 16(6): 861-865.

Niki E. Role of vitamin E as a lipid-soluble peroxy radical scavenger: in vitro and in vivo evidence. *Free Radical Biology and Medicine.* 2014 Jan 8;66:3-12.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature.* 2000;404:787-90.

Nishio H, Dugaiczky A. Complete structure of the human alpha-albumin gene, a new member of the serum albumin multigene family. *Proc Natl Acad Sci U S A* 1996;93: 7557–61.

References

Nishio H, Heiskanen M, Palotie A, Belanger L, Dugaiczuk A. Tandem arrangement of the human serum albumin multigene family in the sub-centromeric region of 4q: evolution and chromosomal direction of transcription. *J Mol Biol* 1996;259:113–9.

O'Gara PT, Kushner FG, Ascheim DD, Casey DE, Chung MK, de Lemos JA, et al. "2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines". *Circulation*. **127** (4): e362–425. Cukierman, T (8 Nov 2005). "Cognitive decline and dementia in diabetes – systematic overview of prospective observational studies". *Diabetologia*. (January 2013).**48** (12): 2460–69. [doi:10.1007/s00125-005-0023-4](https://doi.org/10.1007/s00125-005-0023-4). [PMID 16283246](https://pubmed.ncbi.nlm.nih.gov/16283246/).

Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical biochemistry*. 1979;95(2):351-8.

Ojeda Arredondo ML, Pinilla Betancourt MC, Borrero Yoshida ML, Castro Herrera VM, García Vega ÁS, Rodríguez Rodríguez JC, Sequeda G, Diez O, Lucci P. Relationship between vitamin intake and total antioxidant capacity in elderly adults. *Univ Sci*. 2016; 21 (2): 167-177.

Okado -Matsumoto A., Matsumoto A., Fujii J., Tan_ iguchi N. 2000. Peroxiredoxin IV is a secretable pro_ tein with heparin_ binding properties under reduced conditions. *J. Biochem*. **127**, 493–501.

References

Okado-Matsumoto A, Matsumoto A, Fujii J, Taniguchi N . Peroxiredoxin IV is a secretable protein with heparin-binding properties under reduced conditions. *J Biochem* (2000) ;127:493–501

Orasanu G, Plutzky J. The Pathologic Continuum of Diabetic Vascular Disease. *J [32]Am Coll Cardiol.* 2009;53:S35-42.

Ortega MA, Fraile-Martínez O, Naya I, García-Honduvilla N, Álvarez-Mon M, Buján J, Asúnsolo Á, de la Torre B. Type 2 Diabetes Mellitus Associated with Obesity (Diabesity). The Central Role of Gut Microbiota and Its Translational Applications. *Nutrients.* 2020 Sep;12(9):2749.

Pacifici F, Arriga R, Sorice GP, Capuani B, Scioli MG, Pastore D, Donadel G, Bellia A, Caratelli S, Coppola A, Ferrelli F. Peroxiredoxin 6, a novel player in the pathogenesis of diabetes. *Diabetes.* 2014 Oct 1;63(10):3210-20.

Palanduz, S E. Ademoǵlu, C. G"okkus, u, S, . Tamer, "Plasma antioxidants and type 2 diabetes mellitus," *Research Communications in Molecular Pathology and Pharmacology*, vol. 109, no. 5-6, pp. 309–318, 2001.

Palekar AV, Ray KS. Oxidative stress in patients with diabetes mellitus. *J Diabetes Metab Disord Control.* 2016;3(6):138-43.

Pan H.Z., Zhang L., Guo M.Y., Sui H., Li H., Wu W.H., Qu N.Q., Liang M.H., and Chang D. The Oxidative stress status in diabetes mellitus and diabetic nephropathy. *Acta. Diabetol,*2009 ;47(1): 71- 6.

References

Panahi Y, Khalili N, Sahebi E, Namazi S, Karimian MS, Majeed M, Sahebkar A. Antioxidant effects of curcuminoids in patients with type 2 diabetes mellitus: a randomized controlled trial. *Inflammopharmacology*. 2017 Feb 1;25(1):25-31.

Pandey ,K. B., Mishra ,N., and Rizvi ,S. I., “Protein oxidation biomarkers in plasma of type 2 diabetic patients,” *Clinical Biochemistry*, vol. 43, no. 4-5, pp. 508-511, 2010.

