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Evaluation of Na^+/K^+ ATPase Activity and Gene Polymorphism in Patients with Diabetic Neuropathy in Babylon Governorate

A Thesis Submitted to the College of Science, University of Babylon as a partial fulfillment of the requirements for Degree of Doctorate of philosophy in Science/ Chemistry

By
Eman Hameed Hameedi Ibrahem

B.Sc. Chemistry/ 2006/University of Babylon
M.Sc. chemistry/Biochemistry/ 2012/University of Babylon

Supervised by

Prof. Dr. Oda Mizil Yasser **Assist. Prof. Dr. Mazin Jaafar Mousa**

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Summary

Diabetic neuropathy is one of the most common complications of long-standing diabetes mellitus in both types I and II. The pathogenesis is believed to be directly related to impaired glucose metabolism with resultant derangement of multiple intracellular metabolic pathways resulting in impaired nerve function, mainly in the form of altered nerve conduction and manifested mainly as sensory and autonomic neuropathy, while motor neuropathy is very rare in this setting. One of the blamed metabolic pathways is Na-K ATPase, a trans-membrane transport enzyme responsible for the maintenance of intracellular ion gradient in all cells of the body, specifically in nerve cells where it is also responsible for membrane potential necessary for nerve action potential propagation. Enzyme structure includes four α subunits, three β subunits, and one γ subunit (FXYD-2). The active site is located along the α1, 2, and 3 subunits, while β subunits only provide a site for glycosylation increasing enzyme sodium affinity.

Endogenous digitalis is steroid molecules normally synthesized by different cell types in the body and bind to the extracellular portion of α subunits resulting in its inhibition. In recent years impact of the inhibitory effect of endogenous digitalis has been thoroughly investigated in different disease states, but the role of their effects is still unclear in diabetic neuropathy.

Many studies have shown that C-peptide has a protective effect against the development of diabetic neuropathy, possibly through the activation of Na-K ATPase, induction of nitric oxide synthesis, and protein-kinase C pathway. Nitric oxide acts as a neurotransmitter in the central nervous system and probably in the peripheral nerves.

The goal of this study was to compare the activity of Na-K ATPase in RBC ghosts, plasma endogenous digitalis inhibitory activity of Na-K ATPase

and plasma C-peptide in type 2 diabetic patients with and without neuropathy compared to healthy control subjects. Genetic polymorphism of α 1 (ATP1A1, rs10924081) and α 2 (ATP2A2, rs10924081) were studied by RFLP and gene sequencing respectively. This study was designed as a case-control study, including patients with type II diabetes mellitus, dividing participants into three groups; diabetic neuropathy, diabetics without neuropathy and control healthy individuals.

There was a significant association between diabetic neuropathy with the duration of disease and age of subjects ($p < 0.001$), but no significant association between BMI and diabetic neuropathy (p 0.810). The following parameters: age, Body Mass Index BMI, Glycated Haemoglobin HbA1C, Na-K ATPase activity in RBC ghosts, endogenous digitalis (inhibitory activity of Na-K ATPase), and C-peptide were measured in the study. There were significant differences in the age of diabetic neuropathy (59.39 ± 0.99) when compared with type 2 diabetes mellitus without neuropathy, and control individuals (53.93 ± 1.52, 30.80 ± 0.98 respectively) with $p<0.05$. The results of BMI of diabetic neuropathy patients (DN) were no significant differences (27.91 ± 0.68) when compared with diabetes mellitus without neuropathy (T2DM), and control individuals (28.90 ± 0.80, 27.20 ± 0.95 respectively) with $p=0.444$.

The results of HbA1C of diabetic neuropathy patients (DN) were lower significant (9.42 ± 0.25) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (10.40 ± 0.35, 5.23 ± 0.05 respectively) with $p<0.05$. The results of Erythrocyte Na-K ATPase activity of diabetic neuropathy patients (DN) were lower significant (381.94 ± 18.00) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (498.28 ± 22.98, 837.20 ± 61.43 respectively) with $p<0.05$. The results of Endogenous digitals (plasma inhibitory of Na-K ATPase activity) % of diabetic neuropathy patients (DN) were higher significant (17.88 ± 2.16) as compared with diabetes

mellitus without neuropathy (T2DM), and control individuals (8.79 ± 0.90, 5.35 ±1.34 respectively) with $p<0.05$. The results of plasma C-peptide) of diabetic neuropathy patients (DN) were lower significant (1.17± 0.10) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (1.71 ± 0.19, 1.96±0.28) with $p<0.05$.

In our study, there was a negatively significant correlation between erythrocyte Na-K ATPase activity and endogenous digitalis ($r=-0.167$, $p= 0.042$). The correlation between Na-K ATPase activity and HbA1C was a negatively higher significant correlation ($r=-0.380$, $p<0.01$). In addition, there was a negatively higher significant correlation between Na-K ATPase activity and age of individuals with $r=-0.495$, $p<0.01$. While, no significant positive correlation between Erythrocyte Na-K ATPase activity and plasma C-Peptide ($r=.032$, $p=0.694$). There was no significant negative correlation between erythrocyte Na-K ATPase activity and BMI ($r=-0.009$, $p=0.910$). Also, there was no significant negative correlation between Na-K ATPase activity and duration of diabetes mellitus ($r=-0.138$, $p=0.134$).

Samples of 60 patients with neuropathy, 35 patients without neuropathy, and 30 healthy individuals were submitted to genetic analysis of α1 and α2 genes described above. The genetic association with type 2 diabetic neuropathy for ATP1A1 rs10924081 demonstrated, that there was no significant allelic or genotypic correlation. While the results for ATP1A2 rs373796693 revealed a significant allelic and genotypic association. The findings revealed allele I could be a risk for developing neuropathy in diabetic patients (Odds Ratio=2.857, 95 % confidence interval =1.265-6.452), whereas allele D is possibly a protective allele (OR=0.350, 95% CI=0.2155-0.790). The genotypic (ID, DD) was a significant association in diabetic neuropathy with diabetes without neuropathy (OR= 0.28 and a 95 % CI =0.09-0.82) as compared with other genotypes. This

indicates that D is a protective dominant allele when present as a heterozygote (ID) or homozygote (DD).

When comparing genetic analysis of healthy control subjects to diabetic patients without neuropathy, results showed that there is a significant allele association for allele I, which is a “protective” allele (OR= 0.100, 95 % CI=0.032-0.312). While allele D is a risky allele for diabetes mellitus (OR= 10.000, 95% CI =3.208-31.169). The genotypic association under dissimilar inheritance models showed a significant association of DD genotype (odds ratio 8.62) and 95 % CI (1.60-46.38) when compared between control and patients of diabetes without neuropathy. Also, there was a significant correlation between ID and DD genotypes (OR 18.3, 95 % CI = 3.67-91.23). This suggests that the D allele is the “risk codominant allele” when present as homozygote (DD), and represents the “risky dominant allele” when present as a heterozygote (ID) and homozygote (DD).

Furthermore, when comparing control with diabetic neuropathy samples, allele I was presumably regarded as a “protective” allele (OR=0.286, 95 %CI = 0.086-0.944). On the other hand, allele D is a “risky” allele with an odds ratio of 3.500 and a 95% CI=1.059-11.568). There was no significant correlation of rs373796693 genotypes. In parallel, we studied the link between Na-K ATPase activity in RBC ghosts and C-peptide with ATP1A1 and ATP1A2 gene polymorphism. There was no interaction between ATP1A1 rs10924081 genotypes with erythrocyte Na-K ATPase activity and C-peptide. Patients with ATP1A2 rs373796693 genotype ID had lower erythrocyte Na-K ATPase activity (p=0.049) than other genotypes, but no significant connection between ATP1A2 rs373796693 genotypes and plasma C-peptide.

الخلاصة:

يعد الاعتلال العصبي السكري أحد أكثر المضاعفات شيوعًا لمرض السكري طويل الأمد في كلا النوعين الأول والثاني. يُعتقد أن التسبب في المرض يرتبط ارتباطًا مباشرًا بخلل في استقلاب الجلوكوز مع حدوث خلل ناتج في مسارات التمثيل الغذائي المتعددة داخل الخلايا مما يؤدي إلى ضعف وظيفة الأعصاب ، حيث يحدث التغيير بشكل اساسي في التوصيل العصبي ويتجلى بشكل أساسي في اعتلال الأعصاب الحسي والمستقلي ، في حين أن الاعتلال العصبي الحركي نادر جدًا. يعد Na-K ATPase أحد المسارات الأيضية التي يتم إلقاء الضوء عليها ، وهو إنزيم ناقل عبر الغشاء ومسؤول عن الحفاظ على التدرج الأيوني داخل الخلايا في جميع خلايا الجسم ، وتحديداً في الخلايا العصبية حيث يكون مسؤولاً أيضًا عن إمكانات الغشاء اللازمة لإمكانية عمل الأعصاب. يتضمن هيكل الإنزيم أربع وحدات فرعية α ، وثلاث وحدات فرعية β، ووحدة فرعية واحدة (FXYD-2) زيقع الموقع النشط على طول الوحدات الفرعية α1 و 2 و 3و4 ، بينما توفر الوحدات الفرعيةβ موقعًا للارتباط بالجليكوزيل الذي يزيد من الفة إنزيم الصوديوم.

الديجيتال الداخلي (Endogenous digitals): هو جزيئات الستيرويد التي يتم تصنيعها عادة بواسطة أنواع مختلفة من الخلايا في الجسم وترتبط بالجزء خارج الخلية من الوحدات الفرعية α مما يؤدي إلى تثبيطها. في السنوات الأخيرة ، تم التحقيق بدقة في تأثير التأثير المثبط للديجيتال الداخلي المنشأ في حالات مرضية مختلفة ، لكن دور تأثيراتها لا يزال غير واضح في اعتلال الأعصاب السكري.

أظهرت العديد من الدراسات أن الببتيد C له تأثير وقائي ضد تطور الاعتلال العصبي السكري ، ربما من خلال تنشيط Na-K ATPase ، وتحفيز تخليق أكسيد النيتريك ، ومسار البروتين كيناز سي. يعمل أكسيد النيتريك كناقل عصبي في الجهاز العصبي المركزي وربما في الأعصاب الطرفية.

كان الهدف من هذه الدراسة هو مقارنة نشاط Na-K ATPase في غشاء كريات الدم الحمراء ، وبلازما الديجتيال الداخلي (المثبط لفعالية Na-K ATPase) والببتيد C البلازمي في مرضى السكري من النوع 2 مع أو بدون اعتلال عصبي مقارنة بأشخاص التحكم الأصحاء. تمت دراسة تعدد الأشكال الجيني لـ α 1 (ATP1A1) ، α 2 (ATP2A2وrs10924081) ، (rs10924081 بواسطة RFLP والتسلسل الجيني على التوالي. تم تصميم هذه الدراسة كدراسة حالة وضبط ، بما في ذلك مرضى السكري من النوع الثاني ، وتقسيم المشاركين إلى ثلاث مجموعات ؛ اعتلال الأعصاب السكري ومرضى السكر دون اعتلال الأعصاب والسيطرة على الأفراد الأصحاء.

كان هناك ارتباط معنوي بين الاعتلال العصبي السكري مع مدة المرض وعمر الأشخاص (p <0.001)، ولكن لم يكن هناك ارتباط كبير بين مؤشر كتلة الجسم والاعتلال العصبي السكري (ص 0.810). تم قياس المتغيرات التالية: العمر ، مؤشر كتلة الجسم BMI، الهيموجلوبين الجلوكوز HbA1C، فعالية Na-K ATPase في غشاء RBC ، الديجيتال الداخلي (المثبط لفعالية Na-K ATPase)، والببتيد C في الدراسة. كانت هناك فروق ذات دلالة إحصائية في عمر الاعتلال العصبي السكري (59.39 ± 0.99) بالمقارنة مع داء السكري من النوع 2 دون اعتلال الأعصاب ، والأفراد الضابطين (53.93 ± 1.52 ، 30.80 ± 0.98 على التوالي) مع .p <0.05 .لم تكن نتائج مؤشر كتلة الجسم لمرضى الاعتلال العصبي السكري (DN) فروق ذات دلالة إحصائية (27.91 ± 0.68) عند مقارنتها بمرض السكري بدون اعتلال الأعصاب (T2DM) ، والأفراد الضابطين (28.90 ± 0.80 ، 27.20 ± 0.95 على التوالي) مع .p = 0.444 .

كانت نتائج HbA1C لمرضى الاعتلال العصبي السكري (DN) أقل معنوية (9.42 ± 0.25) مقارنة بمرض السكري بدون اعتلال الأعصاب (T2DM) ، والأفراد الضابطين (10.40 ± 0.35 ، 5.23 ± 0.05 على التوالي) مع .p <0.05 . كانت نتائج فعالية كريات الدم الحمراء Na-K ATPase لمرضى الاعتلال العصبي السكري (DN) أقل أهمية (381.94 ± 18.00) مقارنة بمرض السكري بدون اعتلال الأعصاب (T2DM) ، وأفراد التحكم (498.28 ± 22.98 ، 837.20 ± 61.43 على التوالي) مع .p <0.05 . كانت نتائج بلازما الديجيتال الذاتية (مثبطات فعالية Na-K ATPase) ٪ من مرضى الاعتلال العصبي السكري (DN) أعلى معنوية (17.88 ± 2.16) مقارنة بداء السكري بدون اعتلال عصبي (T2DM) ، وأفراد التحكم (8.79 ± 0.90 ، 5.35 ± 1.34 على التوالي) مع p<0.05. كانت نتائج البلازما (C-peptide) لمرضى الاعتلال العصبي السكري (DN) أقل أهمية (1.17 ± 0.10) مقارنة بداء السكري بدون اعتلال الأعصاب (T2DM) ، والأفراد الضابطين (1.71 ± 0.19 ، 1.96 ± 0.28) مع. p <0.05

في دراستنا ، كان هناك ارتباط ذو دلالة سلبية بين فعالية كريات الدم الحمراء Na-K ATPase والديجيتال الذاتية (r = -0.167، p = 0.042). كان الارتباط بين فعالية Na-K ATPase و HbA1C ارتباطًا ذا دلالة سالبة أعلى (r = -0.380، p <0.01). بالإضافة إلى ذلك ، كان هناك ارتباط معنوي أعلى بشكل سلبي بين فعالية Na-K ATPase وعمر الأفراد مع r = -0.495، p <0.01. بينما لا يوجد ارتباط إيجابي معنوي بين فعالية كريات الدم الحمراء Na-K ATPase والببتيد C في البلازما (r = .032، p = 0.694). لم يكن هناك ارتباط سلبي معنوي بين فعالية كريات الدم

الحمراء Na-K ATPase ومؤشر كتلة الجسم (r = -0.009، p = 0.910). أيضا ، لم يكن هناك ارتباط سلبي معنوي بين فعالية Na-K ATPase ومدة داء السكري (r = -0.138، p = 0.134).

تحتوي الدراسة على عينات من 60 مريضًا يعانون من اعتلال الأعصاب ، و 35 مريضًا بدون اعتلال عصبي ، و 30 فردًا سليمًا للتحليل الجيني لجينات α1 و α2 الموصوفة أعلاه. أظهر الارتباط الجيني مع الاعتلال العصبي السكري من النوع 2 لـ ATP1A1 rs10924081 أنه لا يوجد ارتباط أليلي أو وراثي معنوي. بينما أظهرت نتائج ATP1A2 rs373796693 علاقة أليلية وراثية معنوية. كشفت النتائج أن الأليل الأول يمكن أن يكون خطرًا للإصابة باعتلال الأعصاب لدى مرضى السكري (نسبة الأرجحية = 2.857 ، فاصل الثقة 95٪ = 1.265-6.452) ، في حين أن الأليل D ربما يكون أليلًا وقائيًا(OR = 0.350 ، 95٪ .(0.790 -CI = 0.2155 كان النمط الجيني (ID) ، (DD) ارتباطًا مهمًا في الاعتلال العصبي السكري مع مرض السكري بدون اعتلال عصبي (OR = 0.28 و (95%CI = 0.09-0.82) مقارنة بالأنماط الجينية الأخرى. يشير هذا إلى أن D هو أليل وقائي سائد عندما يكون موجودًا على شكل زيجوت متغاير الزيجوت (ID) أو متماثل الزيجوت.(DD)

عند مقارنة التحليل الجيني لموضوعات التحكم الصحية مع مرضى السكري الذين لا يعانون من اعتلال الأعصاب ، أظهرت النتائج أن هناك ارتباط أليل مهم للأليل I ، وهو أليل "وقائي" (OR=0.100) ، 95)٪ .(CI = 0.032-0.312 بينما الأليل D هو أليل خطير لمرض السكري (OR=10.000) ، 95)٪(CI = 3.208-31.169 . حيث أظهر الارتباط الجيني تحت نماذج الوراثة المختلفة ارتباطًا مهمًا بين النمط الجيني DD (نسبة الأرجحية 8.62) و (95%CI =1.60-46.38)عند المقارنة بين مرضى السكري ومرضى السكري غير المصابين باعتلال عصبي. أيضا ، كان هناك ارتباط كبير بين الأنماط الجينية ID و DD ، (OR 18.3, 95 ٪, .(CI = 3.67-91.23 يشير هذا إلى أن الأليل D هو "أليل المخاطرة المشفر" عندما يكون موجودًا على هيئة زيجوت متماثل (DD) ، ويمثل "الأليل السائد بالخطر" عند وجوده على شكل زيجوت متغاير الزيجوت (ID) ومتماثل الزيجوت (DD).

علاوة على ذلك ، عند مقارنة السيطرة مع عينات اعتلال الأعصاب السكري ، يُفترض أن الأليل I , أليل "وقائي" (OR = 0.286، 95٪CI = 0.086-0.944). من ناحية أخرى ، الأليل D هو أليل " خطر" مع نسبة أرجحية 3.500 و 95 CI = 1.059-11.568%). لم يكن هناك ارتباط معنوي بين الطرز الجينية rs373796693. بالتوازي مع ذلك ، درسنا الارتباط بين نشاط Na-K ATPase في غشاء RBC والببتيد C مع تعدد الأشكال الجيني ATP1A1 و ATP1A2. لم يكن هناك تفاعل بين

الأنماط الجينية ATP1A1 rs10924081 مع فعالية خلايا الدم الحمراء Na-K ATPase و C-peptide. بينما كان لدى المرضى الذين لديهم معرف النمط الجيني ATP1A2 rs373796693فعالية أقل في كريات الدم الحمراء Na-K ATPase (p = 0.049) من الأنماط الجينية الأخرى ، ولكن لا يوجد ارتباط كبير بين الأنماط الجينية ATP1A2 rs373796693 والببتيد C البلازمي.

بسم الله الرحمن الرحيم

﴿ يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَ الَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ﴾

(صدق الله العلي العظيم)

(سورة المجادلة / الآية 11)

Dedication

To my loved mother, an invitation for her to open the door of heaven,

So she ascends, settles in the shadow of the throne.

To my mother, may God have mercy on you and put you in peace.

Eman

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Eman Hameed

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Abbreviations

Symbol	Definition
ACTH	Adrenocorticotropic Hormone
ADP	Adenosine diphosphate
AGE	Advanced Glycation ends products
AKt	Protein kinase B
AR	Aldose Reductase
ATP	Adenosine Tri-Phosphate
ATPlAl	Adenosine Triphosphatase-Alpha-1 subunit
ATP1A2	Adenosine Triphosphatase-Alpha-2 subunit
BMI	Body Mass Index
Ca^{+2}-ATPase	Calcium Adenosine Tri Phosphatase
CAD	Coronary artery disease
CD4+	Helper T lymphocytes
CD8+	Cytotoxic T lymphocytes
COX2	Cyclooxygenase 2
DAN	Diabetic Autonomic Neuropathy
DAG	Diacylglycerol
DHA-3-P	Dihydroxyacetone-3-Phosphate
DKA	Diabetic ketoacidosis
DM	Diabetes Mellitus
DME	diabetic macular edema
DN	Diabetic Neuropathy
DPN	Diabetic Peripheral Neuropathy

DR	Diabetic Retinopathy
DSPN	Distal symmetric polyneuropathy
EGFR	Epidermal Growth Factor Receptor
FFA	Free Fatty Acids
GDM	Gestational Diabetes Mellitus
GR	Glutathione Reductase
GSH	Reduced Glutathione
HDL	High Density Lipoprotein
IDDM	Insulin-Dependent Diabetes Mellitus
IGF-1	Insulin Growth Factor-1
IR	Insulin Resistance
H-K ATPase	Hydrogen-Potassium Adenosine Tri Phosphatase
HNF	Hepatocyte nuclear transcription factor
LDL	Low Density Lipoprotein
MAPK	Mitogen- Activated Protein kinase
MODY	Maturity Onset Diabetes of the Young
NAD^+	Nicotinamide Adenine Dinucleotide.
NADH	Reduced Nicotinamide Adenine Dinucleotide.
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenosine Dinucleotide Phosphate
Na-K ATPase	Sodium- and Potassium Adenosine 5-Triphosphatase
NF-kβ	Necrosis Factor –Kappa Beta

NGF	Nerve growth factor
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NJK	C-Jun N-terminal Kinase
NKA	Sodium-Potassium Adenosine Tri phosphatase Na-K ATPase
NKHHS	Non-ketogenic hyperosmolar hyperglycaemic state
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NT-3	Neurotrophin 3
PAD	peripheral arterial disease
PAI-1	plasminogen activator inhibitor-1
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction- Restriction Fragment Length Polymorphism
PKC	Protein Kinase C
PLC	Phospholipase C
PI	phosphatidylinositol
PI3K	Phosphoinositol 3-Kinase
PTx	Pertussis Toxin
ROS	Reactive Oxygen Species
SDH	Sorbitol Dehydrogenase
SNP	Single Nucleotide Polymorphism
Sp1	Specific protein -1
Src.	Tyrosine-protein kinase

T1DM	Type 1 Diabetes Mellitus
T2DM	Type2 Diabetes Mellitus
TGF-β1	Transforming Growth Factor-β1
TNF	Tumor Necrosis factor –α
UDP-GlcNAc	Uridine diphosphate *N*-acetylglucosamine
WHO	World Health Organization

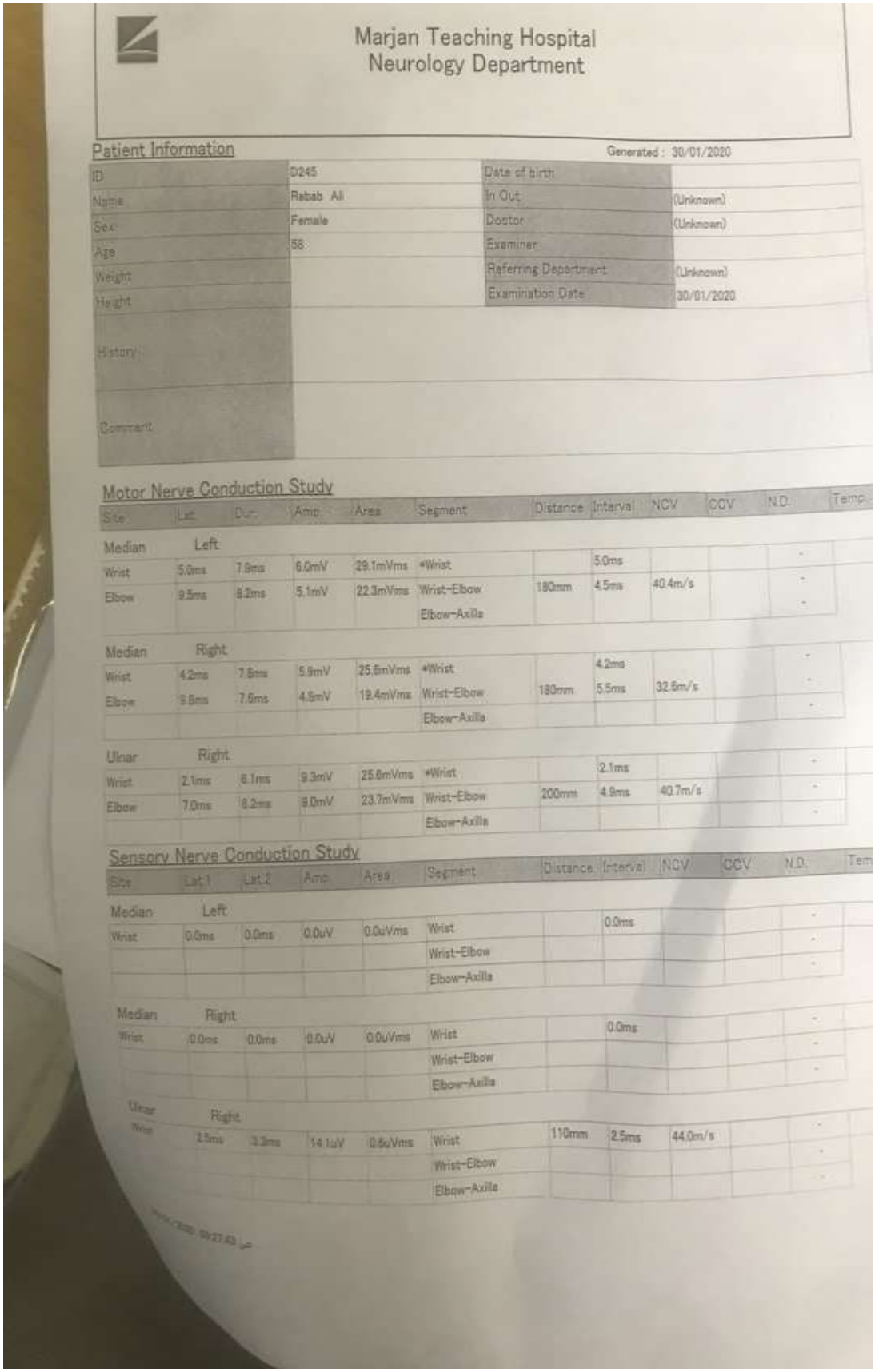
Appendix:

1. **Criteria Area**:

Sample ID:
Patients name : Age : Sex: male : female
Duration of DM
Type of neuropathy :
Sensory polyneuropathy
Motor neuropathy
Autonomic neuropathy:
Diabetic foot Yes No

Na-K ATPase activity
Na-K ATPase inhibitory percent :
C- peptide concentration:
Na-K ATPase gene polymorphism : α1, α2
HbA1C:
Blood pressure
BMI:

2. Nerve conduction study NCS:



Marjan Teaching Hospital
Neurology Department

Patient Information

Generated : 30/01/2020

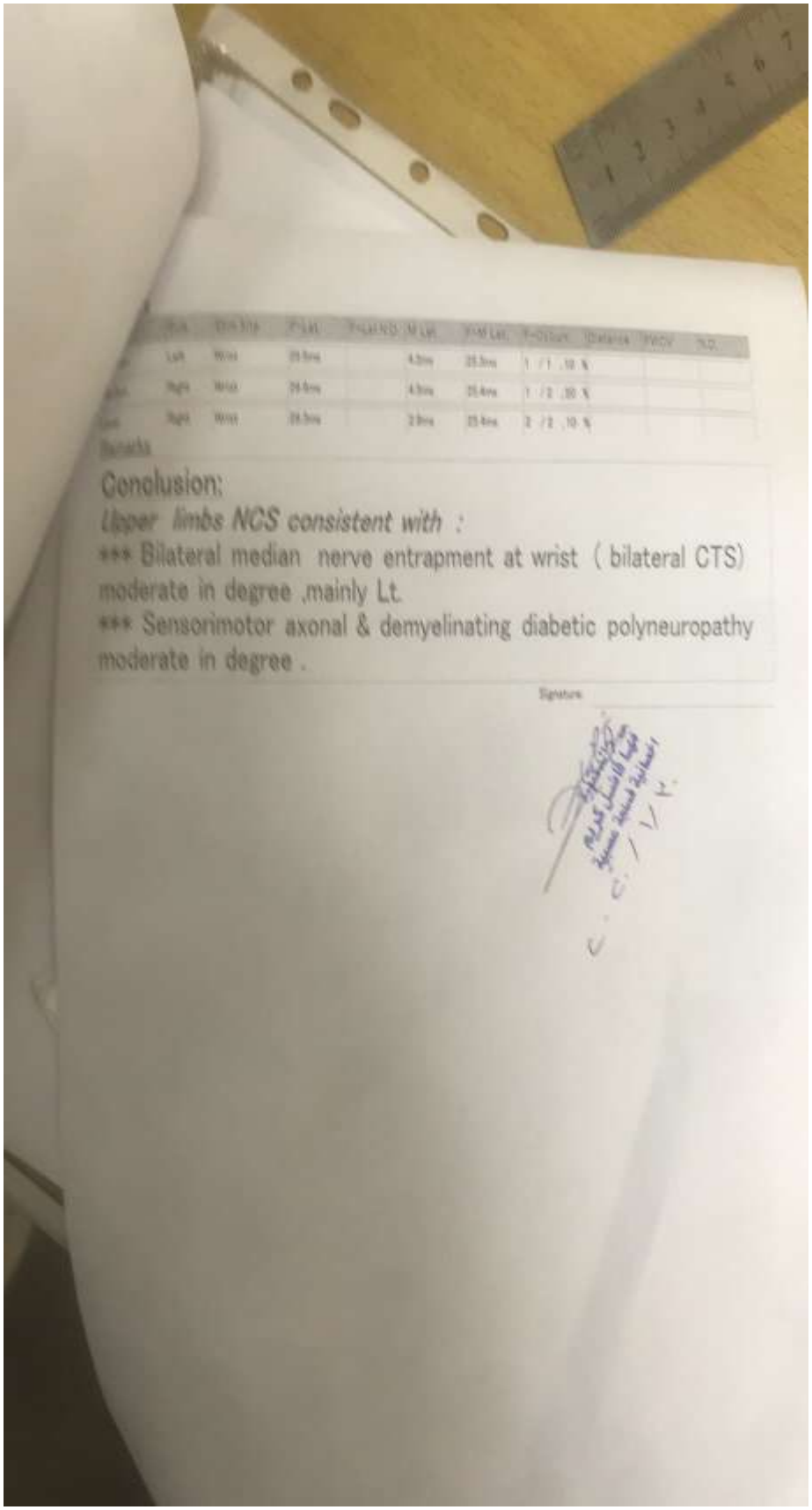
ID	0245	Date of birth	
Name	Rebab Ali	In Out	(Unknown)
Sex	Female	Doctor	(Unknown)
Age	58	Examiner	
Weight		Referring Department	(Unknown)
Height		Examination Date	30/01/2020
History			
Comment			

Motor Nerve Conduction Study

Site	Lat.	Dur.	Amp.	Area	Segment	Distance	Interval	NCV	CCV	N.D.	Temp.
Median	Left										
Wrist	5.0ms	7.9ms	6.0mV	29.1mVms	*Wrist		5.0ms			-	
Elbow	9.5ms	8.2ms	5.1mV	22.3mVms	Wrist-Elbow	180mm	4.5ms	40.4m/s		-	
					Elbow-Axilla					-	
Median	Right										
Wrist	4.2ms	7.8ms	5.9mV	25.6mVms	*Wrist		4.2ms			-	
Elbow	9.8ms	7.6ms	4.5mV	19.4mVms	Wrist-Elbow	180mm	5.5ms	32.6m/s		-	
					Elbow-Axilla					-	
Ulnar	Right										
Wrist	2.1ms	8.1ms	9.3mV	25.6mVms	*Wrist		2.1ms			-	
Elbow	7.0ms	8.2ms	9.0mV	23.7mVms	Wrist-Elbow	200mm	4.9ms	40.7m/s		-	
					Elbow-Axilla					-	

Sensory Nerve Conduction Study

Site	Lat.1	Lat.2	Amp.	Area	Segment	Distance	Interval	NCV	CCV	N.D.	Tem
Median	Left										
Wrist	0.0ms	0.0ms	0.0uV	0.0uVms	Wrist		0.0ms			-	
					Wrist-Elbow					-	
					Elbow-Axilla					-	
Median	Right										
Wrist	0.0ms	0.0ms	0.0uV	0.0uVms	Wrist		0.0ms			-	
					Wrist-Elbow					-	
					Elbow-Axilla					-	
Ulnar	Right										
Wrist	2.5ms	3.3ms	14.1uV	0.6uVms	Wrist	110mm	2.5ms	44.0m/s		-	
					Wrist-Elbow					-	
					Elbow-Axilla					-	



Conclusion:

Upper limbs NCS consistent with :

*** Bilateral median nerve entrapment at wrist (bilateral CTS) moderate in degree ,mainly Lt.

*** Sensorimotor axonal & demyelinating diabetic polyneuropathy moderate in degree .

1-Introduction

1.1 Diabetes Mellitus (DM)

Diabetes mellitus (DM): is a series of metabolic or heterogeneous disorders caused by way of an abnormality of insulin production, and insulin action, or both, and is an endocrinological illness [1]. High blood glucose levels (hyperglycemia), polydipsia, and polyphagia are the hallmarks of diabetes mellitus (DM). Diabetes mellitus (DM) is one of the most prevalent metabolic illnesses, and its prevalence is rising at an alarming rate across the world[2].

1.2 Classification of Diabetes Mellitus

The etiology and clinical signs of diabetes mellitus have been used to classify the disease. There are four kinds: Type 1 diabetes mellitus, Type 2 diabetes mellitus, gestational diabetes, and other specific types[3], as shown in table (1-1)

Table (1-1): Etiologic classification of diabetes mellitus[3].

Type of diabetes	Reason	Subclass
1. Type 1 diabetes	Due to autoimmune β-cell destruction, usually leading to absolute insulin deficiency)	a) Immune mediated (type 1A) b) Idiopathic (type 1B)
2. Type 2 diabetes	Due to a progressive loss of β-cell insulin secretion frequently on the background of insulin resistance	Impairment of insulin and/or secretion (Insulin resistance)
3. Gestational diabetes mellitus (GDM)	There is an insulin resistance and insulin deficiency	During pregnancy
4. other specific types	Genetic defects of β-cell function	a) Hepatocyte nuclear transcription factor (HNF) α1&α4. (MODY3& MODY1) b) Chromosome7, glucokinase (MODY2) c) Chromosome 13,

		insulin promoter factor-1 (IPF-1; MODY4)
	Genetic defects in insulin action	a) Type A insulin resistance b) Leprechaunism c) Rabson-Mendenhall syndrome
	Diseases of the exocrine pancreas Hemochromatosis Fibrocalculous pancreatopathy	a) Pancreatitis b) Trauma/pancreatectomy c) Neoplasia d) Cystic fibrosis
	Endocrinopathies	a) Acromegaly b) Cushing's syndrome c) Glucagonoma d) Pheochromocytoma e) Hyperthyroidism f) Somatostatinoma
	Drug- or chemical-induced	a) Vacor b) Pentamidine c) Nicotinic acid d) Glucocorticoids e) Thyroid hormone f) Diazoxide g) B-adrenergic agonists h) Thiazides
	Infections	a) Congenital rubella b) Cytomegalovirus
	Other genetic syndromes sometimes associated with diabetes	a) Down's syndrome b) inefelter's syndrome c) Myotonic dystrophy d) Porphyria e) Prader-Willi syndrome

1.2.1 Type 1 diabetes Mellitus

Type 1 diabetes is also called Insulin-Dependent Diabetes Mellitus (IDDM): is defined as a result of an autoimmune response, which occurs by the destruction of beta cells which generally results in absolute insulin deficiency. Type 1 diabetes impacts about 20 million individuals globally and accounts for roughly 10% of all diabetes occurrences [4]. Insulin-

dependent diabetes (type 1) is the most common kind of diabetes, affecting 10 to 15% of those under the age of 20[5].

1.2.2 Type 2 diabetes Mellitus

Type 2 diabetes also called Non-Insulin-Dependent Diabetes Mellitus (NIDDM): is far more common and results from a combination of defects in insulin secretion and insulin action, either of which may predominate. People with type 2 diabetes are not dependent on exogenous insulin, but may require it for the control of blood glucose levels if this is not achieved with diet alone or with oral hypoglycemic agents[6]. Type 2 diabetes has multifactorial causes. These include: anxiety, stress, advance age, obesity, sedentary life style, irregular diet etc. Obesity has been found to contribute to approximately 55% type 2 diabetes and decreasing consumption of saturated fats [7]. It is more frequent in women, especially those who have had gestational diabetes in the past. Type 2 diabetes is more common after the age of 50 and accounts for 85 to 90% of all diabetes types[5]. Clinical characteristics of patients with type 1 and type 2 diabetes are presented in Table 1-2 [8].

Table (1-2): Clinical features of patients together with Type 1 and Type 2 diabetes mellitus [8]

Features	Type 1	Type2
Age of onset	Usually less than 20 years	Usually greater than 30 years
Body mass	Low(wasted) to normal	Obese
Plasma insulin	Low or absent	Normal to high initially
Plasma glucagon	High, can be suppressed	High, resistant to suppressed
Plasma glucose	Increased	Increased
Insulin sensitivity	Normal	Reduced
Therapy	Insulin	Weight loss, thiazolidinedione, metformin, sulfonylurea, insulin.

1.2.3 Gestational Diabetes Mellitus

Gestational Diabetes Mellitus (GDM) is a kind of glucose intolerance that originates or is first seen during pregnancy. GDM is thought to impact 7-10 % of all pregnancies, with a global frequency of 6-13 %. GDM has been linked to poor maternal and fetal outcomes. The adverse maternal complications include gestational hypertension, pre-eclampsia, and cesarean section. Within ten years after pregnancy, GDM can lead to type 2 diabetes. In addition, children born to mothers with GDM have high risks of developing macrosomia, preterm birth, neonatal hypoglycaemia, intensive care, and jaundice. Not only when they are children, but when adolescents also, they have a risk of being obese or having type 2 diabetes. Lifestyle changes are the most important component in GDM management [9].

1.3 Epidemiology of Diabetes Mellitus

Diabetes disease burden is high and growing in every country, fuelled by an increase in the prevalence of obesity and unhealthy lifestyles throughout the world. According to the most recent projects, 11.1 % of people in Northern America and the Caribbean had diabetes in 2019, with that number predicted to grow to 13 % by 2045 [10,11].

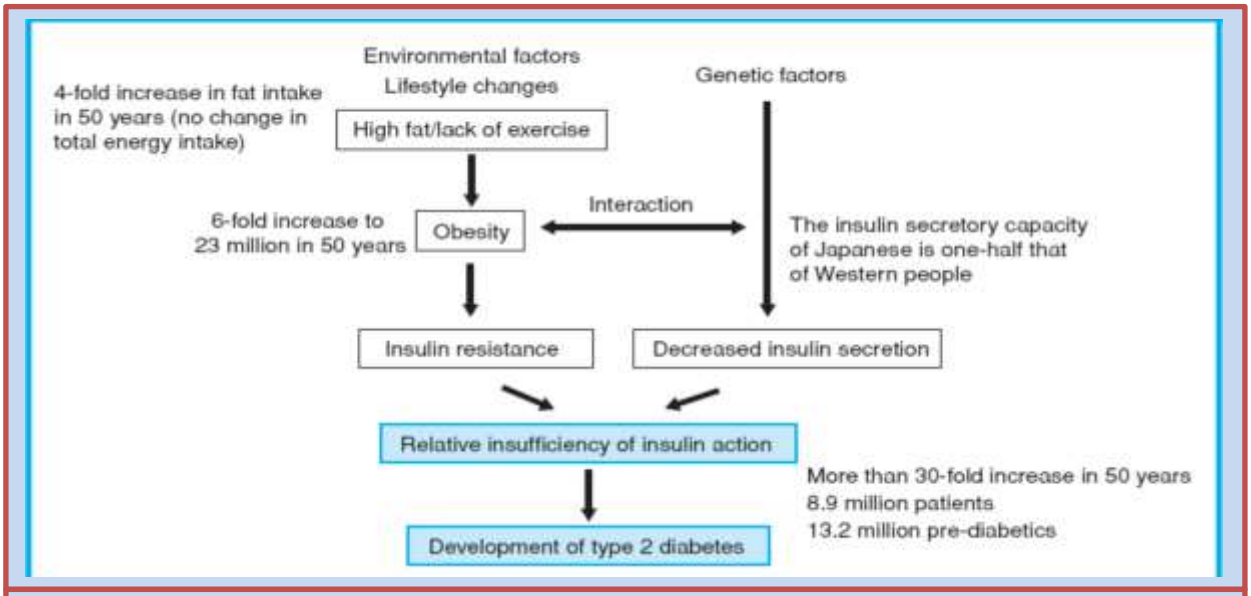
In general, South-East Asian and South American nations have high or moderate occurrences [10,11]. According to research published in 2019 by Saeedi et al. [10], 463 million individuals was lived with diabetes globally, representing a 9.3% prevalence rate. This prevalence rate is expected to reach 10.2 % by 2030, and 10.9 % by 2045. The diabetes prevalence by area is assessed for various nations, resulting in a list of the countries with the largest number of diabetic patients in 2019 [12]. China, with 116 million diabetes patients, has the greatest

proportion of diabetic patients among these countries. India comes in the second with 77 million people, followed by the United States of America with 31 million. Pakistan, Brazil, and Mexico are expected to have the highest numbers of diabetes patients, with 19 million, 16 million, and 12 million diabetic people, respectively[12]. The prevalence rate is highest in the Middle East and North Africa, where it is expected to increase by 13.9 % by 2045. Africa has the lowest prevalence rate (4.7 %), which is predicted to rise to 5.2 % by 2045 [10]. Based on studies from 2006-2007, the International Federation of Diabetes announced in December 2011 that Iraq had a medium prevalence (8.5%) of diabetes in the Middle East[13].

1.4 Pathogenesis And Physiopathology of Diabetes Mellitus:

Hyperglycemia and physiological and behavioral reactions are closely connected[5,14]. The autoimmune death of insulin secretory cells known as β-cells causes type 1 diabetes. The autoimmune process which causes pancreatic "insulitis" occurs over 5 to 10 years or more before the onset of diabetes. This autoimmune reaction happens as a result of triggering events in a field of genetic vulnerability, and blood autoantibody tests can detect it before hyperglycemia develops[5]. Islets of Langerhans infiltration by helper T lymphocytes (CD4) and cytotoxic T lymphocytes (CD8) is the major cause of β-cell destruction. This process might go on for years without showing any clinical signs. Autoantibodies to specific pancreatic antigens are generated during this response. These autoantibodies are not harmful in themselves, but they are accurate indicators of the progression of the pathological autoimmune process[5].

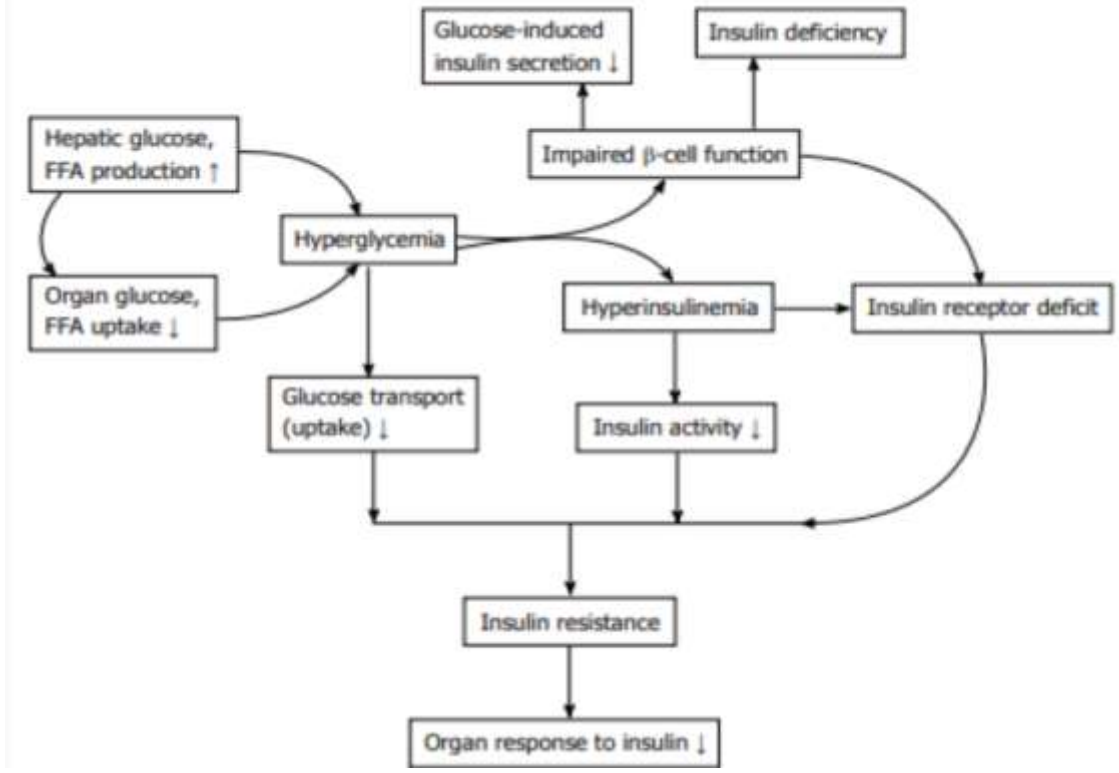
In type 2, genetic, metabolic, and environmental variables are a unique interplay that interconnects to increase the incidence of the disease. However, there is a substantial genetic background for individual propensity to T2DM due to non-modifiable risk variables (ethnicity and family history/genetic predisposition), researchers have noted that many incidences of T2DM can be avoided by reducing the key modifiable risk variables (obesity, low physical activity, and an unhealthy diet)[15]. Dysfunction of the biological processes between insulin action and insulin secretion leads to abnormally elevated blood glucose levels[16]. Insulin production is diminished in the case of β-cell dysfunction, restricting the body's ability to maintain physiological glucose levels. Insulin Resistance (IR), on the other hand, causes increased glucose synthesis in the liver as well as reduced glucose absorption in muscle, liver, and adipose tissue. β-cell dysfunction is frequently more severe than IR, even if both processes occur early in the pathogenesis and contribute to the development of the illness. Hyperglycemia is increased when both β-cell dysfunction and IR are present, contributing to the advancement of T2DM [17,18], as shown in Scheme 1-1:



Scheme(1-1): Etiology and pathophysiology of type 2 diabetes [19].

The three main causes of insulin deficiency conditions are 1. impaired insulin synthesis by β-cells, 2. insulin antagonists in the plasma as a result of counter-regulatory hormones or non-hormonal chemicals that alter insulin receptors or communication, and 3. reduced insulin responsiveness in target tissues [20,21]. Chemicals, such as growth hormone and Insulin Growth Factor-1(IGF-1), interact with insulin function in the fed state. While fasting, glucagon, glucocorticoids, and catecholamines reduce the insulin response to avoid insulin-induced hypoglycemia. This regulation is influenced by the insulin/glucagon ratio, which regulates the amount of phosphorylation for downstream enzymes in regulatory signaling pathways. Glucocorticoids stimulate muscle catabolism, gluconeogenesis, and lipolysis, while catecholamines stimulate lipolysis and glycogenolysis.

As a result, increased release of these hormones might be the cause of IR [22,23], eventually leading to T2DM [24]. Skeletal muscle, adipose tissue, and the liver are the three primary extra-pancreatic insulin-sensitive organs involved in the aforementioned processes. Increased glucose, Free Fatty Acids FFA, and insulin levels cause an increase in reactive oxygen species (ROS) generation, oxidative stress, and the activation of stress transduction factor pathways. As demonstrated in Scheme (1-2), this can result in inhibition of insulin activity and secretion, accelerating the onset of T2DM[25].



Scheme (1-2): Action of insulin resistance[25].

1.5 Dyslipidaemia

Hypertriglyceridemia, lower HDL cholesterol levels, and higher LDL particle concentrations were the most frequent dyslipidemia characteristics in T2DM patients. T2DM is associated with dyslipidemia as a result of insulin resistance and increased free fatty acid flow . Hypertriglyceridemia is caused by insulin resistance and hyperglycemia, which causes an overproduction of triglyceride-rich lipoproteins in the liver, impaired clearance of triglyceride-rich lipoproteins, and, in rare circumstances, abnormal postprandial lipoprotein metabolism[26]. Insulin resistance in T2DM is linked to a reduction in insulin's ability to inhibit hormone-sensitive lipase in adipose tissue, resulting in increased lipolysis and, as a result, increased portal flow of free fatty acid to the liver. Increased free fatty acid levels can cause lipoprotein activity to be

disrupted[26,27]. Consequently, the increased hepatic availability of free fatty acids leads to decreased degradation of apoB, thus causing an overproduction of very-low-density lipoprotein in insulin-resistant states. An increase in triglyceride-rich lipoproteins is commonly associated with a reduction in HDL and an increase in small dense LDL levels[26,28].

1.6 Complication of Diabetes Mellitus:

1.6.1 Acute complications:

1.6.1.1 Diabetic ketoacidosis:

Diabetic ketoacidosis (DKA): is a common and potentially definitive complication of type 1 diabetes, which occurs most often during the diagnosis process. DKA is less likely as type 2 diabetes is diagnosed. DKA is a complicated dysfunctional metabolic syndrome distinguished by hyperglycemia, acidosis, and ketonemia, due to absolute or relative insulin deficiency, which would be preceded by a rising trend in counter-regulatory hormones (i.e., glucagon, cortisol, growth hormone, catecholamines). These hormons increase glucose supply from glycogenolysis and gluconeogenesis while minimizing glucose consumption. Continuously, lipolysis produces more free fatty acids, which are oxidized to promote gluconeogenesis to produce aceto-acetic and beta-hydroxybutyric acids (ketones). Further, overload buffering ability, resulting in metabolic acidosis (pH 7.3), which has been aggravated by lactic acidosis due to weak tissue perfusion. Hyperglycemia causes osmotic diuresis, which may be commonly linked with DKA, as well as vomiting and, finally, a failure to breathe in fluid due to a reduced level of awareness. The osmotic diuresis contributes to electrolyte changes and degradation [29,30].

1.6.1.2 Non-ketogenic hyperosmolar hyperglycaemic state

Non-ketogenic hyperosmolar hyperglycaemic state (NKHHS): in the absence of substantial ketosis or acidosis, NKHHS is described as high blood glucose > 600 mg/dL (> 33.30 mmol / L) and serum osmolality > 320 mOsm / kg. Ketones can be found in small amounts in the blood and urine [31]. HHS is observed more commonly in type 2 diabetes (T2DM) and occurs in 2% of adolescents at presentation. Nonetheless, HHS can also occur in type 1 diabetes (T1DM) [32]. HHS is initiated by increased levels of counter-regulatory hormones (glucagon, catecholamines, cortisol, and growth hormone), which stimulate hepatic glucose synthesis via glycogenolysis and gluconeogenesis, resulting in hyperglycemia, intracellular water depletion, and osmotic diuresis [32]. The combination of high catecholamine levels and low insulin levels reduces peripheral glucose absorption. Glycosuria results in more water loss than sodium loss, leading to hyperosmolarity and dehydration. Reduced intravascular volume, which is frequently associated with underlying renal illness, lowers the glomerular filtration rate, lowering glucose clearance and raising blood glucose levels[33]. Even though insulin levels are insufficient to regulate blood glucose, it can inhibit lipolysis and ketogenesis[34]. As a consequence to free water loss over electrolytes, hypovolemic, intracellular and extracellular dehydration, and hyper osmolality develop. Persistent hypovolemic leads to counter-regulatory hormone release, which exacerbates hyperglycemia and contributes to insulin resistance[35].

1.6.2 Chronic complications

In diabetes, the resulting chronic complications are grouped under "macrovascular disease" (due to damage to the arteries) and "microvascular disease" (due to damage to small blood vessels) [14].

1.6.2.1 Macrovascular complications:

1.6.2.1.1 Cardiovascular morbidity and mortality

In diabetic individuals, cardiovascular disease is the major cause of morbidity and mortality. Coronary heart disease and atherosclerosis are caused by increased BMI, diabetes, hypercholesterolemia, smoking, family history, and age. Increased pulse pressure induces artery rigidity, which is a risk factor for cardiovascular disease in and of itself [36]. Coronary artery disease (CAD), stroke, and peripheral arterial disease (PAD) are widespread in DM, leading to a high mortality rate among diabetic patients [37]. DM-induced cardiomyopathy is primarily associated with dyslipidaemia and Increased blood pressure. The overproduction of oxidative free radicals that disrupt myocardial cells, contributing to dysregulation of cellular calcium homeostasis, contractile dysfunction, remodeling of the myocardium, and, finally, cardiomyocyte death, exacerbates the development of prominent cardiac fibrosis associated with cardiomyopathy[38].

In addition to coronary artery disease, Cerebral vascular disease increases in individuals with diabetes (stroke increase by three times). The etiology of this disease, is likely multifactorial and includes factors such as myocardial ischemia from atherosclerosis, hypertension, and cardiomyocyte dysfunction secondary to chronic hyperglycemia[39].

1.6.2.2 Microvascular complications

Retinopathy, nephropathy, and neuropathy are the three primary m anifestations of microvascular illness that will be discussed[40].

1.6.2.2.1 Diabetic Retinopathy

Diabetic Retinopathy (DR): is a major complication of diabetes mellitus (DM), and it is still the largest cause of vision loss in working-age people. Clinical signs of vascular abnormalities in the retina are used to diagnose DR. Non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) are the two phases of DR (PDR)[41]. Increased vascular permeability and capillary occlusion are two prominent findings in the retinal vasculature in NPDR, which characterizes the early stage of DR. PDR is characterized by neovascularization, which is a more advanced stage of DR. When the new aberrant vessels bleed during this stage, the patients may have a significant visual impairment. The most common cause of vision loss in patients with DR is diabetic macular edema (DME). DME is characterized by swelling or thickening of the macula due to sub- and intra-retinal accumulation of fluid in the macula triggered by the breakdown of the blood-retinal barrier (BRB) [42]. DME can occur at any stage of DR and cause distortion of visual images and a decrease in visual acuity[43].

The most common cause of blindness and diabetic retinopathy is cataracts[44]. The occurrence of cataracts was also substantially linked with the duration of diabetes, uncontrolled diabetes, and maculopathy (advanced diabetic retinopathy) among these type 2 diabetics [45].

1.6.2.2.2 Diabetic Nephropathy

Diabetic Nephropathy or diabetic kidney disease: is a syndrome characterized by pathological amounts of urine albumin excretion, diabetic glomerular lesions, and loss of glomerular filtration rate (GFR) in diabetics[46], as well as reduced kidney function caused by a variety of factors such as hypertensive nephropathy and unresolved acute kidney failure. Diabetic nephropathy is a long-term consequence of type 1 diabetes (absolute insulin deficiency) and types 2 diabetes (insulin resistance and/or reduced insulin production)[47]. The diabetic milieu leads to the synthesis and circulation of advanced glycation end products, the synthesis of growth factors, and hemodynamic and hormonal alterations, all of which contribute to the development of diabetic nephropathy and end-stage kidney disease. Reactive oxygen species and inflammatory mediators are released as a result. These modifications result in glomerular hyper filtration, glomerular hypertension, renal hypertrophy, and changed glomerular composition, resulting in albuminuria and hypertension in the clinic. Pathological alterations in the kidneys tend to involve extracellular matrix deposition (mainly in the mesangium), glomerular basement membrane thickening, proliferative alterations, and tubular atrophy, all of which lead to interstitial fibrosis and glomerulosclerosis (the final common pathway of many kidney diseases)[48].

1.6.2.2.3 Diabetic Neuropathy

Diabetic Neuropathy (DN): refers to a group of illnesses represented by peripheral nerve system impairment. Patients with Diabetic Neuropathy (DN) often have numbness, tingling, and/or burning in their limbs. Despite the reality that metabolic problems are the most

common source of severe pain produced by a clinical DN-based disorder. Although there are numerous causes of peripheral neuropathy, the most common subtype of diabetic peripheral neuropathy can lead to serious consequences[49]. Mechanical compression (e.g., carpal tunnel syndrome) and lifestyle factors like chronic alcohol consumption and smoking [50] have all been related to the syndrome. Consistently high blood glucose levels appear to cause damage to small blood vessels, limiting oxygen and nutrients to the neurons.

The damage occurs with distal sensory and autonomic nerve fibers, then progresses proximally, resulting in a progressive loss of protective feeling in both the skin and foot joints. [51,52]. When compared to painless diabetic neuropathy, a number of metabolic markers are linked to painful diabetic neuropathy, including poor glycaemic control[53], reduced renal function, and a high BMI (BMI). Some of these variables may be linked to neuropathy progression, while others may be linked to sensory neuron hyper excitability and the development of pain[54].

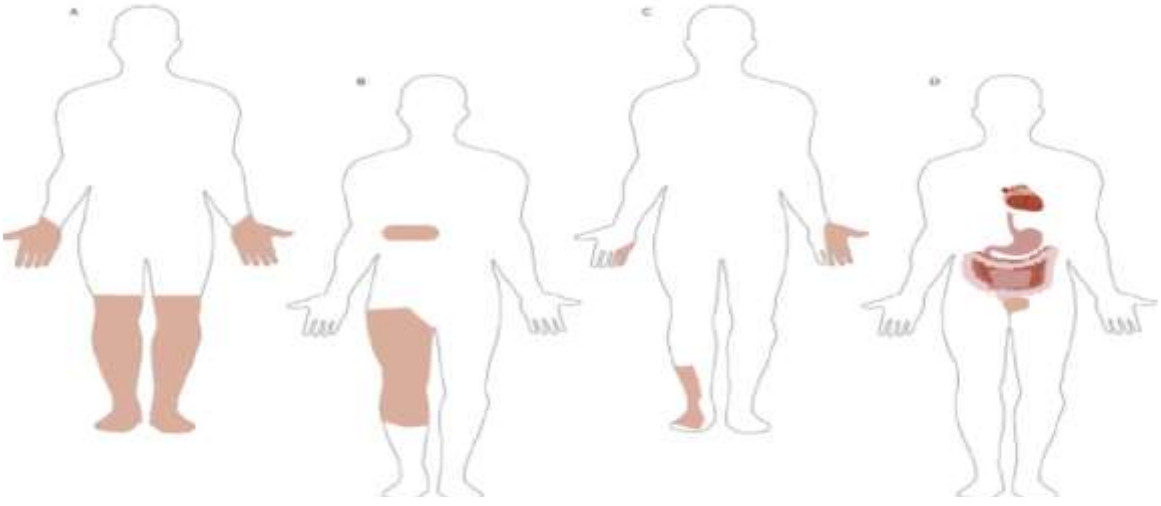
1.7 Classifications of Diabetic Neuropathies

The most common diabetic consequences are a collection of clinical symptoms induced by damage to the peripheral and autonomic nervous systems. These disorders, which are often referred to as distinct types of neuropathy, are caused by diffuse and localized nervous system injury and affect up to half of all diabetics. Diabetic neuropathy symptoms depend on the kind of neuropathy and where and how many nerves are affected[55,56]:

1.7.1 Distal symmetric polyneuropathy

Distal symmetric polyneuropathy (DSPN): Chronic DSPN is the most severe diabetic neuropathy, accounting for around 75% of all diabetic neuropathies. It's the existence of symptoms and/or signs of peripheral nerve dysfunction in patients with diabetes mellitus[57]. It progresses slowly, symmetrically, showing sensory and autonomic symptoms with a predominance of small fiber involvement, progressing with sensory large fiber involvement, and eventually motor fiber involvement in its more severe stages. Multiple variables linked to metabolic, vascular, inflammatory, and neurodegenerative processes are linked to DSP etiology.

Chronic hyperglycemia has a significant role in DSP pathogenic pathways and is the primary triggering factor[58]. Neuropathy duration and the severity of the hyperglycemia are too associated with the risk of ulceration and amputation of foot[59], as shown in the Fig(1-1)[60].



Fig(1-1): Many patterns of nerve injury are seen in patients with diabetes. The following patterns are shown in the figure: (A) diabetic sensorimotor polyneuropathy, small fiber neuropathy, or treatment induced neuropathy; (B) radiculoplexopathy or radiculopathy; (C) mononeuropathy or mononeuritis multiplex; (D) autonomic neuropathy (the most commonly affected organs are shown)[60].

1.7.2 Asymmetric Proximal Diabetic Neuropathy

Diabetic amyotrophy is another name, however, it is more accurate to term it diabetic proximal neuropathy[61]. Thoracic radiculopathy and proximal diffuse lower extremities weakness are two examples of proximal DN that should be grouped together under the term diabetic polyradiculopathy since they are different presentations of the same phenomenon; root or proximal. The majority of these patients have NIDDM. Patients have discomfort in the low back, hip, and front thigh, which is usually unilateral but can also be bilateral. Hip and leg muscular weakening and atrophy occur within days or weeks (Fig. 1-1). Knee reflexes are weak or non-existent. The sensations of numbness and paraesthesia are mild. More than half of the patients lose weight. Months pass in a step-by-step fashion. The discomfort goes away much before the motor symptoms do, which can take months, but mild to moderate weakness might last for years[62].

1.7.3 Diabetic Autonomic Neuropathy

Diabetic Autonomic Neuropathy (DAN): affects the body's multiple organs, inducing cardiac, gastrointestinal, urinary, sweating, pupil, and metabolic disorders. Autonomic nerve intervention will happen as soon as a year after a diagnosis of diabetes mellitus. DAN is a common complication of diabetes. As well, associated with the severity of somatic neuropathy. It ranges from subclinical functional impairment to severe cardiovascular and gastrointestinal, or genitourinary dysfunctions [58].

Table (1-3): Symptoms and tests for aspects of Diabetic autonomic neuropathy[58].

Category	Symptoms/Signs	Diagnostic Tests
Cardiovascular	Orthostatic hypotension Arrhythmia Silent ischemia Reduced exercise tolerance	Heart rate variation to deep breathing, Valsalva maneuver, blood pressure response to standing up , Tilt test cardiac scintigraphy
Gastrointestinal	Nausea Early satiety Constipation/diarrhea	Gastric emptying study Colonoscopy
Genitourinary	Erectile dysfunction Neurogenic bladder	Nocturnal penile plethysmography

1.7.4 Mono neuropathies

Nerve entrapment is more frequent than nerve infarction. Neuropathies with entrapment have an insidious onset, have characteristic electro diagnostic features such as conduction block or segmental nerve conduction slowdown. Carpal tunnel syndrome affects three times as diabetics as it does the general public. Other neuropathies that affect people with diabetes are the ulnar, radial, and lateral femoral cutaneous nerves of the thigh, and the peroneal, medial, and lateral nerves. Asymmetric pain and weakness in the proximal lower limb are symptoms of diabetic lumbosacral radiculoplexus neuropathy. Weight reduction and improved glucose control, such as after starting insulin, are two characteristics that may be linked[63].

1.8 The diabetic foot

Diabetic neuropathy and peripheral arterial disease (PAD) lead to the development of foot ulceration which is frequently complicated by

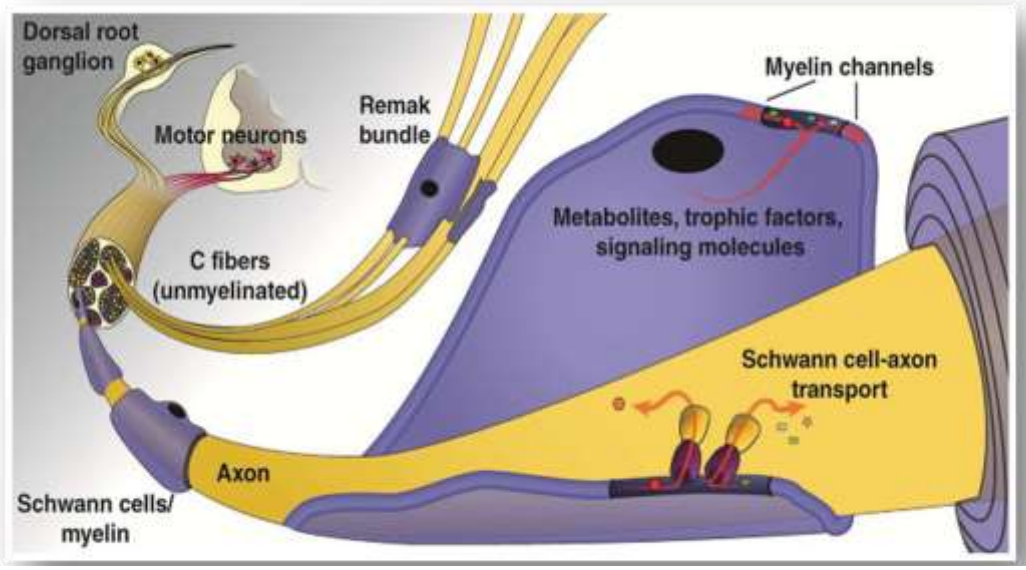
infection. Foot ulcers and amputations account for significantly reduced quality of life and increased morbidity and mortality. Diabetic peripheral neuropathy (DPN) increases the risk of foot ulceration through the loss of protective sensation, in the absence of which patients become vulnerable to trauma [64]. Small-muscle wasting and muscle atrophy related to peripheral neuropathy result in foot deformities, increasing the risk of breakdown of the skin in high-pressure areas [65]. Soft tissue trauma is a major causative factor in the development of diabetic foot ulcers (DFU) in patients with DPN [66]. patients with DPN are more prone to develop, and less likely to notice, wounds in their lower extremities because of the loss of protective sensation[64].

1.9 Epidemiology of Diabetic Neuropathy

According to the literature reports, nearly 10% to 20% of individuals with DM are also identified with DN at the time of their diagnostic DM start. However, research examining patients with DM at various phases of the illness in terms of chronicity has found that the DM and DN link is becoming more prevalent. After five years, 26% of diabetic patients have peripheral neuropathy, and after 10 years, 41% of diabetic patients have neuropathy. According to the research, 50 % to 66 % of persons with DM will acquire DN at some point in their lives[53]. Asymmetric sensory alterations are seen in around half of diabetic PN patients in clinical practice[67,68]. Individuals with a diabetic foot ulcer had 2.5 times higher risk of mortality after five years than patients with diabetes who do not have a foot ulcer. Diabetic foot ulcers and associated infection had a higher prevalence of emergency room visits than congestive heart failure, renal illness, depression, and most types of cancer[69-71].

1.10 Pathogenesis of diabetic neuropathy

Diabetic neuropathy is an unusual peripheral nerve system neurodegenerative disorder that mainly affects sensory axons, autonomic axons, and, to a lesser extent, motor axons. In diabetic neuropathy, terminal sensory axons in the peripheral retract and ‘die back,' while the perikarya (cell bodies) are relatively preserved[72]. Diabetic neuropathy's 'stocking and glove' pattern of involvement indicates damage to the longest sensory axons first, with loss of distal leg epidermal axons occurring before a loss in more proximal limbs; as result, neuropathy is referred to as a length-dependent neuropathy[73]. Diabetes appears to target the whole neuron, from the perikaryon to the terminal, according to extensive experimental data. Damage to peripheral axons and their accompanying Schwann cells, or the neuron perikarya that sit in the dorsal root ganglia (DRG) and function to maintain the axons, is a point of contention[74], Fig(1-2). Even though diabetic neuropathy is not predominantly a demyelinating neuropathy, chronic hyperglycemia attacks Schwann cells, and much more severe instances of diabetic neuropathy in patients contain demyelination[75-77]. Because axons and Schwann cells have such a strong and intimate relationship, Schwann cell injury might cause a variety of changes in the axon[78]. Schwann cells in mice possess ribosome-filled vesicles that can influence axonal protein production when transferred to desomatized axons[79]. This transfer of ribosomes may place a greater emphasis on axon–Schwann cell connections in the presence of axonal injury and stress[79,80].



Fig(1-2): The peripheral nervous system (PNS): The PNS is comprised of both neurons and SCs (Schwan cells), and the structure, location, and interaction of these components have important implications for PNS function. Efferent axons of motor neurons, whose cell bodies are located in the ventral horn of the spinal cord, carry signals from the Central Nerve System (CNS) to muscles and glands, whereas afferent axons of sensory neurons, whose cell bodies are located in the dorsal root ganglia, relay information from peripheral sensory receptors to the CNS. Thin and unmyelinated sensory axons, also known as C fibers or small fibers, are associated with non-myelination SCs and are grouped as Remake bundles and represent a large portion of the PNS neurons. Myelinated sensory axons, on the other hand, are surrounded by myelin sheaths made by SCs that form distinct nodal domains important for saltatory conduction and a tubular network of myelinic channels that connect the SC cytoplasm with the periaxonal space to provide a source of energy to the axonal compartment.[74]

1.11 Mechanisms of Diabetic Neuropathy

The specific processes that contribute to diabetic neuropathy are still unknown. The etiology of diabetic neuropathy is widely considered to be multifactorial, including complicated interactions between glycaemic management, diabetes duration, age-related neuronal attrition, and other variables such as blood pressure, cholesterol levels, and weight [81].

1.11.1 The polyol pathway

The polyol pathway consists of the reduction step of glucose to sorbitol and the oxidation step of sorbitol to fructose: the first reduction

step is catalysed by aldose reductase (AR) in a reduced nicotinamide adenosine dinucleotide phosphate (NADPH)-dependent manner, and the second oxidation step is catalyzed by sorbitol dehydrogenase (SDH) in a NAD-dependent manner, respectively, as shown in Scheme (1-3). Hyperactivity of the polyol pathway causes various metabolic abnormalities, making it a leading cause of diabetic peripheral neuropathy (DPN) [82] and associated complications. Increased sorbitol levels cause myo-inositol and taurine depletion, and decreasing myo-inositol levels cause a decline in phosphatidylinositol (PI) and diacylglycerol (DAG) levels[83]. As a result, the Na-K ATPase activity reduced, causing functional and structural changes in the neuropathy[84]. The polyol pathway hyperactivity and consequent consumption of cofactors of AR (NADPH) and SDH (NAD^+) trigger a cascade of interrelated metabolic imbalances. NADPH is a common cofactor of AR, nitric oxide synthase (NOS), and glutathione reductase (GR), and its depletion due to the increase in AR activity leads to the inhibition of the other enzymes [85]. The reduced NOS activity and NO contents can be a cause of diminished nerve blood flow, whereas the decreases in reduced glutathione (GSH) by GR inhibition results in the excessive production of free radicals and the enhancement of oxidative stress. NAD^+ is transformed to the reduced form NADH during the second stage of the SDH-catalysed pathway[83]. Because NADH is a substrate for NADH oxidase, its conversion causes the activity of NADH oxidase to increase, resulting in the formation of superoxide anions and leading to oxidative stress. Furthermore, redox state abnormalities in NAD^+/NADH may be implicated in the activation of PKC, which is also involved in the development of DPN[82,86].

1.11.2 Hexosamine pathway

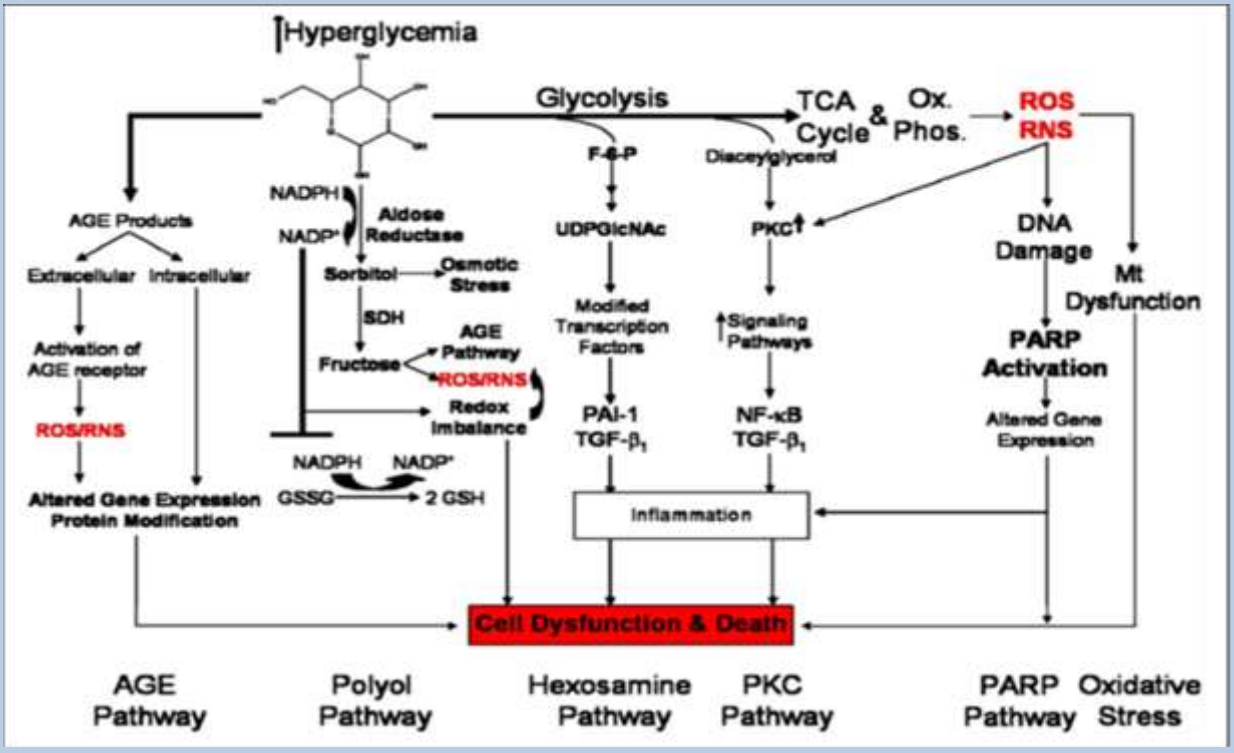
The influence of intracellular Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) on protein modification is a well-accepted mechanism by which the Hexosamine pathway contributes to diabetic neuropathy. The Hexosamine pathway is a minor branch of the glycolytic system under physiological conditions with only 2-5 % of fructose-6-phosphate being converted to glucosamine-6-phosphate by glutamine-fructose-6-phosphate aminotransferase (GFAT), the rate-limiting enzyme. However, in hyperglycemia, the increased generation of mitochondrial ROS inhibits the glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase, preventing fructose-6-phosphate flow via glycolysis. The amino sugar uridine5′-diphosphate-N-acetylglucosamine is then made from glucosamine-6-phosphate, acetyl-CoA, and uridine5′-triphosphate (UDP-GlcNAc)[87]. GlcNac is a glucose moieties that binds to serine/threonine residues on common transcription factors like Sp-1, increasing lipid dyshomeostasis, inflammation, and damage in complication-prone tissues like peripheral nerves [25]. Specific protein -1 Sp1 is responsible for the expression of many glucose-induced genes include transforming growth factor-β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1)[25,88]. Overexpression of TGF-β1 leads to increased collagen matrix production that promotes endothelial fibrosis and decreases proliferation in mesangial cells. PAI-1 overexpression promotes vascular smooth muscle cell mitosis which plays a role in atherosclerosis[86] as shown in Scheme (1-3).