Parellada M, Moreno C, Mac-Dowell K, Leza JC, Giraldez M, Bailón C, Castro C, Miranda-Azpiazu P, Fraguas D, Arango C (March 2012). "Plasma antioxidant capacity is reduced in Asperger syndrome". *J Psychiatr Res*. **46** (3): 394–401. [doi:10.1016/j.jpsychires.2011.10.004](https://doi.org/10.1016/j.jpsychires.2011.10.004). [PMID 22225920](https://pubmed.ncbi.nlm.nih.gov/22225920/).

Pastor-Flores D, Talwar D, Pedre B, Dick TP. Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells. *Proceedings of the National Academy of Sciences*. 2020 Jul 14;117(28):16313-23.

Pavithra D, Praveen D, Chowdary PR, Aanandhi MV. A review on role of Vitamin E supplementation in type 2 diabetes mellitus. *Drug Invent. Today*. 2018 Feb 1;10(2):236-40.

Paynter NP, Mazer NA, Pradhan AD, Gaziano JM, Ridker PM, Cook NR .Cardiovascular risk prediction in diabetic men and women using hemoglobin A1c vs diabetes as a high-risk equivalent. *Arch Intern Med* (2011) ;171:1712–1718.

References

Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, et al. "Human gut microbes impact host serum metabolome and insulin sensitivity".(July 2016). **535** (7612): 376–55.

Peh, H.Y.; Tan, W.S.; Liao, W.; Wong, W.S. Vitamin E therapy beyond cancer: Tocopherol versus tocotrienol. *Pharmacol. Ther.* **2016**, 162, 152–169.

Pham-Huy LA, Hua He, Pham-Huy C. Free Radicals, Antioxidants in Disease and Health. *Int J Biomed Sci.* 2008;4(2):89–96.

Pieme, C. A. Tatangmo, J. A. Simo ,G. et al., “Relationship between hyperglycemia, antioxidant capacity and some enzymatic and non-enzymatic antioxidants in African patients with type 2 diabetes,” *BMC Research Notes*, vol. 10, no. 1, p. 141, 2017.

Piper JT, Singhal SS, Salameh MS, Torman RT, Awasthi YC, Awasthi S (1998) Mechanisms of anticarcinogenic properties of curcumin: the effect of curcumin on glutathione linked detoxification enzymes in rat liver. *Int J Biochem Cell Biol* 30:445–456

Poynton RA, Hampton MB . Peroxiredoxins as biomarkers of oxidative stress. *Biochim Biophys Acta* (2014) ;1840:906–912

Qadri, S.S.; Biswas, A.; Mir, N.A.; Mandal, A.B. Biswas. Physicobiochemical and microbial characteristics of broiler chicken meat fed diet incorporated with *Kappaphycus alvarezii*. *J. Appl. Phycol.*, **2019**, 1-7.

Quaife ML, Scrimshaw NS, Lowry OH: A micromethod for assay of total tocopherols in blood serum. *J Biol Chem*, 1949, 180, 1229–1235.

References

Quilliot D, Walters E, Bonte JP, Fruchart JC, Duriez P, Ziegler O. Diabetes mellitus worsens antioxidant status in patients with chronic pancreatitis. *The American journal of clinical nutrition*. 2005 May 1;81(5):1117-25.

R.P. Robertson, Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes, *J. Biol. Chem.* 279 (October (41)) (2004) 42351–42354.

Rafighi Z, Shiva A, Arab S, Yusuf RM. Association of dietary vitamin C and E intake and antioxidant enzymes in type 2 diabetes mellitus patients. *Global Journal of Health Science* 2013; 3: 183-187.

Rahbani-Nobar, M.E.; Rahimi-Pour, A.; Rahbani-Nobar, M.; Adi-Beig, F., and Mirhashemi, S.M. Total Antioxidant Capacity Superoxide Dismutase and Glutathione Peroxidase in Diabetic Patients. *Medical Journal of Islamic Academy of Sciences*, 1999; 12(4): 1-7.

Ramond A, Godin-Ribuot D, Ribuot C, Totoson P, Koritchneva I, Cachot S, Levy P, Joyeux-Faure M (June 2013). "Oxidative stress mediates cardiac infarction aggravation induced by intermittent hypoxia". *Fundam Clin Pharmacol.* **27** (3): 252–61.

Rani AJ, Mythili S. Study on total antioxidant status in relation to oxidative stress in type 2 diabetes mellitus. *Journal of clinical and diagnostic research: JCDR*. 2014 Mar;8(3):108.

Rawshani A, Rawshani A, Sattar N, Franzén S, McGuire DK, Eliasson B, Svensson AM, Zethelius B, Miftaraj M, Rosengren A, Gudbjörnsdottir S. Relative prognostic importance and optimal levels of risk factors for mortality and cardiovascular outcomes in type 1 diabetes mellitus. *Circulation*. 2019 Apr 16;139(16):1900-12.

References

Rees K, Hartley L, Day C, Flowers N, Clarke A, Stranges S (2013). "Selenium supplementation for the primary prevention of cardiovascular disease" (PDF). The Cochrane Database of Systematic Reviews. **22** (4): 439–14.

Reutens AT. Epidemiology of diabetic kidney disease. *Med Clin North Am* 2013;97:1–18.

Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic BiolMed* 2005; 38: 1543-1552, doi: 10.1016/j.freeradbiomed.2005.02.026.

Rhee SG, Woo HA. Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger H₂O₂, and protein chaperones. *Antioxid Redox Signal*. 2011 Aug 1;15(3):781-94.

Rhee. SG, Woo HA, Kil IS, Bae SH. Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. *Journal of Biological Chemistry*. 2012 Feb 10;287(7):4403-10.

Richters L, Lange N, Renner R, Treiber N, Ghanem A, Tiemann K, Scharffetter-Kochanek K, Bloch W, Brixius K. Exercise-induced adaptations of cardiac redox homeostasis and remodeling in heterozygous SOD2-knockout mice. *Journal of Applied Physiology*. 2011 Nov;111(5):1431-40.

Willett WC, Hu FB. "Dietary fats and prevention of type 2 diabetes". *Progress in Lipid Research*. (January 2009).**48** (1): 44–51.

Risérus U, Diabetes mellitus worsens antioxidant status in patients with chronic pancreatitis. *Am J Clin Nutr* 2005; 81: 1117-1125.

References

Rolo ,A.P. Palmeira ,C.M. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress, *Toxicol. Appl. Pharmacol.* 212 (April (2)) (2006) 167–178.

Romá-Mateo C, Aguado C, García-Giménez JL, Ibáñez-Cabellos JS, Seco-Cervera M, Pallardó FV, Knecht E, Sanz P (2015). "Increased oxidative stress and impaired antioxidant response in Lafora disease". *Mol. Neurobiol.* **51** (3): 932–46.

Rubio CP, Hernández-Ruiz J, Martínez-Subiela S, Tvarijonavičiute A, Ceron JJ. Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update. *BMC Vet Res.* 2016; 12(1):166.

S. Fourquet, M.E. Huang, B. D'Autreaux, M.B. Toledano, The dual functions of thiol- based peroxidases in H₂O₂ scavenging and signaling, *Antioxid. Redox Signal.* 10 (2008) 1565–1576.

S. Naidu, M.L. Peterson, B.T. SpearAlpha-fetoprotein related gene (ARG): a new member of the albumin gene family that is no longer functional in primates *Gene*, 449 (2010), pp. 95-102.

S.J. Cho, G. Roman, F. Yeboah, Y. Konishi, The road to advanced glycation end products: a mechanistic perspective, *Curr. Med. Chem.* 14 (June (15)) (2007) 1653–1671.

S.S. Chung, E.C. Ho, K.S. Lam, S.K. Chung, Contribution of polyol pathway to diabetes-induced oxidative stress, *J. Am. Soc. Nephrol.* 14 (August (suppl. 3)) (2003) S233–6.

Sahebkar A, Serban MC, Ursoniu S, Banach M (2015) Effect of curcuminoids on oxidative stress: a systematic review and metaanalysis

References

of randomized controlled trials. *J Funct Foods* 18:898–909. doi:10.1016/j.jff.2015.01.005

Sahlin K, Shabalina IG, Mattsson CM, Bakkman L, Fernström M, Rozhdestvenskaya Z, Enqvist JK, Nedergaard J, Ekblom B, Tonkonogi M . Ultraendurance exercise increases the production of reactive oxygen species in isolated mitochondria from human skeletal muscle. *J Appl Physiol* (2010) ;108:780–787.