1.11.3 Activation of Protein kinase C

Protein Kinase C (PKC) belongs to a family of proteins that modulate the activities of other proteins through the process of

phosphorylation in a cascade of reactions. The enzyme is a key element in cellular signaling pathways involving diacylglycerol (DAG), phosphatidylserine, and calcium Accumulation of glyceraldehydes-3-Phosphate due to inhibition of glyceraldehyde-3-Phosphate dehydrogenase in hyperglycemic condition[89]. leads to an elevated level of dihydroxyacetone-3-Phosphate (DHA-3-P) which is a triose isomer of the former. DHA-3-Phosphate is subsequently reduced to glycerol-3-Phosphate which in turn combines with fatty acids to drive the de novo synthesis of diacylglycerol (DAG) through the actions of 1-acylglycerol-3-P-acyl transferase and phosphatidate phosphohydrolase [90]. The increased cellular level of DAG may also result from the hydrolysis of the phosphatidate particularly phosphatidylcholine and phosphatidylserine up-regulates PKC pathway/isoforms [89] which also have been noted to be stimulated by the interaction of AGEs with their extracellular receptors (RAGE) [91]. Elevated activities of the PKC pathway have been reported to stimulate ROS-generating enzymes such as NADPH-oxidases and lipoxygenase which all together exacerbate the cellular oxidative environment [92]. Increased production of the PKC β-isoform, in particular, has been implicated in overexpression of the angiogenic protein vascular endothelial growth factor (VEGF), PAI-1, nuclear factor-kappa B (NF-κB), TGF-β, and the development of diabetic complications such as retinopathy, nephropathy, and cardiovascular disease. [93]. Activation of PKC inhibits insulin-stimulated expression of the messenger RNA for endothelial nitric oxide synthase (eNOS) in cultured endothelial cells[94,95]. PKC pathway activation causes hypoxia, angiogenesis, basement membrane thickening, and endothelial proliferation, as well as altering vasoconstriction and capillary permeability[86]. Different PKC isoforms have been demonstrated to

inhibit Na-K ATPase activity in smooth muscle cells and restore activity in peripheral neurons when activated[86], as shown in Scheme (1-3).



Scheme (1-3): Hyperglycemic effects on biochemical pathways in diabetic neuropathy.. NF-κB: Nuclear factor kappa B; PARP: Poly(ADP-ribose) polymerase; PKC: Protein kinase C; AGE: Advanced glycation endproducts; RNS: Reactive nitrogen species; ROS: Reactive oxygen species, GSH: glutathione; GSSG: oxidized glutathione; UDPGlcNAc: UDP-N-Acetyl glucosamine; VEGF: Vascular endothelial growth factor[86].

1.11.4 Pathway of advanced glycation end products

During the Maillard process, glucose interacts with amino groups on proteins to make Amadori products, which produce irreversible glycation products called AGEs throughout time. Essential proteins are cross-linked by AGEs, which alter their function and cause cellular destruction. AGEs bind to cell surface receptors, principally the Receptor for AGE (RAGE), which trigger harmful downstream signaling pathways controlled in part by nuclear factor-kappa B (NF-B) activation[96]. Endothelin-1, tissue factor, and thrombomodulin gene transcription is

regulated by NF-kB, and so is the generation of pro-inflammatory cytokines including interleukin-1a (IL-1a), interleukin-6 (IL-6), and tumor necrosis factor-α (α -TNF) [97]. In addition to increased vascular permeability, there is increased expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM1)[98]. AGEs have been demonstrated to decrease sensory-motor conduction velocities, nerve action potentials, and blood flow in peripheral nerves in animal investigations, but the significance of glycation in diabetic neuropathy is unknown [99].

1.11.5 Poly (ADP-ribose) polymerase pathway

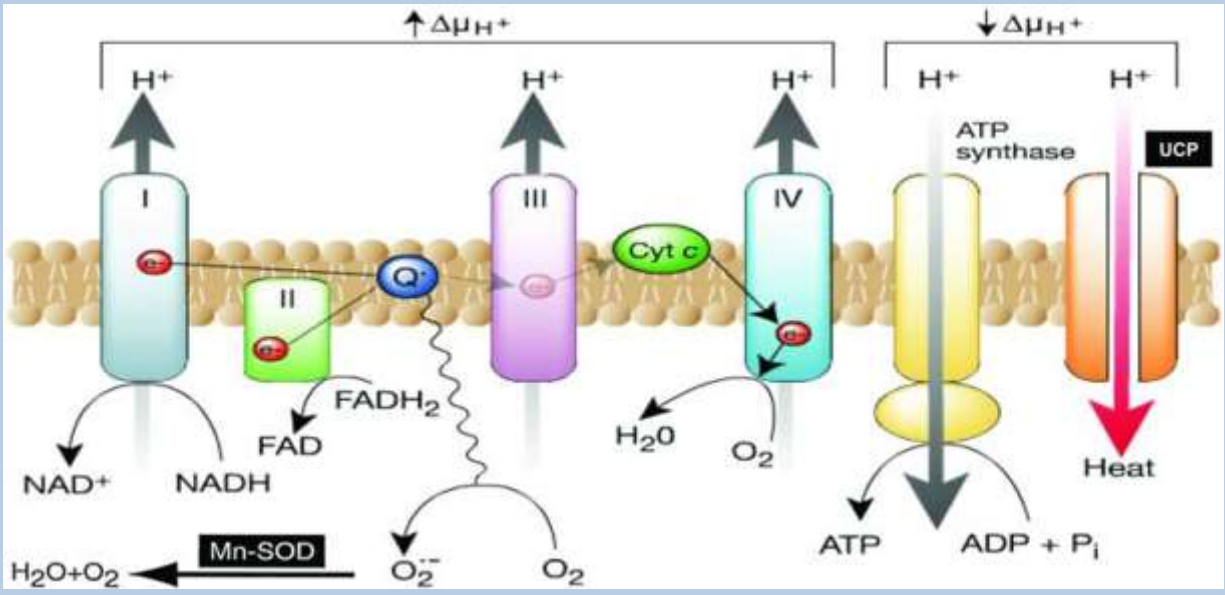
Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant nuclear enzyme playing a prominent role in DNA damage repair, genomic integrity maintenance, and execution of cell differentiation and death [100]. PARP-1, regarded as the prototypical PARP enzyme, cleaves NAD^+ and forms negatively charged poly(ADP-ribose) (PAR) polymers on a variety of target proteins. The poly(ADP-ribosyl)ation reaction (PARylation) is divided into three steps: initiation, elongation, and branching [101]. In the initial stage, the glycosidic bonds between nicotinamide (DAM) and adenosine diphosphate (ADP) ribose are broken down by enzymes, and the ADP ribose is linked to the acceptor proteins [102], and DAM is released during the enzymatic reaction. In the case of PARP-1, ADP ribose is elongated and branched, and finally forms PAR [101]. PARP-1 participates in a series of biological functions of cells, including DNA repair, gene transcription regulation, inflammation, energy metabolism, cell proliferation, and apoptosis [103]. In DM, hyperglycemia leads to the generation of reactive oxygen and nitrogen species, which initiate single-strand DNA breaks and activate the PARP

enzyme.5However, when the PARP enzyme is highly activated, it causes depletion of intracellular NAD+/NADPH and generation of ADP ribosylation of glyceraldehydes 3-phosphate dehydrogenase (GAPDH). This process causes energy failure, alteration of transcriptional regulation and gene expression that up regulates the release of pro-inflammatory mediators, and a shift of the glycolytic flux into several pathways associated with nerve blood vessel damage[104].

1.11.6 Oxidative stress–induced vascular pathology in diabetes

Superoxide is the initial oxygen free radical formed by the mitochondria, which is then converted to other more reactive species that can damage cells in numerous ways [105]. Normally, electron transfer through Complexes I, III, and IV extrudes protons outwards into the intermembrane space, generating a proton gradient that drives ATP synthase (Complex V) as protons pass back through the inner membrane into the matrix. In contrast, in diabetic cells with high intracellular glucose concentration, there is more glucose-derived pyruvate being oxidized in the TCA cycle, increasing the flux of electron donors (NADH and $FADH_2$) into the electron transport chain. As a result, the voltage gradient across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked [106], causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide (Fig. 1-3). The mitochondrial isoform of the enzyme superoxide dismutase degrades this oxygen-free radical to hydrogen peroxide, which is then converted to H_2O and O_2 by other enzymes. In primary arterial endothelial cells in culture, intracellular hyperglycemia increases the voltage across the mitochondrial membrane

above the critical threshold necessary to increase superoxide formation [107] and, subsequently, increases the production of ROS. It has been also demonstrated that dynamic changes in mitochondrial morphology are associated with high glucose-induced overproduction of ROS. Inhibition of mitochondrial fission prevented periodic fluctuation of ROS production during high glucose exposure [108]. The mitochondria are required for the initiation of hyperglycemia-induced superoxide production, much evidence indicates that this, in turn, can activate several other superoxide production pathways that may amplify the original damaging effect of hyperglycemia. These include redox changes, NADPH oxidases, and uncoupled eNOS[106].



Fig(1-3): Hyperglycemia-induced production of superoxide by the mitochondrial electron transport chain: Production of ROS by the mitochondrial electron transport chain. In cultured endothelial cells the electron donors NADH and $FADH_2$ are generated by the oxidation of glucose-derived pyruvate. The flow of the donated electrons (e−) through the electron-transport chain in the inner mitochondrial membrane pumps H+ ions into the intermembrane space. When the voltage gradient is high due to increased flux of electron donors, more superoxide is generated. H+ ions can pass back across the inner membrane along their concentration gradient, either via ATP synthase (to produce ATP) or via uncoupling proteins (UCP), which dissipate the energy of the proton gradient as heat. ADP, adenosine diphosphate; Cyt c, cytochrome c; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide[106].

1.11.7 Inflammatory processes

C-reactive protein and TNF-alpha are inflammatory agents appear in the blood of both T1DM and T2DM [36,110]. Alternative metabolic pathways like fructose-6 phosphate or di-acyl-glycerol, the signaling intermediates and modified transcription factors cause increases in TGF-β and NF-κB when excess glucose is shunted[111].

Cyclooxygenase -2 (COX-2) is a fundamental enzyme that is activated by NF-κB. This up-regulation can be seen in peripheral nerves and vascular tissues in diabetes of experiments and in turn it generates prostaglandin E2 and ROS. Another inflammatory enzyme regulated by NF-κB is inducible nitric oxide synthase (iNOS). The NO generated by iNOS directly modulates the blood supply to nerves and participates in microvascular changes following injury. In diabetic nerves, the cytokines created by NF-κB in endothelial cells, Schwann cells, and neurons also affect macrophage recruitment[112]. Macrophages facilitate diabetic neuropathy via several pathways, including the development of reactive oxygen species (ROS), cytokines, and proteases, both of which cause myelin breakdown and cellular oxidative damage[92].

1.11.8 Growth factors

Because diabetic neuropathy is marked by neuronal degeneration and disruption to supporting Schwann cells, changes in growth factors like nerve growth factor (NGF), insulin-like growth factor (IGF), and neurotrophin 3 (NT-3) have been proposed to play a role in the disease. These proteins bind to tyrosine kinase receptors that are heterodimeric[92].

1.12 P-type ATPases:

Enzymes that carry ions through cell membranes are known as transport enzymes. Transport occurs against an ion gradient, and the energy needed for the process is supplied by ATP, which is hydrolyzed to ADP and Pi during the process. These are a broad class of evolutionarily conserved ion pumps present in bacteria, archaea, and higher eukaryotes which belong to the P-type ATPases (includes Na^{+}, K^{+}, Ca^{2+}, H^{+}, K^{+} -ATPases),

Besides are involved in a variety of essential biological and medical processes, including membrane potential production, muscle contraction, toxic ion elimination, and cell maintenance proper acidity inside cells, etc.[110]. Three P-type of ATPases, provide three-dimensional configurations at atomic resolution: Ca-ATPase[111] Na-K ATPase[112,113], and H-ATPase[112].

1.12.1 Ca^{+2}-ATPase or Ca^{+2}pump

Ca^{2+}-ATPases are key components of Ca^{2+} extrusion machinery and thus are pivotal for the preservation of neuronal function. Among three main calcium pumps, the plasma membrane Ca^{2+}-ATPase (PMCA) and Sarco/endoplasmic Ca^{2+}-ATPase (SERCA) is known for decades while the secretory pathway Ca^{2+}-ATPase has been discovered in the 2000s by two independent laboratories that described novel mutations leading to Hailey-Hailey disease [114]. All pumps have a high affinity for Ca^{2+} and function to restore cytosolic Ca^{2+} concentration [Ca^{2+}] to the resting, Nano molar level following neuronal stimulations. They belong to the superfamily of mammalian P-type ATPases and are characterized by the formation of a phosphorylated enzyme intermediate during the catalytic cycle [115]. Calcium Ca^{2+}, which interacts with the outer of the

cellular membrane as though it was a hormone or growth factor, could also function as a real first messenger [116].

Plasma membrane Ca^{2+}-ATPase (PMCA) is one of the most important and sensitive players in maintaining low resting Ca^{2+} concentration[117]. Structurally, PMCA comprises ten trans membrane segments with N- and C- terminal tails both located on the cytosolic site [118]. Most of the regulatory regions including acidic phospholipids, protein kinase C (PKC), and protein kinase A (PKA)[119].

The Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) The SERCA pump is the product of a multigene family. It is a 110-kDa single polypeptide located in the Sarco/endoplasmic reticulum (SR/ER) which primary role is to transport Ca^{2+} back to the internal stores. Mammals express three isoforms of the pump, called SERCA1 through SERCA3, and post-translational modifications increase the total number of identified subtypes to 10 [120]. The expression profile of individual variants is not only tissue-dependent but also changes development. The distribution of SERCA1 is limited to fast- and slow-twitch skeletal muscles, and the role of this pump is to accumulate calcium in the SR of skeletal muscle.SERCA2 exists in two variants—SERCA2a and SERCA 2b [121]. SERCA2a is mostly found in cardiac and skeletal muscle with slow contractile characteristics but also exhibits minor expression in the brain, where it is almost exclusively restricted to the Purkinje neurons of the cerebellum [122]. SERCA2a is considered to be involved in the contraction and relaxation of cardiac muscle. SERCA2b is the most abundant variant expressed widely in all tissue types, including neurons. In the brain, SERCA2b expression has been identified in both, cerebrum, and cerebellum [123]. Moreover, SERCA2b is the only SERCA variant present in astrocytes [124]. This enzyme translocates two of Ca^{2+} from

the cytoplasm to the lumen of the reticulum by using the chemical energy derived from ATP hydrolysis. After that Ca^{2+} is accumulated inside the reticulum, a Ca^{2+} gradient is formed across the membrane and this promotes the reversal of the catalytic cycle of the enzyme during which Ca^{2+} leaves the reticulum in a process coupled with the synthesis of ATP from ADP and Pi.)[125].

Secretory Pathway Ca^{2+}-ATPase (SPCA): The Golgi-Resident Ca^{2+}/Mn^{2+} Pump SPCA pump, as a member of the P2A subfamily, shares some common structural and mechanistic properties of SERCA [126], yet in addition to high Ca^{2+} affinity, SPCA also represents a strong preference for Mn^{2+} ions. This Ca^{2+}/Mn^{2+} transporter possesses unique structural elements in the N-terminus and trans membrane (TM) region determining the orientation and selectivity of the ion transport during phosphoryl-transfer reactions. Particularly, the SPCA pump is crucial for maintaining the sufficient supply of Mn^{2+} for glycosyltransferases and sulfotransferases in the Golgi lumen [127]. On the other hand, in the cytosol, the SPCA pump prevents excessive accumulation of Ca^{2+} and Mn^{2+}, while the overload of these ions may trigger neurotoxicity resulting in several neurological disorders [128].

1.12.2 Proton Pump (H^+-K^+ ATPase)

The proton pump (H^+-K^+ ATPase) is composed of the α-subunit and β-subunit[129,130]. The proton pump is primarily located in the parietal cells of the stomach, where it secretes hydrogen ions (protons) into the gastric lumen. H^+-K^+ ATPase is a member of the P2-type ATPase family, such as Na^+-K^+ and Ca^{2+}-ATPases. The production of acid with the exchange of K^+ for H^+ is an ATP-consuming process. The α-subunit plays a catalytic role, due to its binding site and hydrolysis of ATP[129].

The α-subunit is composed of 10 trans membranes (TM) helices, in which the cation-binding sites are located, and three cytoplasmic domains (the nucleotide (N), phosphorylation (P), and actuator (A) domains) that catalyze ATP hydrolysis and the auto-phosphorylation reaction. The β-subunit is a single-span membrane protein with a short N-terminal cytoplasmic tail and a large C-terminal ectodomain, and it is involved in correct membrane integration and targeting of the complex to the cell surface[131].

1.12.3 Sodium-Potassium Adenosine 5-Triphosphatase (Na-K ATPase) (NKA)

The sodium- and potassium-activated adenosine 5-triphosphatase (NKA) (EC 3.6.1.37), or Na-K pump is a ubiquitous enzyme, structurally consists of α, β and γ subunits[132], and is responsible for maintenance of the Na^+ and K^+ gradients across the cell membrane by transporting 2 K^+ into the cell and extrudes 3 Na^+ when hydrolyzing adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate (Pi), against the concentration gradients [133]. The sodium pump can also activate secondary active co-/counter transporters, such as the Na^+/Ca^{2+} exchanger, which are activated by the gradient of extracellular to intracellular [Na^+][134]. This enzyme is found in all mammalian cell membranes and it helps in maintaining osmotic balance and membrane potential[135]. Na-K ATPase is a signal transducer as well as an ion pump. Both the α and β-subunits bind to signaling molecules such as tyrosine-protein kinase (Src.), phosphoinositide 3-kinase (PI3K), caveolin-1, protein phosphatase 2, and EGFR, activating several intracellular signaling pathways such as MAPK and Akt signaling to regulate cell polarity, cell growth, cell motility, and

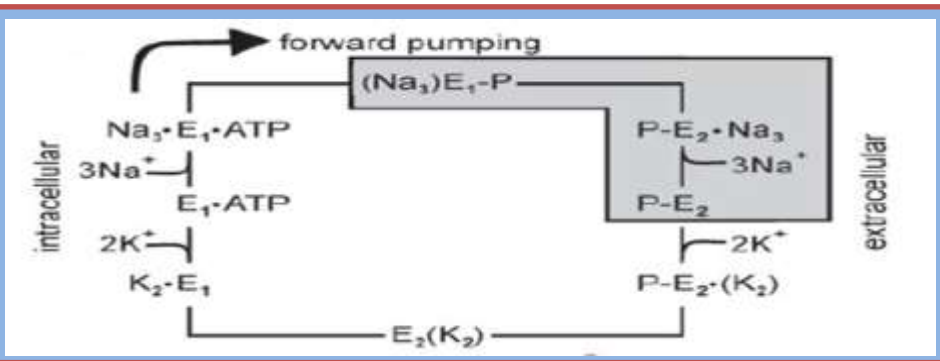
gene expression[136]. The cardiac glycoside ouabain, a particular pump inhibitor, acts as a receptor for Na-K ATPase. Ouabain studies at doses that do not block the Na-K ATPase pump function have been especially valuable in identifying the Na-K ATPase pump-independent signaling pathways. Na-K ATPase also regulating the development and stability of intercellular junctions. As well, the β1 and β2-subunits of Na-K ATPase operate as cell adhesion molecules[137]. The etiology of certain pathological processes is believed to be linked to the regulation of this enzyme (transporter) and its isoforms.

Other physiological processes involving it include the processing of waste products in the nephrons (kidneys), sperm motility, and the generation of a neuronal action potential[138]. Similarly, the physiologic effects of inhibiting the Na-K ATPase are beneficial and the focus of many pharmacologic applications[139].

1.12.3.1 Functional Properties with Structural Implications

Ion movements through the Na-K ATPase have been the subject of biophysical investigations employing several methods in recent years [140-142]. The so-called Post Albers cycle (Scheme 1-4) provides a conceptual framework for such research. It is a widely acknowledged system for describing the sequence of reaction stages in linked ion transport and enzymatic processes. It has been established that Na^+ and K^+ transport occur by a "Ping-Pong" mechanism, meaning that the two ion species are transported sequentially. Na^+ movement out of the cells is associated with phosphorylation by ATP and then the conformational change E1-P → E2-P, while the counter transport of K^+ ions is associated with rapid dephosphorylation of E2-P and the conformational change E2(2K) → E1 which is accelerated by binding of ATP with a low affinity

[143]. The occurrence of occluded states, E1-P(3Na) and E2(2K), in which the ions are unable to interchange with either aqueous phase is a distinctive attribute of the sequence reaction [144]. The pump creates an electric current in which a net positive charge is extruded from the cell due to uneven quantities of charge transferred in opposing directions. Electrogenicity is the name given to this characteristic of the ion pump[143]. The measurement of charge changes throughout the transport process is an excellent approach to examine ion transport. The kinetics, energetics, and electrogenicity of the ion-trans locating partial processes have been studied extensively, and the experimental findings impose numerous restrictions on any mechanistic model of the Na-K ATPase. The charge-trans locating stages of the transport process are mostly associated with ion binding and release[142], as shown in Scheme (1-4).



Scheme (1-4): Albers–Post scheme for the NaK-ATPase reaction cycle. The enzyme can assume two distinct conformations: E1 with ion binding sites facing the cytoplasm, and E2 with ion binding sites open to the extracellular space. The main electrogenic event was assigned to Na transport steps that are kinetically coupled to the E1P–E2P transition[145].

1.12.3.2 Na-K ATPase Gene Polymorphism

The ubiquitous Na-K ATPase maintains the sodium and potassium gradients in animal cells. It controls basic cellular homeostasis, assures excitability of neuronal and contractile tissues and drives sodium reabsorption in renal epithelia, which controls the extracellular volume

and blood pressure. The X-ray crystal structure of the oligomeric Na-K ATPase has recently been determined[146], containing the catalytic α subunit, the β subunit and a FXYD protein (Fig. 1-4) [49,146-148]. The catalytic α subunit has 10 trans-membrane segments, transports the cations and is represented by four isoforms[149]. It is a multi-spanning membrane protein with a molecular mass of ~112,000 Da that contains the binding sites for the cations and ATP and acts as the receptor for specific inhibitors, cardiac glycosides such as ouabain, which are bound to the extracellular side of the protein at very high affinity and lead to the inhibition of enzymatic activity [150-152]. The β subunit is a type II glycoprotein, represented by three isoforms [149,153], that crosses the membrane once and has a molecular weight between 40,000 and 60,000 Da, depending on the degree of glycosylation in various tissues. The β subunit is essential for the normal activity of the enzyme [131], as well as, it appears to be involved in the deformation of K^+ and a modulation of the K^+ and Na^+ affinity of the enzyme [137]. Furthermore, the γ-subunit, an FXYD protein, is tissue-specific and modulates Na^+, K^+, and ATP affinity, pump kinetics, and transport characteristics, as well as stabilizing the Na-K ATPase[136].