Sambrook, J. and Russell, D.W., 2001. *Molecular cloning: A laboratory manual*, the third edition.

Santin I, Eizirik DL. Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and beta-cell apoptosis. *Diabetes Obes Metab* 2013;15(Suppl. 3):71–81.

Sarkar P, Nath K, Banu S. Modulatory effect of baicalein on gene expression and activity of antioxidant enzymes in streptozotocin-nicotinamide induced diabetic rats. *Brazilian Journal of Pharmaceutical Sciences*. 2019;55.

Sarvajeet, S.G. and T. Narendra, 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Bioch.*, 48: 909-930. Doi:10.1016/j.plaphy.2010.08.016.

Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, Ingelsson E, Lawlor DA ,et al. "Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies". (June 2010). **375** (9733): 2215–22.

References

Satchell SC, Tooke JE. What is the mechanism of microalbuminuria in diabetes: a role for the glomerular endothelium? *Diabetologia* 2008;51:714–25.

Schett G, Kiechl S, Weger S, Pederiva A, Mayr A, Petrangeli M, Oberhollenzer F, Lorenzini R, Redlich K, Axmann R, Zwerina J, Willeit J. High-sensitivity c-reactive protein and risk of nontraumatic fractures in the bruneck study. *Arch Intern Med.* 2006;166:2495-2501.

Schrader M, Fahimi HD. Review Peroxisomes and oxidative stress. *Biochim Biophys Acta.* 2006;1763(12):1755–1766.

Schulte J, Struck J, Bergmann A, Köhrle J. Immunoluminometric assay for quantification of peroxiredoxin 4 in human serum. *Clin Chim Acta.* 2010;411:1258–1263.

Schulte J, Struck J, Köhrle J, Müller B . Circulating levels of peroxiredoxin 4 as a novel biomarker of oxidative stress in patients with sepsis. *Shock* (2011) ;35: 460– 465.

Schulte J, Struck J, Köhrle J, Müller B . Circulating levels of peroxiredoxin 4 as a novel biomarker of oxidative stress in patients with sepsis. *Shock* (2011).35:460–465.

Seeber B, Morandell E, Lunger F, Wildt L, Dieplinger H: Afamin serum concentrations are associated with insulin resistance and metabolic syndrome in polycystic ovary syndrome. *Reproductive Biology and Endocrinology.* 2014 Dec;12(1):1-7.

Segal AW (2005). "How neutrophils kill microbes". *Annu. Rev. Immunol.* **23**: 197–223.

References

Sharapov, M. G., V. K. Ravin, and V. I. Novoselov. "Peroxiredoxins as multifunctional enzymes." *Molecular biology* 48.4 (2014): 520-545.

Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of botany*. 2012;2012.

Shastri A, Srivastava R, Jyoti B, Gupta M. The antioxidants-scavengers of free radicals for immunity boosting and human health/overall well-being. *International Journal of Contemporary Medical Research*. 2016;3(10):2918-23.

Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract*. 2010; 87(1):4-14.

Shen CT, Wei WJ, Qiu ZL, Song HJ, Luo QY: Afamin promotes glucose metabolism in papillary thyroid carcinoma. *Mol Cell Endocrinol*

SHENOY S, Devi UH, Kumari SN, Subramanya C. A .study on Association of Antioxidant Status of Red Blood Cells with Type 2 Diabetes. *Journal of Clinical & Diagnostic Research*. 2018 Aug 1;12(8).

Shiga K, Urakami T, Suzuki J, Igarashi Y, Tajima H, Amemiya S, Sugihara S. Fulminant type 1 diabetes mellitus in Japanese children and adolescents: multi-institutional joint research of the Japanese Study Group of Insulin Therapy for Childhood and Adolescent Diabetes. *Endocrine journal*. 2018;65(8):795-803.

Shin, S.K.; Cho, H.W.; Song, S.E.; Bae, J.H.; Im, S.S.; Hwang, I.; Ha, H.; Song, D.K. Ablation of catalase promotes non-alcoholic fatty liver *via* oxidative stress and mitochondrial dysfunction in diet- induced obese

References

mice. *Pflugers Arch.*, **2019**, 471(6), 829-843.
<http://dx.doi.org/10.1007/s00424-018-02250-3> PMID: 30617744

Simone Harder, Meike Bente, Kerstin Isermann, and Iris Bruchhaus, Expression of a Mitochondrial Peroxiredoxin Prevents Programmed Cell Death in *Leishmania donovani*, *EUKARYOTIC CELL*. 2006; 861–870 Vol. 5, No. 5 .

Sirdah MM, Reading NS. Genetic predisposition in type 2 diabetes: A promising approach toward a personalized management of diabetes. *Clinical genetics*. 2020 Dec;98(6):525-47.

Skog O, Korsgren S, Melhus A, Korsgren O. Revisiting the notion of type 1 diabetes being a T-cell-mediated autoimmune disease. *Curr Opin Endocrinol Diabetes Obes* 2013;20:118–23.

Sladek R, Rocheleau G, Rung J, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 2007;445:881–5.

So W, Ng M, Lee S, et al. Genetics of types 2 diabetes mellitus. *HKMJ (Hong Kong Med J)* **2018** ;6.
<http://www.hkmj.org/system/files/hkm0003p69.pdf>. [Accessed 22 January 2018].

Sriphairoj K, Na-Nakorn U, Klinbunga S. Species identification of non-hybrid and hybrid Pangasiid catfish using polymerase chain reaction-restriction fragment length polymorphism. *Agriculture and Natural Resources*. 2018.

References

Srivatsan ,R. Das, S. Gadde, R. et al., “Antioxidants and lipid peroxidation status in diabetic patients with and without complications,” *Archives of Iranian Medicine*, vol. 12, no. 2, pp. 121–127, 2009.

Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* 2005;365:1333-46.

Styskal J, Van Remmen H, Richardson A, Salmon AB. Oxidative stress and diabetes: what can we learn about insulin resistance from antioxidant mutant mouse models?. *Free Radical Biology and Medicine*. 2012 Jan 1;52(1):46-58.

Sue GR, Sang WK, Tong SC, Woojin J, Kanghwa K. Peroxiredoxin, a novel family of peroxidases. *IUBMB Life* 2001;52:35-41.

Sultana A. Outcome of Vitrectomy in Proliferative Diabetic Retinopathy Patients with Diabetic Nephropathy--A Retrospective Study at Tertiary Eye Centre, Telangana State, in South India. *Journal of Evolution of Medical and Dental Sciences*. 2020 Mar 9;9(10):711-6.

Sun B, Luo Z, Zhou J. Comprehensive elaboration of glycemic variability in diabetic macrovascular and microvascular complications. *Cardiovascular Diabetology*. 2021 Dec;20(1):1-3.

Tabaei BP, Al-Kassab AS, Ilag LL, Zawacki CM, Herman WH. Does microalbuminuria predict diabetic nephropathy? *Diabetes Care*. 2001; 24(9):1560-6.

Tajiri Y, Grill VE: Interactions between vitamin E and glucose on B-cell functions in the rat: an in vivo and in vitro study. *Pancreas* 18:274 –281, 1999.

References

Tan KC, Mackay IR, Zimmet PZ, et al. Metabolic and immunologic features of Chinese patients with atypical diabetes mellitus. *Diabetes Care* 2010. [http:// dx.doi.org/10.2337/diacare.23.3.335](http://dx.doi.org/10.2337/diacare.23.3.335).

Taniyama M, Katsumata R, Aoki K, Suzuki S. A Filipino patient with fulminant type 1 diabetes. *Diabetes Care* 2004;27:842–3.

Tasaki E, Kobayashi K, Matsuura K, Iuchi Y (2017). "An Efficient Antioxidant System in a Long-Lived Termite Queen". *PLoS ONE*. **134** (4): 167–92.

Tavender TJ, Bulleid NJ .Peroxiredoxin IV protects cells from oxidative stress by removing H₂O₂ produced during disulphide formation. *J Cell Science*(2010) ; 123: 2672–2679.

Teodoro, J. S. Rolo, A. P. and Palmeira, C. M. “The NAD ratio redox paradox: why does too much reductive power cause oxidative stress?,” *Toxicology Mechanisms and Methods*, vol. 23, no. 5, pp. 297–302, 2013.

Thakur P, Kumar A, Kumar A. Targeting oxidative stress through antioxidants in diabetes mellitus. *J Drug Target*. 2018: 1-11.