The alpha subunit has three cytoplasmic domains, the nucleotide binding (N), the phosphorylation (P) and the actuator (A) domains, which function as the kinase, the substrate and the phosphatase, respectively, in the catalytic cycle, when ATP is hydrolyzed (Figure 1-4). A conserved aspartate in the P-domain is phosphorylated and dephosphorylated during the cycle, and the movements of the cytoplasmic domains cause the connected trans-membrane TM helices to bind and release the pump's substrates in response. The Na-K ATPase was the founding member of the P-type ATPase family[154].

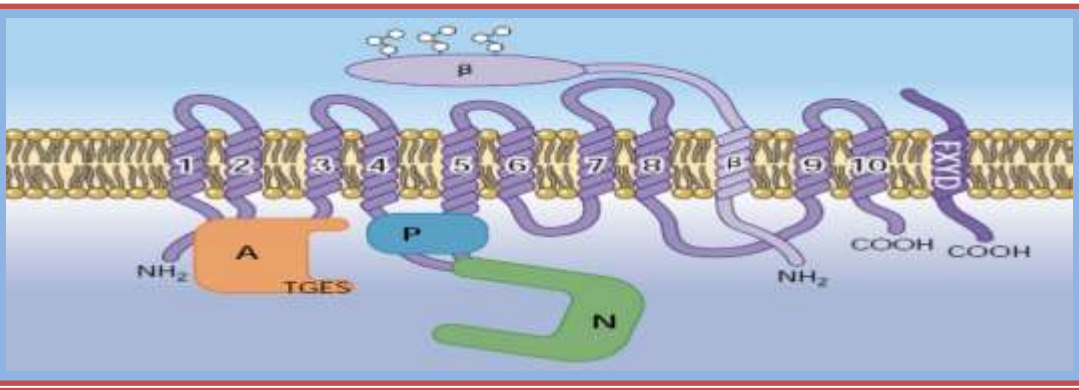


Fig.(1-4): Structure of the Na-K-ATPase. Ten trans-membrane segments are represented in an "unfolded" disposition, but they actually form a bundle around the 3 central 4th, 5th, and 6th trans-membrane segments. Color coding shows the actuator (A; orange) domain, which is constituted by the NH_2-terminal segment preceding the 1st trans-membrane segment and the intracellular loop between the 2nd and 3rd trans-membrane segments. It contains the TGES motif that is involved in the dephosphorylation step. The phosphorylation (P; blue) domain is made up of the proximal and distal parts of the large intracellular loop between the 4th and 5th trans-membrane segments. In its initial section, it contains the DKTGT motif with the aspartyl residue D376, on which the phosphate of ATP is transiently transferred. The nucleotide (N; green) domain is made up of the main part of the large intracellular loop and forms a kind of pocket for ATP binding. The β-subunit has a single trans-membrane segment, a short intracellular NH_2 terminal, and a large extracellular domain with several glycosylation sites (3 are shown). In this scheme the β-subunit trans-membrane segment is located close to the 7th and 8th trans-membrane segments because the early part of the extracellular domain of the β-subunit is known to interact with a conserved SYGQ motif located in the relatively large extracellular loop between the 7th and 8th trans-membrane segments. The Na-K-ATPase α- and β- subunits may also associate with a third subunit belonging to the "FXYD" protein family[138].

The smaller beta-subunit have recently been cloned using cDNA. They used Southern filters containing panels of rodent X human somatic cell hybrid lines to hybridize rat brain and human kidney cDNA probes, as well as human genomic isoform-specific DNA fragments. The results obtained have allowed us to assign the loci for the *ATPlAl* gene that has been located on the short arm of human chromosome 1, region lp2l+cen. *ATPlA2* gene is located on the long arm of chromosome 1, region cen+q32. ATP1A3 maps on chromosome 19. The fourth gene of unknown function ((YD) that was isolated by molecular cloning (*ATPlALl*) was mapped to chromosome 13. Regardless of the fact the historic reports indicated, the B-subunit was encoded by a single gene, their revealed hybridizing restriction fragments originated from two

separate human chromosomes. They hypothesize that the coding gene *ATPlB* is situated on the long arm of human chromosome 1 and so the sequences on human chromosome 4 (ATPlBLZ) are either a related gene or a pseudogene based on knowledge of conserved linkage groups on human and murine chromosomes[155]. The genes coding for each Na pump isoform have been mapped to specific chromosomal regions. This, as well as the intron/exon composition of the isoforms of human, rat, and mouse[155], are shown in Table 1-4.

Table (1-4): Chromosomal Localization and Number of Exons and Introns of Na, K-ATPase Isoform Genes From Various Species[156,157].

Isoform	α1	α2	α3	α4	β1	β2	β3	βm (β4)
Gene nomenclature	**ATP1A**	**ATP1A2**	**ATP1A3**	**ATP1A4**	**ATP1B1**	**ATP1B2**	**ATP1B3**	**ATP1B4**
Human								
Chromosome	1p21	1q21-q23	19q13	1q21-q23	1q24	17p13	3q22-q23	Xq25
Exon/intron	23/22	23/22	23/22	22/21	7/6	7/6	7/6	8/7
Rat								
Chromosome	2q34	13q24-q26	1q21	13q24	13q22	10q24	8q31	Xq11
Exon/intron	21/20	23/22	23/22	22/21	7/6	6/5	7/6	8/7
Mouse								
Chromosome	3F3	1H3	7A3	1H3	1H2.2	11B3	9E3.3	XA3.1
Exon/intron	23/22	23/22	23/22	22/21	7/6	7/6	7/6	8/7

Data obtained from Ensembl Genome Data Resources (www.ensembl.org/) and the Nationaɑl Center for Biotechnology Information (www.ncbi. nlm.nih.gov/entrez/).

1.12.3.3 Major Alpha Subunit Distribution And Disease Association

1. Alpha1: (ATP1A1 gene) is expressed in all tissues; The major organ that expresses alpha1 subtype is the heart as well as 2 and 3. Alpha 1 subunit is also expressed in the proximal convoluted tubules of the kidney[137]. Although deleterious mutations in ATP1A1 are unlikely to be life-threatening, somatic mutations in ATP1A1 can lead to disturbed hormone balance in a subset of aldosterone-producing adenomas (APAs) in the adrenal gland[158,159].

2. Alpha2: (ATP1A2 gene) is the predominant isoform in skeletal muscle, but is also found in the brain (astrocytes), heart, eyes, adipose tissue, and distal tubules of the kidney[137]. Following migraine attacks, patients with Familial Hemiplegic Migraine (FHM) have aura and weakness on one side of the body[160]. Three genes encoding a calcium channel (FHM1), a sodium channel (FHM3), and the alpha2 subunit have been identified as having FHM-causing mutations (FHM2). FHM2[137] is caused by at least 80 mutations in the ATP1A2 gene.

3. Alpha3: Is highly expressed in the brain (neurons and astrocytes)[161]. Rapid-onset Dystonia Parkinsonism (RDP) was discovered to be caused by autosomal dominant mutations in ATP1A3, followed by Alternating Hemiplegia of Childhood (AHC) and CAPOS [137].

4. Alpha4: Is expressed in spermatozoa. Male mice are entirely infertile if they lack alpha4, even though sperm cells express both alpha1 and alpha4[162,163].

5. Betas: The Na-K ATPase beta subunit is essential for the pump's trafficking to the plasma membrane and is part of its functional core. The three human isoforms have a sequence similarity of 39 percent (beta1 and 2), 36 percent (beta1 and 3), and 47 percent (beta1 and 4). (beta2 and 3). It has a short N-terminal intracellular domain (30 amino acids), a TM helix, and a larger C-terminal extracellular domain (240 amino acids). The various beta isoforms, like the alpha isoforms, have diverse tissue and cell-type-specific expression patterns. The beta2 isoform was first discovered in glia, where it plays a role in cell-cell interaction, and was dubbed Adhesion Molecule On Glia (AMOG). Functionally, when compared to beta1 and 3, beta2 has the greatest impact on the pump's kinetic characteristics, lowering the apparent potassium affinity while

increasing the extracellular sodium affinity. The TM helix, not the N- or C-terminal domains, was discovered to be responsible for beta's influence on the pump, and more particularly, a variation in the tilting degrees of the 3 isoforms' TM helices. The various beta isoforms and post-translational modifications enable controlled Na, K-ATPase activity that is adaptable to diverse tissues and environmental changes[137].

6. FXYDs: The heart, skeletal muscle, and brain all have high levels of FXYD1. FXYD2, also known as gamma, was the first FXYD to be linked to the Na-K ATPase, and it is abundant in the kidney. FXYD2 inhibits pump action in the same way as FXYD1 does, and this inhibition is likewise alleviated when the protein is knocked out in mice. Mice missing FXYD2 are alive, but their reproductive is reduced, probably due to a metabolic state in which glucose is tolerated well. FXYD3 (Mat-8) and FXYD5 (dysadherin) have unknown functions, although they are overexpressed in some cancer cells, while FXYD6 and 7 are expressed in the brain. There are no confirmed human genetic disorders associated with mutations in the FXYD component at this time[137,164].

1.12.3.4 Pathophysiological significance of Na-K ATPase in diabetes: Diabetes has a substantial impact on a variety of tissues' metabolism, and because the NKA is required for membrane potential and numerous transports, a change in its activity in diabetes would have a major effect on these tissues. Diabetes has a powerful effect on diabetic erythrocyte metabolism [134], as well as a significant decrease in NKA activity in alloxan-induced diabetic rats [165]. Das et al [166] observed a decline in NKA enzyme activity in the sciatic nerve of diabetic rats in 1976, but an increase in enzyme activity in the small intestine mucosa of diabetic rats in 1976 [167]. These two instances show how diabetes affects NKA differently depending on the tissues. The sciatic nerve, lens,

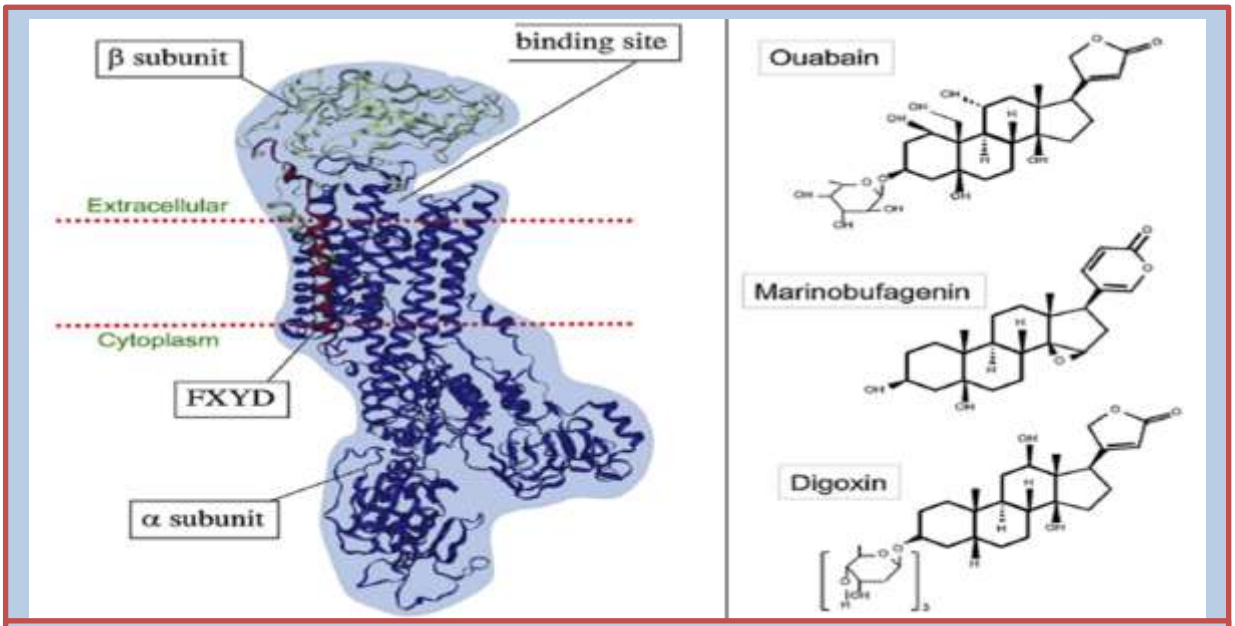
heart, and erythrocyte are among the tissues that have seen a decrease in NKA activity due to diabetes; another group has seen an increase in enzyme activity caused by diabetes, including the mucosa of the small intestine; and a third group has seen no change in NKA activity or has differences between studies. Depletion of the intracellular pool of myo-inositol increased flux through the aldose reductase pathway, and a change in protein kinase-C (PKC) activity have all been postulated as reasons for the reduction in NKA activity [168].

Other diabetes-related metabolic changes, such as an increase in oxidative stress, the formation of advanced glycation products, nerve growth factor metabolism [169], and disruption in essential fatty acid metabolism leading to an abnormal red blood cell membrane [170], can all reduce enzyme activity. Deficits in insulin and C-peptide can also affect the long-term control of enzyme units produced on the cell surface. Isoform overexpression cannot be linked to an increase in enzyme activity since β-isoforms are required to form an active complex. [171].

1.13 Endogenous Digitalis-like Factors

Endogenous Digitalis-like Factors (EDLF): are a kind of steroid that circulates in the bloodstream and is excreted through the urine. Cardenolides (plant-derived compounds such as ouabain and digoxin), and bufadienolides (animal-derived compounds as marinobufagenin) are two categories based on their chemical structure. A five-membered unsaturated lactone ring is linked to the steroid nucleus at position 17 in cardenolides, while a doubly unsaturated six-membered lactone ring is attached to the steroid nucleus in bufadienolides (Figure 1-5)[172]. They function as ubiquitous enzyme Na-K ATPase regulators. They had proposed as vasoconstrictors, natriuretic-hormones(NH), involved in the

physiological control of renal sodium excretion, cell growth, and blood pressure [173]. As well, contribute to certain forms of hypertension when pathologically elevated [174]. Humans with myocardial infarction, renal failure, congestive heart failure, hepatic failure, and the human placenta have been reported to have endogenous digoxin-like factors EDLF. All of these diseases have significant sodium balance or intravascular fluid status abnormalities[175]. Recently, they established that in investigational rats and patients through type 1 and type 2 DM, inhibition of NKA is attended by elevated levels of endogenous digitalis [176].



Fig(1-5): Structure of the NKA and the chemical structures of its commonly described ligands ouabain, marinobufagenin and digoxin. Conserved endogenous digitalis binding site is found within the α subunit. Three commonly studied endogenous digitalis are shown on the right[177].

1.13.1 Biosynthesis of endogenous cardiotonic steroids

The brain, the heart, and the adrenal gland have all been used to produce endogenous cardiotonic steroids[178]. Despite evidence that cardenolides may be produced in the heart [179] and the hypothalamus [180], most evidence refers to the zona fasciculata and glomerulosa of adrenal glands as the site of cardenolide production, particularly endogenous ouabain [181]. The precursors in the production of endogenous ouabain and endogenous digoxin are pregnenolone and progesterone. It has been shown that radioactive acetate and cholesterol may be converted into endogenous digoxin. The essential step in cardenolide production in plants is the conversion of progesterone's A/B trans rings to the A/B cis ring conformation of 5 preganane-3,20-dione by progesterone 5β reductase. This enzyme is found in mammals as well [178], however it has yet to be proved to be involved in cardenolide or bufadienolide production in mammals. As a consequence, the adrenal cortex can produce both bufadienolides and cardenolides from cholesterol, although their metabolic routes are different, under the control of catecholamines, ACTH, and angiotensin II As the concentration of endogenous cardiac glycosides is higher in the midbrain than in blood plasma, it is feasible that brain cells are also able to synthesize endogenous cardiotonic steroids as a neurotransmitter [182,183].

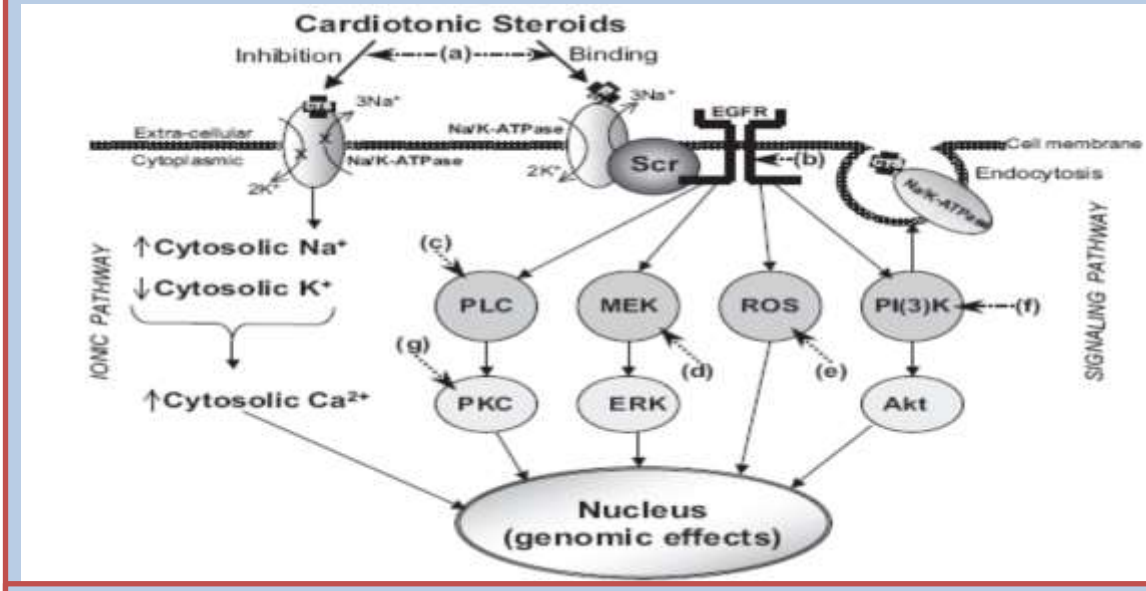
1.13.2 Endogenous Cardiotonic Steroids and Cell Signaling

Na-K-ATPase(NKA) inhibitors comprise a group of drugs that bind to the extracellular portion of the NKA pump and prevent its activity. The extracellular regions between helices 1 and 2 and helices 7 and 8 on the alpha-subunit tend to be important amino acids in inhibitor

binding. Ouabain, marinobufagenin, digitalis, digoxin, and digitoxin are just examples[181]. The different alpha-isoforms of the NKA have different affinities for each class of cardiac glycoside. Therefore, they have different effects largely based on the expression of the α-isoforms for which they have higher affinity[184]. The binding site, also known as the "ouabain binding site," is located within the catalytic domain of the NKA subunit [185], as shown in Fig. 1-5 [177]. It is strongly conserved across species, indicating that it has a physiological function.

As in the Scheme 1-5, The two pathways via which binding of cardiotonic steroids to the Na-K ATPase exerts genomic and non-genomic effects. In the classic 'ionic' pathway (left), inhibition of the pump function of the Na-K ATPase by endogenous digitalis results in an increase in cytosolic sodium concentration and a decrease in cytosolic potassium concentration[187]. This increase, in turn, alters the transport function of the Na^{+}/Ca^{2+} exchanger, which then results in increases in cytosolic calcium. With this connection to cytosolic [Ca^{2+}], it is easy to see how innumerable cellular functions might be influenced. These changes induce an increase in cytosolic calcium level, which in turn activates a variety of pathways with genomic and non-genomic effects[186]. Endogenous digitalis binding to the Na-K ATPase, on the other hand, activates Na-K ATPase-associated non-receptor protein kinase Src, according to the researchers. The activated Src then trans activates receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor EGF receptor (EGFR), and phospholipase C PLC. This leads to a cascade that involves generation of ROS, activation of extracellular regulated kinase ERK through activation of mitogen-activated protein kinase MEK, activation of PI(3)K, activation of Akt (protein kinase B) via PI(3)K, stimulation of endocytosis and activation of PKC[187]. These steps induce the genomic and non-

genomic effects of endogenous digitalis. Ouabain, for example, can activate protein kinase cascades and control cell development in cardiac myocytes, renal epithelial cells, vascular smooth and endothelial cells, and skeletal muscle cells. Both “signaling” and “ion pumping” functions of the Na-K ATPase may work in concert in the regulation of cellular functions, and the total effect may be dependent on cell type, expression of different α subunits, and interaction with other receptors[187].



Scheme (1-5): The binding of endogenous digitalis to Na-K ATPase. Abbreviations: EGFR; epidermal growth factor receptor; ERK, extracellular regulated kinase; MEK, mitogen-activated protein kinase; PI(3)K, phosphoinositide-3 kinase; PKC, protein kinase C; PLC, phospholipase C[186].

1.13.3 Role of Endogenous Digitalis in Diabetic Mellitus

Endogenous Digitalis levels were shown to be elevated, as well as the activities of the Na-K ATPase in diabetic humans and experimental animals. Because insulin resistance is frequently associated with NaCl-sensitive hypertension, it has been theorized that endogenous digitalis rises as a result of insulin-dependent renal salt retention in type 2 diabetes

and that excessive endogenous digitalis complexity leads to hypertension[186]. According to additional data, rats with type 1 diabetes had higher MBG levels and more severe sodium pump blockage than rats with type 2 diabetes[188]. This evidence suggested that endogenous digitalis may play a role in insulin sensitivity determination[189]. Ouabain and MBG enhance glycogen synthesis in skeletal muscle, according to Kotova et al. [190]. This impact was additive to insulin and was mediated by activation of a signaling cascade including Src, ERK1/2, and glycogen synthase kinase 3 [190]. As a result, endogenous digitalis might be involved in physiological systems that regulate carbohydrate metabolism and glucose tolerance[186].

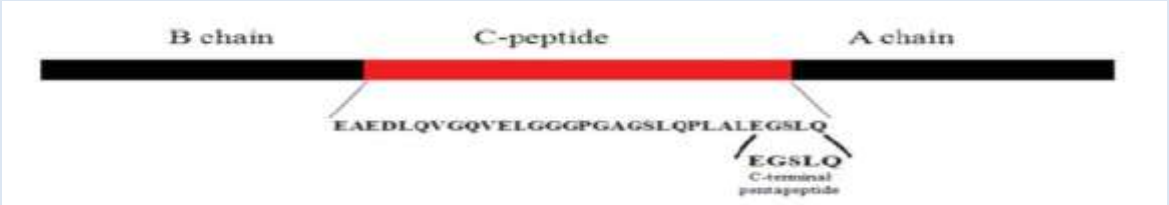
1.14 C-Peptide:

C-Peptide is a 31-amino-acid peptide that connects the portions of the proinsulin molecule that form the A and B chains of insulin. It is an essential component in insulin production. It is separated from proinsulin and secreted in equimolar quantities with insulin[191]. Much new knowledge on C-peptide physiology has emerged in the last 20 years [192]. C-peptide has been demonstrated to bind selectively to cell membranes[193], and to activate and enhance the production of endothelial nitric oxide, Na-K ATPase, and many transcripts via intracellular signaling via G-protein and Ca^{2+} dependent pathways, as well several transcription factors of importance for antioxidative[191].

1.14.1 C-Peptide Synthesis and Secretion

The precursor molecules pre-proinsulin and proinsulin are cleaved by enzymes to generate C-peptide Proinsulin is transported in vesicles from the rough endoplasmic reticulum to the Golgi apparatus in

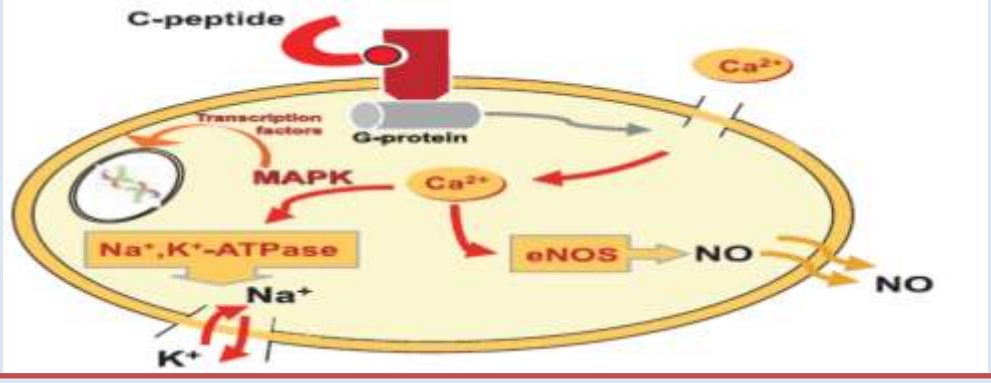
pancreatic beta cells, where the vesicles are guided into controlled secretion. Three peptidases participate in proinsulin post-translational processing to produce insulin and C-peptide during this vesicle transition[194]. Prohormone convertase type 2 cleaves proinsulin at the A-chain/C-peptide junction, whereas prohormone convertase type 1/3 cleaves it at the B-chain/C-peptide junction. Two pairs of amino acids are removed from both cleaved junctions by the carboxypeptidases H enzyme, resulting in proinsulin forms. Finally, endo-peptidase recognizes proinsulin, prompting proinsulin to release insulin and C-peptide. C-peptide assists in the proper twisting of proinsulin, allowing two disulfide bridges to form between the A- and B-chains of insulin, and thus works an essential part in insulin biosynthesis[195], as seen in fig (1-6). Only one kind of proinsulin has been identified in most animals. When blood glucose levels rise, an equimolar quantity of insulin and C-peptide is secreted into the portal circulation[194]. Because of the single-pass effect, the liver rapidly absorbs insulin, and only approximately half of it enters the systemic circulation, with a half-life of about 4 minutes. On the other hand, C-peptide is largely processed by the kidney and has a circulating half-life of about 30 minutes, which explains why it has a greater plasma concentration than insulin[194,196]. The blood C-peptide level is an indication of residual beta-cell activity that can help differentiate T1DM from T2DM patients[197].