Tiedge M, Lortz S, Munday R, Lenzen S. Protection against the cooperative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. *Diabetologia* 1999;42: 849–855

Tiwari BK, Pandey KB, Abidi AB, Rizvi SI. Markers of oxidative stress during diabetes mellitus. *Journal of biomarkers*. 2013;2013.

Touma C, Pannain S ."Does lack of sleep cause diabetes?". *Cleveland Clinic Journal of Medicine*. (August 2011). **78** (8): 549–58. doi:10.3949/ccjm.78a.10165. PMID 21807927.

References

Turk, H.M., Servinc .A., Camci, C., Cigli, A. Buyuk berber, S., Savli, H. et al (2002). Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 DM. *Acta Diabetologica* **39**(3):117-122

Umpierrez GE, Smiley D, Kitabchi AE. Narrative review: ketosis-prone type 2 diabetes mellitus. *Ann Intern Med* 2006;144(5):350–7. <http://dx.doi.org/10.7326/0003-4819-144-5-200603070-00011>.

Upritchard JE, Sutherl WHF, Mann JI. Effect of supplementation, vitamin E and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. *Diabetes Care* 2008; 23: 733-738.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007). "Free radicals and antioxidants in normal physiological functions and human disease". *Int. J. Biochem. Cell Biol.* **39** (1): 44–84.

Veal E, Day A (2011) .Hydrogen peroxide as a signaling molecule. *Antiox. Redox Signal* 15: 147-151.

Vigersky, R. A. Going beyond HbA1c to understand the benefits of advanced diabetes therapies. *J. Diabetes* **11**, 23–31 (2019).

Voegele AF, Jerkovic L, Wellenzohn B, Eller P, Kronenberg F, Liedl KR, Dieplinger H: Characterization of the vitamin E-binding properties of human serum afamin. *Biochemistry* 41:14532-14538, 2002.

Volpe CM, Villar-Delfino PH, Dos Anjos PM, Nogueira-Machado JA. Cellular death, reactive oxygen species (ROS) and diabetic complications. *Cell death & disease.* 2018 Jan 25;9(2):1-9.

Wahlen, J.; De Vos, D.E.; Groothaert, M.H.; Nardello, V.; Aubry, J.M.; Alsters, P.L.; Jacobs, P.A. Synergism between molybdenum and

References

lanthanum in the disproportionation of hydrogen peroxide into singlet oxygen. *J. Am. Chem. Soc.*, **2005**, *127*(49), 17166-17167. <http://dx.doi.org/10.1021/ja0547026> PMID: 16332047.

Wang, Y., 2005. The Bioavailability, Potency and Distribution of Dietary Palm Tocopherols and Tocotrienols in the Tissues of Red Hybrid Tilapia, *Oreochromis sp.* University Sains Malaysia.

West, I. (2000) Radicals and oxidative stress in diabetes. *Diabetic medicine* **17**:171-180

Willemsen, Gonneke; Ward, Kirsten J.; Bell, Christopher G.; Christensen, Kaare; Bowden, Jocelyn; Dalgård, Christine; Harris, Jennifer R.; Kaprio, Jaakko; Lyle, Robert; Magnusson, Patrik K. E.; Mather, Karen A. (December 2015). "The Concordance and Heritability of Type 2 Diabetes in 34,166 Twin Pairs From International Twin Registers: The Discordant Twin (DISCOTWIN) Consortium". *Twin Research and Human Genetics*. **18** (6): 762–771.

Wilson PW, Meigs JB, Sullivan L, Fox CS, Nathan DM, D'Agostino RB Sr .Prediction of incident diabetes mellitus in middle-aged adults: the Framingham Offspring Study. *Arch InternMed*(2007) ; *167*:1068–1074.

Winter WE, Maclaren NK, Riley WJ. Maturity-onset diabetes of youth in black Americans. *N Engl J Med* 1987;*316*(6):285–91.

Winter WE, Schatz DA. Autoimmune markers in diabetes. *Clin Chem* 2011;*57*: 168–75.

Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*. 2008;*4*:278–286.

Winterbourn, C.C., Hampton, M.B. Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.*2008. *45* (5), 549–561.