Fig(1-6)[206]: Linear structure of human proinsulin showing the A- and B-chains of insulin separated by C-peptide. The amino acid sequence of C-peptide is shown highlighting the C-terminal pentapeptide that is particularly important in membrane interactions and signalling[198].

1.14.2 Molecular Effects of C-Peptide

Na-K ATPase is a ubiquitous protein complex that activates the counter- transport of Na-K ions through the plasma membrane with energy produced by ATP hydrolysis. Na-K ATPase activity is reduced in diabetes in a variety of cells, and this diminished activation appears to play a role in the occurrence of diabetic complications [198]. C-peptide controls Na-K ATPase by molecular mechanisms that are now becoming clear. C-peptide binds to a cell membrane receptor that is coupled to a pertussis toxin (PTX) sensitive G-protein. Activation of G-protein-coupled receptor(GPCR) stimulates both, phospholipase C (PLC) and , phosphoinositide 3-kinase(PI3K). PLC activation evokes an increase in [Ca^{2+}], resulting in the concomitant activation of both endothelial NOS(eNOS) and, protein kinase C(PKC). Together with increased de novo synthesis of diacylglycerol DAG, as well, stimulates the activation of the Na-K ATPase via PKCα translocation to the cell membrane. Increased Na-K ATPase activity and activation of different transcription factors arise from stimulation of the mitogen-activated protein kinase MAPK pathway. MAPK-induced transcription factor expression is enhanced in conjunction with high PI3K levels, and consequences such as decreased apoptosis and increased eNOS and CD36 levels are shown in the scheme (1-6) [199].



Scheme (1-6): The molecular effects of C-peptide[199].

1.14.3 C-peptide and Diabetes Mellitus

Diabetic neuropathy is a common and severe complication of long-term diabetes that, is caused by a combination of metabolic, vascular, and hormonal causes as a result of high blood sugar[200]. C-peptide has been shown to prevent, or improve neuropathy in T1D patients [195].

Reduced nitric oxide production and decreased Na-K ATPase activity are thought to have a role in the development of diabetic neuropathy. C-peptide has been used to avoid a Na-K ATPase imbalance in the brain and a reduction in nerve conduction velocity[201].

The importance of acidic residues on the N-terminus of proinsulin C-peptide is verified inside a study for the folding of insulin precursors. In vitro refolding studies with C-peptide mutant proinsulin genes that contain both an alanine substitution mutation and the loss of three highly conserved acidic residues at the N-terminus C-peptide have demonstrated severe aggregation during refolding[202]. The relevance of normal C-peptide was established by refolding studies it not only aids in the folding of human proinsulin but also controls its kinetic folding route. When diabetes is first discovered and it is unclear whether it is type 1 or type 2 diabetes, a C-peptide test can be used[203,204].

Aim of the research :

1. To distinguish the specific activity of Na-K ATPase in red blood cell ghosts in type 2 diabetic neuropathy patients compared with type 2 without neuropathy and control individuals.

2. To assess the percentage inhibition of endogenous digitalis in type 2 diabetic neuropathy patients compared with type 2 without neuropathy and control individuals.

3. To determine plasma C-peptide in type 2 diabetic neuropathy patients compared with type 2 without neuropathy and control individuals.

4. Investigation the relationship between enzymatic activity of Na-K ATPase with the level of C-peptide and plasma inhibitory of Na-K ATPase in diabetic with and without neuropathy patients compared with control groups.

5. Detection of erythrocyte Na-K ATPase alpha 1 & alpha 2 gene polymorphism, and evaluate the association of erythrocyte Na-K ATPase gene polymorphism with diabetic neuropathy.

6. To estimate the correlation of erythrocyte Na-K ATPase gene polymorphism with the activity of erythrocyte Na-K ATPase and plasma C-Peptide.

2.1 Patients and Methods:

2.1.1 Subjects and Sampling

The study design a case- control study conducted between January to July 2020 in the Biotechnology and Genetic Engineering Laboratory, Department of Chemistry, College of pharmacy, University of Babylon. In addition, blood samples were collected from patients in Department of diabetic central at Merjan Teaching Hospital in Babylon Governorate, Iraq.

2.1.2 Study individuals

The present study was included One-hundred fifty individuals. They were involving patients with type II diabetes mellitus, dividing participants into three groups; G1:diabetic neuropathy DN (N=80), G2: diabetics without neuropathy T2DM (N=40), and G3: control healthy individuals (N=30), for evaluation activity of Na-K ATPase in membranes (ghosts), Endogenous digitalis and plasma C-peptide. Patients with diabetic neuropathy were selected carefully according to their clinical manifestations and the nerve conduction study results. The patients were admitted to the Merjan Teaching Hospital's Diabetic Center. The control group, was consisted of 30 healthy persons, was not matched with the type 2 diabetes mellitus T2DM or DN diabetic neuropathy groups. For the genetic study, the study involved 125 subjects. They were divided into three groups , G1 patients of DN (N=60), G2 patients of T2DM (N= 35) and G3 of healthy controls (N=30).

2.1.3 Data collection

The following were the study's inclusion and exclusion criteria:

1. **Criteria for inclusion*:*** A professional physician examined each of the patients, The present study included diabetic neuropathy type 2 patients (based on clinical symptoms and nerve conduction study findings), as well as a control group of apparently healthy individuals who were accepted to participate.

2. Criteria for exclusion:

Any subject that has received any of the following was excluded from the further study:

- Patients with type 1 diabetes mellitus.

-Patients with Gestational DM.

-Patients with hepatitis, heart disease, or kidney illness

2.1.4 Research and Sampling Ethics

The project and sampling method were approved by the Research Ethics Committee of Babylon Health Directorate, also the project achieve the permission of research ethics in Marjan teaching hospital. Verbal consent agreement from all patients was obtained before to the collection of samples.

2.1.5 Blood samples:

Samples were collected during the period 7/1/2020 to 1/6/2020 , from the patients of diabetic central in Merjan Teaching hospital. According to the criterion area, the persons' age, sex, diabetes mellitus duration, clinical symptoms, and nerve conduction testing results were collected. The weight and height of the individuals were assessed using an electronic balance and a measuring tape, respectively.

Five milliliters of venous blood were drawn from all subjects by using a disposable syringe (5 mL) in the sitting position with a tourniquet.

Two millilitres of blood were withdrawn and shaken gently into Eppendorf tubes, which were then stored at -20 C and utilized for Extraction of DNA.

The rest 3 ml of blood was obtained and were collected into the EDTA-K_3 tube and mixed gently. Blood was centrifuged at 3000 RPM for 10 minutes. After centrifuge, the plasma was transferred to a plain tube and stored at 4˚C

until the analysis of endogenous digitalis and C-Peptide. Whereas immediately, the red blood cell was used for the measurement of Na-K ATPase activity.

2.1.6 Material and instruments

To perform out all the investigations in this study, the instruments, chemicals, biological materials, and software program sequences, in Tables (2-1), (2-2), (3-2), and(2-4) were used.

Table (2-1): Instruments And Tools Used In The Study

Instruments and Tools	Manufacturer Com. and Origin
Autoclave	Haramaya- Japan
Centrifuge	Hettich - Germany
ELISA reader and washer	Biotech – USA
Horizontal electrophoresis unite	Cleaver Scientific - UK
Hot plate with magnetic stirrer	Heidolph - Germany
TRIO Thermal Cycler	Biometra-Germany
Aerosol resistant pipette tips	Bio Basic -Canada
EDTA tubes	ASCO - Jordan
Eppendorf tubes	Bioneer - Korea
Gel loading tips	Bio Basic - Canada
Micropipette in different sizes (µl) (0.5-10), (5-50), (10-100) & (100-1000)	Huawei - Germany
Multi-channel pipette (20-200)	Huawei - Germany
PCR thin wall tubes 0.2 ml	Bioneer – Korea
Spectrophotometer (UV-1800)	GFL / Germany
Water bath	GFL / Germany

Table (2-2): Chemicals were used in the Study.

Chemical materials	Manufacturer Com. and Origin
Adenosine tri phosphate ATP	Bio Basic – Canada
Tris (hydroxyethyl) Hydrochloride (Tris-acid)	Bio Basic – Canada
Magnesium Chloride $MgCl_2$	BDH
Potassium chloride KCl	Fluka
Sodium chloride NaCl	Fluka
Ethylene diamine tetra acetic acid (EDTA-Na2)	Fluka
Agarose	Bio-Basic - Canada
Ammonium per sulphate	Bio-Basic - Canada
Boric acid	Bio Basic - Canada
Ethanol CH3CH2OH	Merck - Germany
Ethedium bromide	Promega – USA
Tris (hydroxyethyl) aminomethane (Tris-base)	Thoms baker-India
Bromophenol blue	Sigma - USA

Table (2-3): Kits and Enzyme were used in the Study

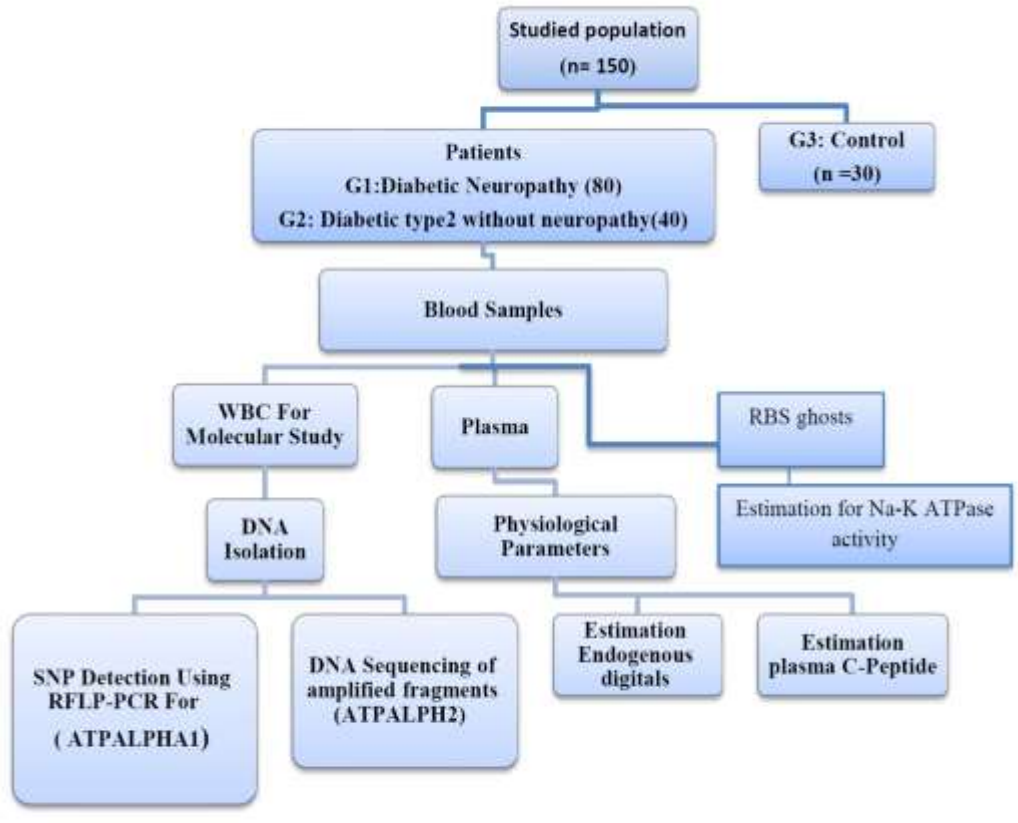
Kits and Enzymes	**Manufacturer Com. and Origin**
C-Peptide Kit	CalBiotech
DNA Extraction Kits From Blood	Favorgen- Taiwan
DNA Ladder(100bp)	Cyuto-Russia
PCR Master Mix	Cyuto-Russia
Primers (lyophilized)	Bioneer - Korea
Proteinase K	Biolabs- England
Restriction Enzyme: *MSPI* (5' C$^{\downarrow}$CGG 3')	Bio-Labs

Table (2-4): Software Programs Used To DNA Editing, And Protein Folding.

Software	Online reference	Application
Bio Edit for DNA	Access to bioinformatics	DNA Sequencing

2.2 Methods:

2.2.1 Design of the study and methodology:



Scheme (2-1): Design of the Study, the study involve 150 individuals. Their divided into three group G1 DN(N=60), G2 T2DM (N=40), and G3 healthy subjects(N=30). Five milliliters of Blood were drawn from all subjects. Two milliliters for DNA isolation and then estimation ATP-α1 by RFLP-PCR and ATP-α2 by sequencing method. The rest 3 ml for determination Na-K ATPase activity in membranes(ghosts) and plasma C-peptide and endogenous.

DN : Diabetic Neuropathy, T2DM: Type 2 Diabetic Mellitus

2.2.2 Clinical Parameters

2.2.2.1 Determination of Na-k ATPase Activity

A. Principle:

The activity of Na-k ATPase in RBCs membranes (ghosts) was determined as inorganic phosphate produced during enzymatic hydrolysis of ATP and expressed in micrograms of phosphate per gram of protein released during 30 minutes of incubation (Pi), according to a modification of the method described by Kaššák, P., et al (2006) [205].

B. Preparation of ATPase Reagent*:*

The ATPase reagent was produced by placing the following ingredients at 37 °C for 30 minutes: (100 mM Tris-HCl, 10 mM MgCl2, 15 mM KCl, 85 mM NaCl, 1 mM EDTA, 2 mM ATP, pH 7.4) to observe both non-enzymatic and enzymatic ATP hydrolysis. 1.2 g was used for Tris - HCl (100 mM). A 0.095 g of MgCl2 (10 mM) was produced. 15 mmol KCl formed using 0.11 g. 85mM NaCl 85 mM created by a weighting of 0.4 g. 1Mm Na2-EDTA was prepared by 0.036 g. 2mM ATP was prepared with a weight of 0.1 g of ATP. All components were dissolved in 100 mL of distilled water, Then the reagent's pH was adjusted to 7.4 by adding NaOH[205].

C. Preparation of Red Blood Cell Ghosts*:* Fresh human blood was collected in EDTA-K_3 tubes as an anticoagulant, and centrifuged at 3000 rpm for 10 min yielded erythrocytes. Excessive plasma and platelets were discarded, and erythrocytes were washed three times in ice-cold PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4). To get a suspension of separated erythrocyte membranes (ghosts), one volume of erythrocytes was hemolyzed in five volumes of 20 mM Tris-EDTA-HCl buffer (pH 7.4) and centrifuged at 4000 rpm for 10 minutes. Following that, a double washing with 20 mM, 10 mM, and 5 mM Tris-EDTA-HCl buffer were performed by Hanahan and Ekholm (1974).

Adding EDTA as a calcium ion chelator caused the inactivation of Ca^{2+}-ATPase during the preparation and incubation of membranes[206].

1. Determination of inorganic phosphorus

The inorganic phosphate was determined spectrophotometrically according to the method described by Baykov et al. (1988)[207].

2. Principle

As demonstrated in equations 1 and 2, inorganic phosphate interacted with molybdic acid to create a phosphomolybdic complex. Its subsequent reduction in alkaline media results in a blue molybdenum colour, the strength of which is related to the quantity of phosphorus contained in the samples.

$$PO_4^{3-} + H^+ + (NH_4)_6\,Mo_7O_{24} \xrightarrow{Pi} \text{Phosphomolybdic Complex} \quad \text{------ (1)}$$

$$\text{Phosphomolybdic Complex} \xrightarrow[\text{Reduction}]{pH > 10} \text{Molybdenum Blue} \quad \text{------ (2)}$$

3. Working Reagent Preparation

Equal quantities of R1 and R2 were mixed to make the working reagent, which was stable for 8 hours.

4. Protocol

Three tubes were constructed, and the method was done as shown in Table (2-5)

Table (2-5): Technique Used for Determination of Inorganic Phosphorus according to linear kit

Regents	Blank	sample	Standard
Working reagent	1ml	1ml	1 ml
CAL (standard)	------	------	50 µl
Sample	------	50 µl	------
All tubes were mixed and let to stand for 10 min. at room temperature			
R2 developer	0.5 ml	0.5 ml	0.5 ml

5. **Calculation*:*** The results were calculated as follows:

$$\text{Concentration of Inorganic Phosphorus (mg/L)} = \frac{\text{A. Sample}}{\text{A. Standard}} * \text{Concentration of Standard}$$

6. **Concentration of Standard** = 100 mg/L

D. Total Protein Estimation

Total protein was determined using the Lowry et al. (1951)[208] standard technique, which included the proper procedures:

1. Principle:

Cupric ions were complexes with functional groups in the protein's peptide bonds in the Biuret technique. To create a Cu^{2+}-protein complex and produce a violet-colored chelate product, two peptide bonds or longer were required, as determined by absorption spectroscopy at 540 nm.

2. Formulation of Working Reagent:

The working reagent was produced by adding 3 ml of R2 to a container of R1, and this reagent remained stable for 6 months.

3. Procedure:

Three tubes were constructed, and the method was done as shown in Table (2-6).

Table (2-6): Procedure Used for Determination of Total Protein

Reagents	Blank	sample	Standard
Working reagent	1ml	1ml	1 ml
Reagent 3 (standard)	------	------	20 µl
Sample	------	20 µl	------
All tubes were mixed and let to stand for 2 min. at room temperature, then the absorbance was reading at 540 nm against the blank.			

4. Calculation: The results were calculated as follows:

$$\text{Concentration of Total Protein(g/L)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} * \text{Concentration of Standard}$$

5. Concentration of Standard = 50 g/L

E. Measurements of Na-K ATPase Activity

Ten microliters of red cell ghosts was added to 500 µL of ATPase reagent which prepared in section B, and incubated for 30 min. After that, the samples were centrifuged at 4000 RPM for 10 min. Then pulled 50 µL from the supernatant to determine inorganic phosphates. The inorganic phosphates were

determined spectrophotometrically according to the method illustrated in section D. For the purpose of standardization, protein concentration in red cell ghosts was estimated according to the standard biuret method illustrated in section E[209]. Enzyme activity was expressed as the inorganic phosphorus to red cell ghost protein concentration as follows:

$$\text{Enzyme Activity}(\mu g \backslash g) = \frac{\text{Concentration of Inorganic Phosphorus}}{\text{Concentration of Total Protein}}$$

2.2.2.2 Determination of Endogenous Digitals

1. Red cell membrane ghosts was prepared as mentioned above in section 2.2.2.1 C.
2. Determination of Na-K ATPase in normal red cell ghosts was accomplished.
3. In another tube, mix up 25µl plasma (patient) and 25µl ghosts (control), and then incubate in a water bath for 10 minutes.
4. After the incubation period is over, Na-K ATPase activity was determined as described above[209].

Calculation of results*:* Inhibitory effect of endogenous digitalis was determined as a percentage of inhibition according to the following equation:

$$\% = \frac{\text{activity of normal -activity of patient}}{\text{activity of normal}} * 100$$

2.2.2.3 Measurement of Plasma C-peptide

The steps for detecting plasma C-peptide are as follows:

A. Principle: The C-Peptide ELISA technique was a solid phase direct sandwich ELISA. The standards, samples, and controls were placed in the antic-Peptide monoclonal anyone-coated wells. Anti-C-Peptide binds to C-Peptide in

the serum of the standards, controls, and patients. Wash buffer is used to remove unbound proteins and horseradish peroxidase HRP conjugate. The enzyme activity is proportional to the concentration of C-Peptide in the samples after the substrate is added. A standard curve was created by connecting color intensity to C-Peptide concentration.

B. Reagent Preparation:

1. Standards: Rehydrate the lyophilized standards with 2.0 mL of distilled water. Allow them to sit undisturbed until dissolved, then gently invert to thoroughly combine them.

2. Wash buffer 1X was prepared by adding the contents of the bottle (25 ml, 20X) to 475 ml distilled or deionized water to wash the buffer. Room temperature (18-26 °C) is recommended for storage.

C. Assay Methodology

Bring all reagents, serum references, and controls to room temperature (18-26 °C) prior starting the experiment.

1. The microplate wells were arranged in duplicate for each reference, control, and patient sample to be tested. Any unused microwell strips were placed in the aluminum bag, sealed, and stored at 2-8°C.

2. A 50 µl of the relevant standard, control, or specimen pipetted into the allocated well (urine samples should be diluted 1:20 in sample diluent).

3. Each well was pipetted with 100 µl Enzyme Conjugate.

4. The plate was mixed gently for 15-20 seconds and incubated at room temperature for 60 minutes.

5. Wells were washed three times with 300 µl of 1X wash buffer each time. On absorbent paper towels, I was bloated.

6. All wells were filled with 100 µl of TMB substrate.
7. The plate was incubated at room temperature for 15 minutes.
8. A 50 µl of stop solution was poured into each well and stir gently for 15-20 seconds.
9. Within 15 minutes of adding the sample, the absorbance of well was measured using an ELISA Reader at 450 nm.

D. Findings calculation: The standard curve is built as follows:

i. On each standard vial, we checked the C-Peptide standard value. This value may differ from one lot to the next. We make sure you know how much each kit is worth.

ii. On a piece of linear graph paper, Curve was plotted using OD for each C-Peptide standard point (vertical axis) against the C-Peptide standard concentrations (horizontal axis) to create the standard curve. As well, drew the best is possible between the spots.

iii. From the curve, we read the concentration (ng/ml) for the controls and each unknown sample. For each control or unknown sample, make a note of the value.

2.3 Methodology of the genetic part

2.3.1 Markers selection:

A. The selection of genotyping technique according to Hashim, H. O., et al. 2019. [210].

B. The PCR-RFLP primers were designed according to the protocol of Hashim, H. O., et al 2015 [211] briefly as following:

The primers were designed by the NCBI-primer BLAST online software(http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?LINK_LOC

=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 pairs were selected according to the demand criteria such as : product length , the similarity of melting temperature , primers length , specificity , etc. Then the mutations was interred according to the design demands. The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www.basic.northwestern.edu/biotools/oligocalc.html) , the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin. Each primers pair was checked for dimer formation by the aid of "Multiple Primer Analyzer" online software from Thermo Fisher Scientific Inc.© , the sensitivity of the software was adjusted to the value 2 , the primer pair would be rejected if it made any dimers in this degree of sensitivity .

By reviewing the resent literatures ,we selected the following genes markers .

AGTCTTCTACTTTGCCCTTGACACAGGCAGCTTGTCCCCAGTCCTGAGACCT/TAAGCATTTTCTTAC

GAATTCTACGGTAATGAAAGGTGCTATAAATGTT/TAATCTCTCACTTGGTATTTTCAGATAAGTAA

CCAGAAGTAGGTAGTTGGGAATAGCATTGGCTTTGAGACTTTTCAGTAGGCCAGGG

G= 52 + 138

A = 52 + 49 + 88

Enzyme : MseI neb

Table (2-7): The forward and reverse primers for SNP of ATP1A1gene

Primers pair 1 of rs10924081

	Sequence (5'->3')	Template strand	Length	Star	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGTCTTCTACTTTGCCCTTGACA	Plus	23	38	60	59.61	43.48	3.00	3.00
Reverse primer	CCCTGGCCTACTGAAAAGTC	Minus	20	226	207	57.88	55.00	4.00	1.00
Product length	189								

Products on intended target

>NC_000001.11 Homo sapiens chromosome 1, GRCh38.p12 Primary Assembly

product length = 189

Features associated with this product:

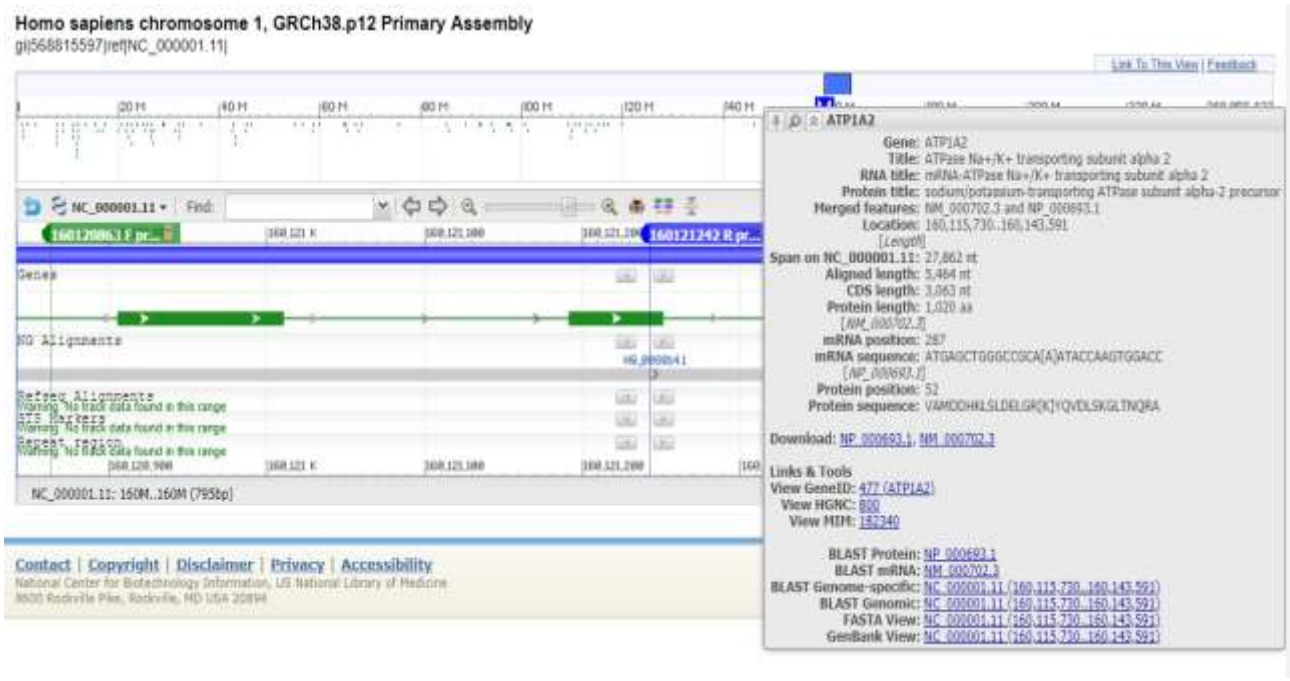
sodium/potassium-transporting ATPase subunit alpha-1 isof...

sodium/potassium-transporting ATPase subunit alpha-1 isof...

Forward primer 1 AGTCTTCTACTTTGCCCTTGACA 23

C. The selection of genotyping technique according to[210].

The PCR for Sanger sequencing was designed according to the protocol of [211].



Table(2-8): The forward and reverse primers for SNPs of ATP1A2gene

Primers pair 5 of rs373796693

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCCCTCTCTTCCCTGACTCT	Plus	20	160120863	160120882	59.66	60.00	3.00	1.00
Reverse primer	GTCCACTTGGTATTTGCGGC	Minus	20	160121242	160121223	59.83	55.00	4.00	3.00
Product length	380								

Products on intended target

>NC_000001.11 Homo sapiens chromosome 1, GRCh38.p12 Primary Assembly

product length = 380

Features associated with this product:

sodium/potassium-transporting ATPase subunit alpha-2 prec...

Forward primer 1 CCCCTCTCTTCCCTGACTCT 20

Template 160120863 160120882

Table (2-9): lists of Variant ID

"Variant ID"	"Chr: bp"	Alleles	"Global MA	Class	"Clin. Sig."	Consequence
rs114284892	1:160120873	C/A/G/T	"< 0.001"	SNP		"intron variant"
rs2854246	1:160120884	T/C	"< 0.001"	SNP		"intron variant"
rs373796693	1:160120895-160120898	TCCT/-/CCTT	0.311	"sequence alteration"	benign	"splice region variant"
COSM898153	1:160120911	COSMIC_MUTATIOI	-	"somatic SNV"		"coding sequence variant"
rs55858252	1:160120918	T/A	"< 0.001"	SNP	"likely benign"	"missense variant"
rs371257019	1:160120937	C/T	"< 0.001"	SNP	"uncertain significance"	"missense variant"
rs201688946	1:160121005	G/A/T	"< 0.001"	SNP		"missense variant"
rs146329414	1:160121093	G/A	0.007	SNP		"non coding transcript exon variant"
rs575480986	"1: between 160121096 & 160121097"	-/C	"< 0.001"	insertion		"non coding transcript exon variant"
rs1272183105	1:160121130	C/T	-	SNP		"non coding transcript exon variant"

2.3.2 Molecular Analysis:

2.3.2.1 DNA Extraction Kit Quick Protocol (Geneaid)

The following methods were followed to extract genomic DNA from white blood cells (WBCs) of both patients and controls using a DNA extraction kit:

Step 1. RBC Lysis

1. A 200 microliter blood sample was placed in a clean 1.5-milliliter micro centrifuge tube. If the sample volume was less than 200 µl, a suitable amount of PBS was added to make up the difference.

2. Proteinase K (10 mg/ml) was added to 20 µl of blood and mixed quickly prior being incubated at 60 ºC for 15 minutes.

3. A 200 µl GSB Buffer solution was added to the blood lysate and forcefully stirred.

4. The mixture was incubated in a water bath at 60°C for 5 min., with the tube inverted every 2 minutes.

5. The needed volume of elution buffer (200 µl/sample) was pre-heated at 60^{o}C. (for Step 4 DNA Elution).

Step 2. DNA Binding

1. Approximately 200 µl of absolute ethanol was added to the sample lysate and stirred violently for 10 seconds, and if precipitately, it was broken up by pipetting.

2. A 2 ml collection tube was used to hold the GS Column.

3. The entire mixture was poured onto the GS column, including any precipitate.

4. The samples were centrifuged for 1 minute at 12,000 RPM

5. The flow-through 2 ml collection tube was discarded, and the GS column was put in a fresh 2 ml collection tube.

Step 3. Washing

1. A total of 400µ l of W1 Buffer was loaded into the GS column and centrifuged at 12,000 RPM for 30 seconds.

2. Discard the flow-through and re-insert the GS column into the 2 ml collection tube.

3. Approximately 600 µl of wash buffer (ethanol) was added to the GS column, which was centrifuged for 30 seconds at 12,000 RPM

4. After discarding the flow-through, the GS column was placed back in the 2 ml collection tube and centrifuged at 12,000 RPM for 3 minutes to dry the column matrix.

Step 4. DNA Elution*:* If less sample volume was used, increase the elution volume (30-50 l) to raise DNA concentration, then repeat the DNA elution stage to enhance DNA recovery and the total elution volume to about 200 µl if higher DNA yield was necessary.

1. The dried GS column was transferred to a clean 1.5 ml microcentrifuge tube, and 100 µl of pre-heated elution buffer or TE was added to the middle of the column matrix.

2. To elute the pure DNA, the micro centrifuge tube containing the GS column was centrifuged at 12,000 RPM for 30 seconds and then kept at -24°C until needed.

2.3.2.2 Polymerase Chain Reaction

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

2.3.2.2.1 Restriction Enzyme Selection

The selection of the suitable restriction enzyme was performed by the aid of WatCut online software (http://watcut.uwaterloo.ca/template), The restriction

enzyme we selected 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 .

2.3.2.2.2 Primers dilution

Bioneer, a South Korean business, provided the primers. Bioneer' primers are frequently sent in lyophilized form. A lyophilized primer's units are expressed as a mass in Pico moles. Reconstitute the primer in sterile, nuclease-free H_2O to make a stock of primers. The company provides the amount of sterile, nuclease-free H_2O that should be added to each primer to make master stock (100mol/ul), which will then be used to make working stock. The following is an example:

1. Prior the tube was spun down opening the cap.

2. To produce a 100 pmol/µl concentration, the appropriate amount of water was added according to the oligos manufacturer's instructions (Master Stock).The tubes was vortex properly for re-suspend the primers evenly.

3. A ten-microliter volume of each primer from the master stock was transferred to a 1.5 µl Eppendorf tube containing 180 ml of sterile, nuclease-free H_2O. (Working Stock).

4. The master stock is kept at -20 ºC.

5. The working stock is kept at -20 ºC.

2.3.2.2.3 PCR Experiments:

This is the first research in the Iraqi population to look at possibly functional polymorphisms in the ATP1A and ATP1A2 genes concerning neuropathy susceptibility. A standard thermal cycler (Biometra - Germany) was used to do PCR amplification as follows: In PCR master mix tubes, template DNA (approximately 50 ng) was added. In PCR master mix tubes, forward and

reverse primers (about 10 Pico moles) were added. A Twenty microliters of DW were added to PCR Premix tubes like shown in table(2-10).

Table (2-10): Protocols for PCR reaction mixture volume and concentration into PCR tubes.

Component	**Volume**(µl)	**Concentration**
PCR master mix	8	2.5X
Forward primer	1	10 pmol/ µl
Reverse primer	1	10 pmol/ µl
Template DNA	2	50 ng
$MgCl_2$	0.5	25mM
Final volume (dH_2O)	20	

2.3.2.2.4 Optimization of PCR

Optimization of PCR reaction for each marker with the thermo-cycling condition listed in table (2-9). Plate was placed in a thermal cycler (Biometra-Germany) which preheated to 95°C and set up to the desired cyclic conditions. The target regions of the investigated genes, including *ATP1A1* and *ATP1A2* were amplified using particular primers following the conditions mentioned in the Table (2-11).

Table (2-11): Optimized PCR condition of Na\K ATPase α1 & α2 .

Stage	Steps	Temperature C*	Time	No. of cycle
1	Initial denaturation	94	5 min.	1
2	DNA denaturation	94	30 sec.	35
	Primer annealing	Gradient 55-67 ºC	30 sec.	
	Extension	72	5 min	
3	Final extension	72	5 min.	1

*Different annealing temperature were record.

After optimization , the best thermo-cycling conditions for all SNPs were as listed in table (2-12) , these conditions produce the most specific and sufficient PCR product.

Table (2-12): Optimized thermo-cycling condition for rs10924081 and rs373796693 PCR product

Stage	Steps	Temperature Cº	Time	No. of cycle
1	Initial denaturation	94	5 min.	1
2	DNA denaturation	94	30 sec.	35
	Primer annealing	60	30 sec.	
	Extension	72	30 min	
3	Final extension	72	5 min.	1

The amplification products were separated on a 2% agarose gel stained with ethidium bromide by electrophoresis. The rs10924081 PCR result was 189 bp long, while the rs373796693 PCR product was 380 bp long.

After that, MSPI enzyme was used to digest the amplified segments of the ATPase encoding gene (ATP1A1) to look for restriction fragment length polymorphisms (PCR-RFLP). The existence of SNPs in the other target areas (ATP1A2) was investigated using the Sanger sequencing method.

2.3.2.3 Restriction Fragment Length Polymorphism (RFLP)

The PCR products of the target SNP rs10924081 were cut using *MSPI* restriction enzyme the RFLP-PCR was accomplished to Promega company protocol. The reaction mixture of *MSPI* composed of the following components as shown in table (2-13).

Table (2-13): The reaction mixture of MSPI .

Materials	Volume (ul)
PCR product	5
Enzyme	0.2
Buffer B	1.5
DH_2O	8
Incubation at 37 °C for overnight.	

The digested product of amplified ATPase fragments was migrated into 2% agarose gel at 75V for 1 h. The DNA bands were visualized after staining with ethidium bromide under UV light. A100 bp ladder was used as a size marker for estimation the size of DNA fragments.

2.3.3 Agarose Gel Electrophoresis Technique

The agarose gel electrophoresis was perfomed according to the method of Robinson and Lafleche (2000)[212]. This technique was used to detect genomic DNA extracts, PCR products and digested products of restriction enzyme of RFLP.

2.3.3.1 Solution And Buffer Preparation

a. Ethidium bromide staining solution: a stock of 10000X (10mg/ml) solution was supplied from intron biotechnology (Korea) .

b. Buffer for Loading: The buffer was made with 0.25% Bromophenol blue and 40% sucrose and kept at 4° C [213].

c. TBE Buffer (5X): to weight (5xTBE), 27g Tris-base, 14g boric acid and 1.8g EDTA were mixed, then dissolved in 500 ml D.W and put on hot plate stirrer.

To prepare 1000 ml from 0.5x, A 100 mililiter of TBE (5X) standard solution was combined through 900ml D.W. With concentrated HCl or 0.5 M tris base solution, the pH was adjusted to 8.

d. Gel electrophoresis protocol

1. Device setup: The casting gates were placed on the gel tray's ends and firmly pressed on the casting tray. This was accomplished by engaging the gate's "claws" in the recess of the side wall of the tray. The sample wells were placed near the cathode by inserting the comb into the slots of the gel tray (1.0 mm above the base of the gel casting tray).

2. Gel dissolving: A 1.6 g of agarose was dissolved in 80 ml of 0.5 X TBE solution by melting in a microwave oven for about 3 minutes, resulting in a 2% agarose gel for separation of PCR product. The PCR product and genomic DNA were separated using a 2% and 1% agarose gel, individually.

3. Gel casting: After the agarose gel was completely dissolved, then it was allowed to cool at around 60°C prior adding 8 µl of the ethidium bromide stock solution. Then slowly pouring the agarose into the gel-casting tray and removing any air bubbles. The comb was placed around 1.5 cm away from one of the gel's edges. Allow the agarose to harden at room temperature for at least 30 min. The claws were then carefully removed from the gel tray and the comb was disengaged. After that, The gel was positioned in the gel tank such that the wells were on end with the cathode. The buffer tank was filled with 0.5 X TBE buffer (depending on the purpose) until it was roughly 5 mm above the top of the gel.

4. Loading the samples: Each 5 µl genomic DNA sample was quickly mixed with 2 µl 5X loading buffer before being put into the wells.

5. Electrophoresis conditions*:* the electric field was activated at 5 V/cm (75 V) for 60-120 minutes, or until the bromophenol blue dye reached the gel's

end edge, after loading the samples, The gel was exposed to UV- light and imaged by digital camera. (Cleaver Scientific - UK).

2.3.4 DNA Sequencing of PCR amplicons

DNA sequencing method was performed for genetic genotyping of *ATP1A2* gene. The PCR products of *ATP1A2*gene were sent to macrogen company in korea for performed DNA sequencing[214,215].

2.3.4.1 Principle, and Protocol:

A DNA primer complementary to the template DNA (the DNA to be sequenced) is used as a starting point for DNA synthesis in Sanger sequencing. The polymerase extends the primer by adding the corresponding dNTP to the template DNA strand in the presence of the four deoxynucleotide triphosphates (dNTPs: A, G, C, and T). Four dideoxynucleotide triphosphates (ddNTPs: ddATP, ddGTP, ddCTP, and ddTTP) tagged with a unique fluorescent dye are employed to terminate the synthesis reaction to determine which nucleotide is integrated into the chain of nucleotides.

ddNTPs have an oxygen atom removed from the ribonucleotide, thus they cannot establish a connection with the next nucleotide like dNTPs. Following synthesis, the reaction products were putted into four lanes of a single gel and subjected to gel electrophoresis, depending on the different chain-terminating nucleotides.. According to their sizes, the sequence of the DNA is thus determined[214,215].

2.3.4.2 DNA sequence data analysis

The resolved PCR amplicons were commercially sequenced according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from Applied Biosystem (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 highly related DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified

2.3.4.3 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). Each detected variant within the *ATP1A2* gene was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapgene.com). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/). Each particular SNP was positioned according to its place in the reference genome.

2.3.5 Checking the details of the targeted SNP

Subsequently, the determination of the presence of the detected SNP was performed by viewing its corresponding dbSNP position. The observed SNPs were submitted to the dbSNP database to check their details (https://www.ncbi.nlm.nih.gov/snp/). Each particular SNP position was checked

in its corresponding reference genome to assess whether it was previously deposited in dbSNP server.

2.4 Statistical analysis

All statistical calculation were performed by the using of SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). All the results were expressed as mean ± SEM. A $p < 0.05$ was considered statistically significant. Analysis of variance (One Way Anova) Test was employed to evaluate the presence of significant differences. Regression analysis to evaluate the presence of correlations. Chi-square test to assess the categorically association variables and genetic association, according to (sole et al.,2006)[216].

3. Results :

The individuals of the study were divided into three groups: G1 Diabetic Neuropathy DN (N: 80, 53.3 %), G2 Type2 Diabetic Mellitus without Neuropathy T2DM (N: 40, 26.7 %), and G3 healthy control individuals (N: 30, 20.0 %).

3.1 Study the association of diabetes duration, age and BMI with diabetic neuropathy.

According to the Chi-square test, there is a significant association of diabetic neuropathy with disease duration of diabetes and age ($p<0.05$), although no such significance exists between BMI and neuropathy ($p=0.810$), as shown in Table (3-1).

Table (3-1): Chi-Square Tests of Diabetes duration, BMI, and Age between patients with neuropathy and control group.

Parameter	Counts	P-value
Duration of diabetes (Years)	<=10 (N: 16, 20%)	<0.05
	>11 (N: 64, 80%)	
BMI(Kg/m^2)	<=25 (N: 30, 37.5%)	0.810
	>26 (N: 50, 62.5%)	
Age (Years)	<= 40 (N: 1, 1.25%)	<0.05
	40+ (N: 79, 98.75%)	

3.2 Sample parameters

By one way ANOVA tests, The results of Age, Body Mass Index (BMI), Glycated Haemoglobin HbA1C, Erythrocyte Na-K ATPase activity, Plasma Endogenous digitalis (inhibitory Na-K ATPase activity) and plasma C-peptide were shown in Tables 3-2.

Table (3-2): The results of Age of subject(years), BMI (Kg/m2), HbA1C (%), Erythrocyte Na-K ATPase activity (µg Pi/g protein/30 min.), Plasma Endogenous digitalis (inhibitory Na-K ATPase activity)%, C-peptide (ng/mL) among G1: diabetic neuropathy (DN), G2: diabetes mellitus without neuropathy (T2DM), and Healthy subjects.

Parameters	G1: DN (Mean ± SE)	G2: T2DM (Mean ± SE)	G3: Healthy (Mean ± SE)	P value
Age of subject(years)	59.39 ± 0.99	53.93 ± 1.52	30.80 ± 0.98	<0.05
BMI (Kg/m^2)	27.91 ± 0.68	28.90 ± 0.80	27.20 ± 0.95	0.444
HbA1C (%)	9.42 ± 0.25	10.40 ± 0.35	5.23 ± 0.05	<0.05
Erythrocyte Na-K ATPase activity (µg Pi/g protein/30 min.)	381.94 ± 18.00	498.28 ± 22.98	837.20 ± 61.43	<0.05
	DN	T2DM	-----	<0.05
		----	Healthy	<0.05
Plasma Endogenous digitalis (inhibitory Na-K ATPase activity)%	17.88±2.16	8.79± 0.90	8.79± 0.90	<0.05
	DN	T2DM	----	<0.05
		----	Healthy	<0.05
C-peptide (ng/mL)	1.17 ± 0.10	1.71 ± 0.19	1.96 ± 0.28	<0.05
	DN	T2DM	----	<0.05
		----	Healthy	<0.05

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

3.2.1 Age:

The results of an age of diabetic neuropathy patients (DN) were higher significantly (59.39 ± 0.99) when compared with type 2 diabetes mellitus without Neuropathy (T2DM), and control individuals (53.93 ± 1.52, 30.80 ± 0.98 respectively) with $p<0.05$, as shown in Table (3-2).

3.2.2 Body Mass Index (BMI) (kg/m^2):

The results of BMI of diabetic neuropathy patients (DN) were no significant differences (27.91 ± 0.68) when compared with diabetes mellitus without neuropathy (T2DM), and control individuals (28.90 ± 0.80, 27.20 ± 0.95 respectively) with p=0.444, as shown in Table (3-2).

3.2.3 Glycated Haemoglobin (HbA1C)%:

The results of HbA1C of diabetic neuropathy patients (DN) were lower significant (9.42 ± 0.25) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (10.40 ± 0.35, 5.23 ± 0.05 respectively) with p<0.05, as shown in Table (3-2).

3.2.4 Erythrocyte Na-K ATPase activity (µg Pi/g protein/30 min.):

The results of Erythrocyte Na-K ATPase activity of diabetic neuropathy patients (DN) were lower significant (381.94 ± 18.00) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (498.28 ± 22.98, 837.20 ± 61.43 respectively) with p<0.05, as shown in Table (3-2).

3.2.5 Endogenous digitals (plasma inhibitory of Na-K ATPase activity)

The results of Endogenous digitals (plasma inhibitory of Na-K ATPase activity) % of diabetic neuropathy patients (DN) were higher significant (17.88 ± 2.16) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (8.79 ± 0.90, 5.35 ±1.34 respectively) with p<0.05, as shown in Table (3-2).

3.2.6 plasma C-peptide:

The results of plasma C-peptide) of diabetic neuropathy patients (DN) were lower significant (1.17± 0.10) as compared with diabetes mellitus without neuropathy (T2DM), and control individuals (1.71 ± 0.19, 1.96±0.28) with $p<0.05$, as shown in Table (3-2).

3.3 Study the correlation of Na-K ATPase activity with other parameters in our study:

For diabetic patients with and without neuropathy and healthy subjects, There was a significant correlation among Erythrocyte Na-K ATPase activity with Endogenous digitalis, HbA1C, and age ($p<0.05$). While, there was no significant correlation between erythrocyte Na-K ATPase activity with C-Peptide, BMI, and duration of diabetes (p-value=0.694, 0.910, as well 0.134 in turn), as shown in Table 3-3.

Table (3-3): Correlation of Na-K ATPase(µg Pi\g protein) with Endogenous digitalis(%), C-peptide(ng/ml), Duration of diabetes (DM/years), HbA1C(%), BMI(Kg/m^2)and Age (years) of subject for and control groups.

Erythrocyte Na-K ATPase activity (µg Pi\g protein/30 min.)	Pearson correlation (r)	p-value
Endogenous digitalis (%)	-.167*	0.042
C-peptide (ng/ml)	.032	0.694
HbA1C (%)	-.380**	<0.01
BMI (Kg/m^2)	-.009	0.910
Duration (DM/Year)	-.138	0.134
Age (Year)	-.495**	<0.01

*. Correlation is significant at the 0.05 level.

**. Correlation is higher significant at the 0.01 level

3.3.1 Correlation of Erythrocyte Na-K ATPase activity with Endogenous digitalis (Na-K ATPase inhibitory present).

In our study, the correlation between erythrocyte Na-K ATPase activity and Na-K ATPase inhibitory (endogenous digitalis) was negatively significant with r=-0.167, and p= 0.042, for patients of diabetic with and without neuropathy and control subjects as shown in Table (3-3).

3.3.2 Correlation of Erythrocyte Na-K ATPase activity with plasma C-Peptide:

As shown in Table (3-3), the correlation between Erythrocyte Na-K ATPase activity and plasma C-Peptide was non significantly positive (r=.032, p=0.694).

3.3.3 Correlation of Erythrocyte Na-K ATPase activity with HbA1C and Age:

As shown in Table (3-3), there was a negatively higher significant correlation between Na-K ATPase activity and HbA1C with r=-0.380, p<0.01. In addition, there was a negatively higher significant correlation between Na-K ATPase activity and age of individuals with r=-0.495, p<0.01.

3.3.4 Correlation of Erythrocyte Na-K ATPase activity with BMI and Duration of DM

In our study groups, there was negatively no significant correlation between erythrocyte Na-K ATPase activity and BMI (r=-0.009, p=0.910). Also there was negatively no significant correlation between Na-K ATPase activity and duration of diabetes mellitus (r=-0.138, p=0.134) as shown in Table (3-3).

3.4 Genetic Association:

3.4.1 Association of rs10924081 with diabetic neuropathy

3.4.1.1 rs10924081 PCR-RFLP genotyping technique optimization:

The optimal PCR product of the proposed primers pair, which would be utilized in ATP1A1 genotyping, was shown in Figure 3-1.