References

Wong CM, Marcocci L, Das D et al . Mechanism of protein decarboxylation. *Free Radic Biol Med*(2013) ; 65:1126–1133

Wood ZA, Poole LB, Hantgan RR, Karplus PA. Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry*. 2002 Apr 30;41(17):5493-504

Wood ZA, Schroder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 2003;28:32-40.

World Health Organization. "Definition, diagnosis and classification of diabetes mellitus and its complications: Report of a WHO Consultation. Part 1. Diagnosis and classification of diabetes mellitus". Archived from the original on 2007-05-29. Retrieved 2007-05-29.

Wright Jr E, Scism-Bacon JL, Glass LC. Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *International journal of clinical practice*. 2006 Mar;60(3):308-14.

Yagi K. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochemical medicine*. 1976;15(2):212-6.

Yaman SO, Ayhanci A. Lipid Peroxidation. In *Lipid Peroxidation 2021* Feb 12. Intech Open.

Yamazaki I, Piette LH. ESR Spin-trapping studies on the reaction of Fe⁺⁺ ions with H₂O₂-reactive species in oxygen toxicity in biology. *J Biol Chem* 1990;265:13589–94.

Yan J, Jiang J, He L, Chen L. Mitochondrial superoxide/hydrogen peroxide: An emerging therapeutic target for metabolic diseases. *Free Radical Biology and Medicine*. 2020 May 20;152:33-42.

References

Yan, L. J. “Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress,” *Journal of Diabetes Research*, vol. 2014, Article ID 137919, 11 pages, 2014.

Yang CS, Luo P, Zeng Z, Wang H, Malafa M, Suh N. Vitamin E and cancer prevention: Studies with different forms of tocopherols and tocotrienols. *Molecular carcinogenesis*. 2020 Apr;59(4):365-89.

Khallouki F, Owen RW, Akdad M, El Bouhali B, Silvente-Poirot S, Poirot M. **Vitamin E:** an overview. *Molecular Nutrition*. 2020 Jan 1:51-66.

Yang Y, Hu X, Zhang Q, Zou R (. "Diabetes mellitus and risk of falls in older adults: a systematic review and meta-analysis". *Age and Ageing*. November 2016). 45 (6): 761–67. [doi:10.1093/ageing/afw140](https://doi.org/10.1093/ageing/afw140). [PMID 27515679](https://pubmed.ncbi.nlm.nih.gov/27515679/)

Z.A. Wood, L.B. Poole, R.R. Hantgan, P.A. Karplus, Dimers to doughnuts: redox sensitive oligomerization of 2-cysteine peroxiredoxins, *Biochemistry* 41 (2002) 5493–5504.

Zeggini E, Weedon MN, Lindgren CM, et al. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science* 2007;316:1336-41.

Zeida A, Manta B, Trujillo M. In vivo observation of peroxiredoxins oligomerization dynamics. *Proceedings of the National Academy of Sciences*. 2020 Aug 11;117(32):18918-20.

Zhao Y, Xu G, Wu W, Yi X. Type 2 diabetes mellitus-disease, diagnosis and treatment. *J Diabetes Metab*. 2015 May 1;6(533):2.

References

Zheng P, Li Z, Zhou Z. Gut microbiome in type 1 diabetes: A comprehensive review. *Diabetes/metabolism research and reviews*. 2018 Oct;34(7):e3043.

Zielinski ZA, Pratt DA. Lipid peroxidation: Kinetics, mechanisms, and products. *The Journal of organic chemistry*. 2017 Mar 17;82(6):2817-25.

Zito E, Melo EP, Yang Y, Wahlander Å, Neubert TA, Ron D. Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. *Mol Cell*. 2010 Dec 10;40(5):787-97.

I certify that this thesis entitled (**The association of Afamin and Peroxiredoxin 4 genes polymorphisms with antioxidant status in diabetic patients with nephropathy**) was prepared under my supervision at the Department of chemistry/ College of Science / University of Babylon, in partial requirements for the Degree of Doctor of Science in chemistry and this work has never been published anywhere.

Signature:

Name: **Dr. Mahmoud Hussein Hadwan**

Title: Professor

Address: Department of chemistry -College of Sciences - University of Babylon

Date: / / 2021

In the view of the available recommendation, I forward this thesis for debate by the Examination Committee.

Signature:

Name: **Dr. Saadon Abdulla Aowda**

Title: Professor

Address: Head of Department of Chemistry-College of Sciences- University of Babylon

Date: / /2021