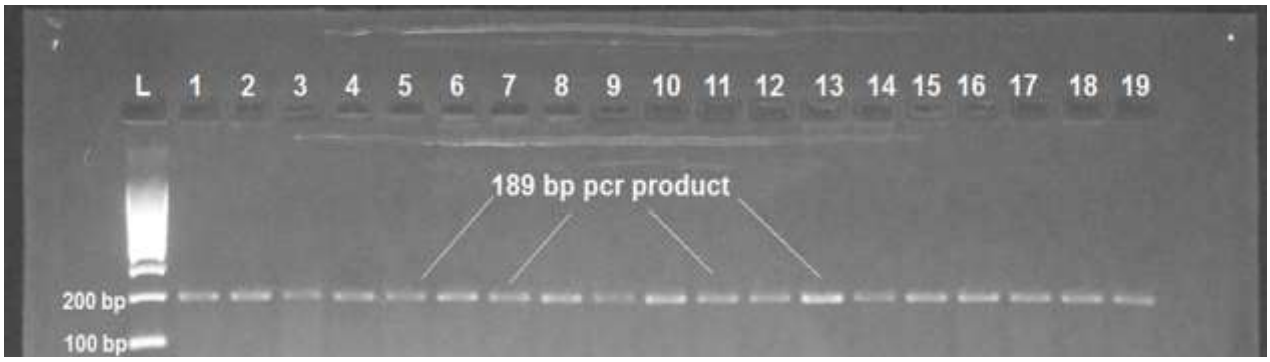


Figure (3-1) : Optimized PCR product (189 bp) of the designed primers pair for *rs10924081* genotyping . the electrophoresis carried on agarose gel (2%) , lane L= 100bp step ladder , other lanes= 189 pbPCR product.

In our result, there was identified a rs10924081 SNP at chromosome 1 in intro 9 (corresponding to nucleotide 189 of the PCR result) using *MSPI* digestion, which yielded 189 bp.

MSPI restriction enzyme was used to digest the PCR result. Only if the G allele of rs10924081 is present would this enzyme cleave the product, in this case, it will split the 189 bp product into two polynucleotide chains with a molecular weight of 52bp and 137 bp, besides if the allele A of rs10924081 presents the PCR product will split the 189 bp product into three polynucleotide chains with a molecular weight of 52bp, 49bp, and 88bp. the figure (3-2) shows the electrophoresis bands pattern of *MSPI* digested PCR-product.

G= 52 + 137

A = 52 + 49 + 88

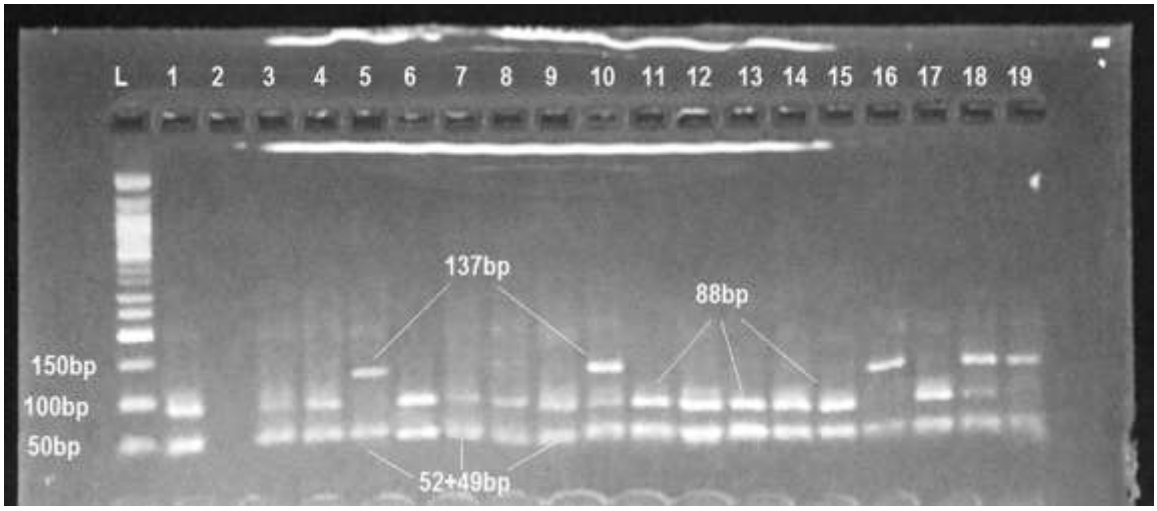


Fig.(3-2): PCR-RFLP genotyping of rs10924081 ATP1A1 gene polymorphisim , lane L 50bp step DNA ladder ; lanes 5,16,and 19 AA genotype ; lanes 10 and 18 AG genotype ; lane 2 empty lane ; other lanes GG genotype .

3.4.1.2 Allelic association of rs10924081 with diabetic neuropathy:

As shown in Table 3-4, Alleles frequency and allelic association of rs10924081 for type 2 diabetic neuropathy vs. type 2 diabetes mellitus without neuropathy. The results showed no significant allelic association between diabetic neuropathy (DN) and diabetes mellitus without neuropathy (T2DM).

Table (3-4): Alleles frequency and allelic association of rs10924081 for diabetic without neuropathy (T2DM) vs. diabetic neuropathy (DN).

	T2DM		DN		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion		
A	63	0.9	113	0.94	1.794 (0.602-5.346)	0.28895
G	7	0.1	7	0.06	0.558 (0.187-1.662)	

As shown in Table 3-5, Alleles frequency and allelic association of rs10924081 for type 2 diabetic without neuropathy vs. control subjects. The results showed no significant allelic association between type 2 diabetic without neuropathy (DM) and control individuals.

Table (3-5): Alleles frequency and allelic association of rs10924081for diabetic without neuropathy (T2DM) vs. control individuals.

	Control		T2DM		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion		
A	55	0.92	63	0.9	0.818 (0.246-2.725)	0.74
G	5	0.08	7	0.1	1.222 (0.367-4.071)	

Alleles frequency and allelic association of rs10924081 for diabetic neuropathy vs. control subjects as shown in Table 3-6. The results showed no significant allelic association between diabetic neuropathy(DN) and control individuals.

Table (3-6): Alleles frequency and allelic association of rs10924081 for diabetic neuropathy (DN) vs. control individuals.

	Control		DN		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion		
A	55	0.92	113	0.94	1.468 (0.446-4.834)	0.538
G	5	0.08	7	0.06	0.681 (0.207-2.244)	

3.4.1.3 Genotypic association of rs10924081 with diabetic neuropathy:

The data was then assessed for an association between each genotype and diabetic neuropathy using several inheritance models, there was no significant genotypic association between diabetic neuropathy (DN) and type 2 diabetes mellitus without neuropathy (T2DM), as shown in Table (3-7). Also, no significant genotypic association between Type 2 diabetes mellitus without neuropathy (T2DM) and control, as shown in the Table (3-8). The same information was obtained when the samples were divided into diabetic neuropathy vs. control individuals (Table 3-9).

Table (3-7): The association of rs10924081 genotypes with diabetic neuropathy (DN) vs. diabetes mellitus without neuropathy (T2DM) under different models of inheritance.

Model	Genotype	T2DM	DN	OR (95% CI)	P-value
Codominant	A/A	29 (82.9%)	54 (90%)	1.00	0.35
	A/G	5 (14.3%)	5 (8.3%)	0.54 (0.14-2.01)	
	G/G	1 (2.9%)	1 (1.7%)	0.54 (0.03-8.91)	
Dominant	A/A	29 (82.9%)	54 (90%)	1.00	0.31
	A/G-G/G	6 (17.1%)	6 (10%)	0.54 (0.16-1.82)	
Recessive	A/A-A/G	34 (97.1%)	59 (98.3%)	1.00	1.000*
	G/G	1 (2.9%)	1 (1.7%)	0.58 (0.03-9.51)	
Overdominant	A/A-G/G	30 (85.7%)	55 (91.7%)	1.00	0.37
	A/G	5 (14.3%)	5 (8.3%)	0.55 (0.15-2.04)	

Table (3-8): The association of rs10924081 genotypes with diabetes mellitus without neuropathy (T2DM) vs. control individuals under different models of inheritance.

Model	Genotype	Control	T2DM	OR (95% CI)	P-value
Codominant	A/A	26 (86.7%)	29 (82.9%)	1.00	0.9*
	A/G	3 (10%)	5 (14.3%)	1.49 (0.32-6.87)	
	G/G	1 (3.3%)	1 (2.9%)	0.90 (0.05-15.07)	
Dominant	A/A	26 (86.7%)	29 (82.9%)	1.00	0.742*
	A/G-G/G	4 (13.3%)	6 (17.1%)	1.34 (0.34-5.30)	
Recessive	A/A-A/G	29 (96.7%)	34 (97.1%)	1.00	1.000*
	G/G	1 (3.3%)	1 (2.9%)	0.85 (0.05-14.25)	
Overdominant	A/A-G/G	27 (90%)	30 (85.7%)	1.00	0.716*
	A/G	3 (10%)	5 (14.3%)	1.50 (0.33-6.88)	

*Fisher Exact Test.

Table (3-9): The association of rs10924081 genotypes with diabetic neuropathy (DN) vs. control under different models of inheritance.

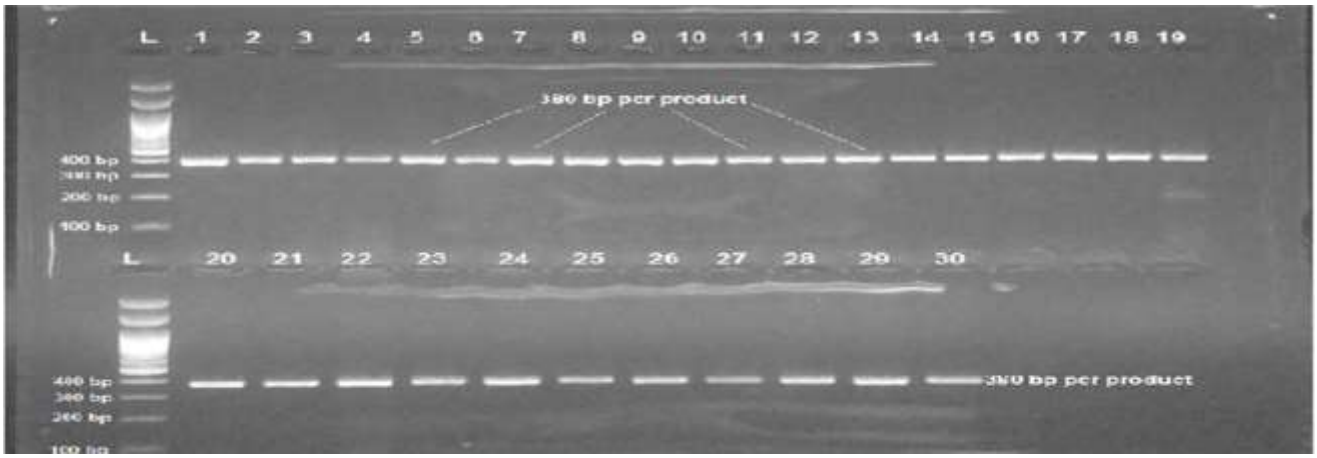
Model	Genotype	Control	DN	OR (95% CI)	P-value
Codominant	A/A	26 (86.7%)	54 (90%)	1.00	0.91
	A/G	3 (10%)	5 (8.3%)	0.80 (0.18-3.62)	
	G/G	1 (3.3%)	1 (1.7%)	0.48 (0.03-8.01)	
Dominant	A/A	26 (86.7%)	54 (90%)	1.00	0.726*
	A/G-G/G	4 (13.3%)	6 (10%)	0.72 (0.19-2.78)	
Recessive	A/A-A/G	29 (96.7%)	59 (98.3%)	1.00	1.000*
	G/G	1 (3.3%)	1 (1.7%)	0.49 (0.03-8.14)	
Overdominant	A/A-G/G	27 (90%)	55 (91.7%)	1.00	1.000*
	A/G	3 (10%)	5 (8.3%)	0.82 (0.18-3.68)	

*Fisher Exact Test

3.4.2 Association of rs373796693 with diabetic neuropathy

3.4.2.1 Optimization of rs373796693 PCR genotyping–Sequencing method:

The optimal PCR amplification of the proposed primers pair that would be utilized in rs373796693 genotyping was seen in Figure 3-3.



Figure(3-3): Optimized PCR product (380bp) of the designed primers pair for *rs373796693* genotyping . the electrophoresis carried on agarose gel (2%) , lane L= 100bp step ladder ; other lanes = 380 pb PCR product .

The polymorphism of the Na-K ATPase transporting subunit alpha 2 (*ATP1A2*) gene was examined in this work. This gene encodes for a protein that transports charged atoms into and out of cells using energy from adenosine triphosphate (ATP) (https://ghr.nlm.nih.gov/gene/ATP1A2).

After using NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequencing processes revealed the exact identification of this genomic fragment. The NCBI BLASTn engine found 99.0 % sequence similarity between the sequenced samples and the targeted reference target sequences for the 380 bp amplicons, Within the *ATP1A2* gene, this includes the whole of exon 2, intron 2, and the majority of exon 3. The precise locations and other characteristics of the recovered PCR fragments were discovered by comparing the observed DNA

sequences of these studied samples with the retrieved DNA sequences (GenBank acc. NG 008014.1) (Fig 3-4).

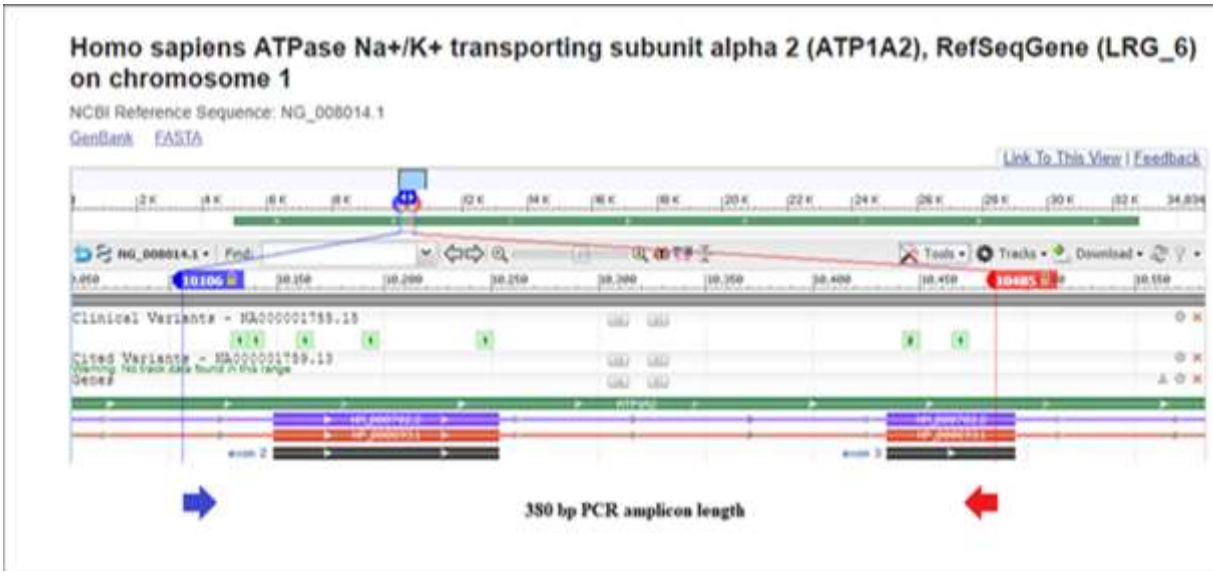


Figure (3-4): the exact location of the 380-bp amplicon that partially spans exon 2, intron 2, and the bulk of exon 3 of the ATP1A2 gene on chromosome 1 (GenBank accession number NG 008014.1). The amplicon's beginning point is shown by the cyan arrow, while its terminus is indicated by the red arrow.

The matching location of the ATP1A2 gene was downloaded from the dbSNP website (https://www.ncbi.nlm.nih.gov/projects/SNP/) to reveal the position of the targeted SNP in their deposited SNP database of the sequenced 380 bp segment. A graphical depiction of the ATP1A2 dbSNP database inside chromosome no. 1 (GenBank Acc. No. NG_008014.1) was used to determine the nature of this SNP.

This discovered SNP was previously known as rs373796693, and it was deposited with various patterns of allelic polymorphisms, according to the dbSNP engine (Fig. 3-5). On the other hand, this SNP was found in intron-2 of the ATP1A2 gene (https://www.ncbi.nlm.nih.gov/snp/rs373796693).

Furthermore, this SNP was quite common, as documented in both the ClinVar database and the literature. (https://www.ncbi.nlm.nih.gov/snp/rs373796693#publications)

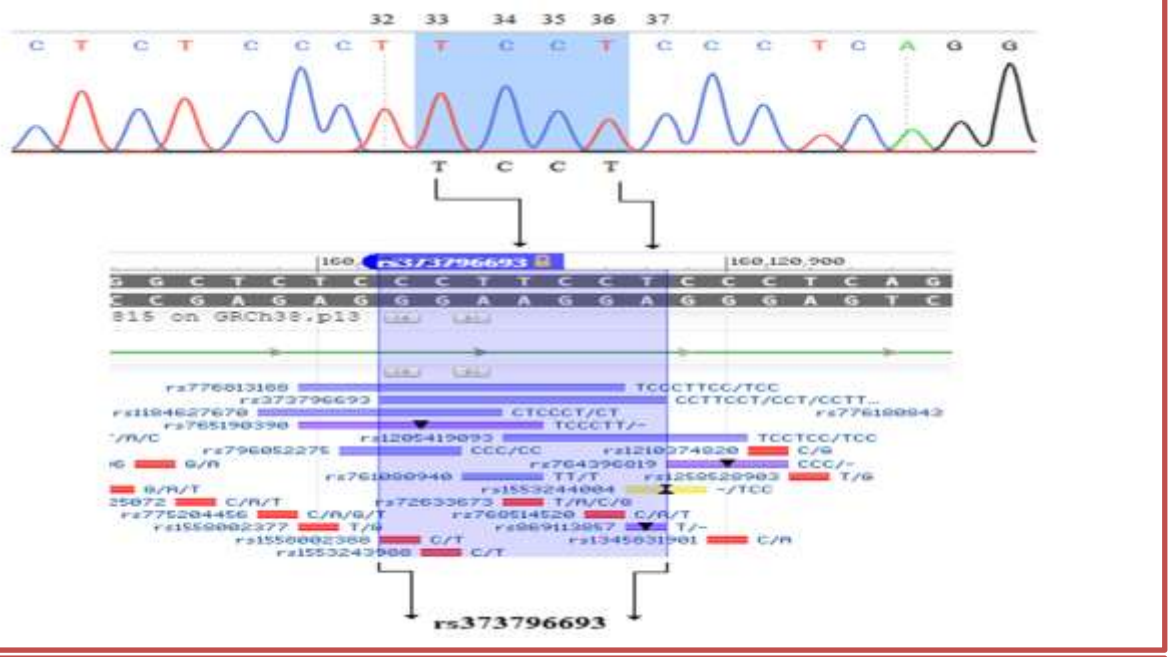


Fig. (3-5): The SNP's novelty checks of *ATP1A2* genetic single nucleotides polymorphisms using the dbSNP server. The identified rs373796693SNP marks with blue color. The GenBank acc. no. NG_008014.1 uses in the positioning of the highlighted deletion SNP. The position of the targeted sequences present in the positive strand.

3.4.2.2 Allelic association of rs373796693 with diabetic neuropathy

The allele frequencies and allelic association for diabetic neuropathy (DN) vs. type 2 diabetes mellitus without neuropathy (T2DM) are listed in Table 3-10. The results showed a significant allelic association between diabetic neuropathy (DN) and diabetes mellitus without neuropathy (T2DM) patients for allele I (OR=2.857,95 % CI=1.265-6.452). For allele D, there was a significant allelic association between diabetic neuropathy (DN) and type 2 diabetes mellitus without neuropathy (T2DM) patients (OR=0.350, 95 % CI =0.155-

0.790). This indicates that the patient's carrier allele (I) could be pathogenic for the development of diabetic neuropathy, But the D allele could be protective.

Table (3-10): Alleles frequency and allelic association between diabetic neuropathy (DN) and diabetes mellitus without neuropathy (T2DM) individuals of rs373796693.

	T2DM		DN		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion		
I	35	0.58	48	0.8	2.857 (1.265-6.452)	0.01
D	25	0.42	12	0.2	0.350 (0.155-0.790)	

I:insertion, D: deletion, OR: Odds Ratio. (95%CI): Confidence Interval.

Table 3-11 showed the alleles frequencies and the allelic association between diabetes mellitus without neuropathy (T2DM) and control individuals. The results indicate was a significant allelic association between type 2 diabetes mellitus without neuropathy (T2DM) and control groups for allele I (OR= 0.100, 95% CI =0.032-0.312). There was a significant allelic association between diabetes mellitus without neuropathy (T2DM) and control groups for allele D (OR= 10.000, 95%CI =3.208-31.169). This explains that I could be a diabetes protective allele, and D could be a risk allele.

Table (3-11): Alleles frequency and allelic association between diabetic without neuropathy (T2DM) and control groups of rs373796693

	Control		T2DM		OR (95% CI)	P-value
Allele	Count	Proportion	Count	Proportion		
I	56	0.93	35	0.58	0.100 (0.032-0.312)	7.532e-06
D	4	0.07	25	0.42	10.000 (3.208-31.169)	

I: Insertion, D: Deletion, OR: Odds Ratio, 95% CI : Confidence Interval.

Also in table 3-12 showed the alleles frequencies and the allelic association between diabetic neuropathy (DN) and control individuals. The results indicate was a significant allelic association between diabetic neuropathy (T2DN) and control groups for allele I (OR=0.286, 95% CI =0.086-0.944). There was a significant allelic association between diabetes neuropathy (DN) and control groups for allele D (OR=3.500, 95% CI =1.059-11.568). This means that I could be a diabetes protective allele, and D could be a risk allele.

Table (3-12): Alleles frequency between control and diabetic neuropathy (DN) groups of rs373796693.

	Control		DN		OR (95%CI)	P-value
Allele	Count	Proportion	Count	Proportion		
I	56	0.93	48	0.8	0.286 (0.086-0.944)	0.032
D	4	0.07	12	0.2	3.500 (1.059-11.568)	

I: Insertion, D: Deletion, OR: Odds Ratio, 95% CI : Confidence Interval

3.4.2.3 Genotypic association of rs373796693 with diabetic neuropathy:

The genotypic association between diabetic neuropathy (DN) and diabetes mellitus without neuropathy (T2DM) was studied under different models of inheritance. The results showed were significant association between the I/D-D/D genotype in patients with diabetic neuropathy (DN) and diabetes mellitus without neuropathy (T2DM) (OR=0.28, 95% CI= 0.09-0.82). This indicated, D allele could be a protective dominant allele, which confers resistance to the carrier individual when present as a heterozygote (ID) or homozygote (DD) when compared to other genotypes as shown in table (3-13).

Table (3-13) : The association of rs373796693 genotypes with diabetic neuropathy (DN) vs. diabetes mellitus without neuropathy (T2DM) under different models of inheritance

Model	Genotype	T2DM	DN	OR (95% CI)	P-value
Codominant	I/I	13 (43.3%)	22 (73.3%)	1.00	0.059*
	I/D	9 (30%)	4 (13.3%)	0.26 (0.07-1.03)	
	D/D	8 (26.7%)	4 (13.3%)	0.30 (0.07-1.18)	
Dominant	I/I	13 (43.3%)	22 (73.3%)	1.00	**0.017**
	I/D-D/D	17 (56.7%)	8 (26.7%)	**0.28 (0.09-0.82)**	
Recessive	I/I-I/D	22 (73.3%)	26 (86.7%)	1.00	0.33*
	D/D	8 (26.7%)	4 (13.3%)	0.42 (0.11-1.60)	
Overdominant	I/I-D/D	21 (70%)	26 (86.7%)	1.00	0.21
	I/D	9 (30%)	4 (13.3%)	0.36 (0.10-1.33)	

*Fisher Exact Test.

In table 3-14, the results showed a significant association of D/D genotype in diabetic without neuropathy (T2DM) and healthy individuals (OR= 8.62, 95% CI =1.60-46.38). In comparison to other genotypes, the D allele could be the risk codominant when present as a homozygote (DD).

Also, there was a significant association of I/D-D/D genotype in T2DM and healthy individuals (OR=18.3, 95% CI =3.67-91.23). The D allele could be a risk dominant allele when it presents as the heterozygote (ID) and homozygote (DD) as compared to other genotypes.

Table (3-14): The association of rs373796693 genotypes with diabetic without neuropathy (T2DM) vs. control under different models of inheritance.

Model	Genotype	Control	T2DM	OR (95% CI)	P-value
Codominant	I/I	28 (93.3%)	13 (43.3%)	1.00	<0.0001
	I/D	0 (0%)	9 (30%)	NA (0.00-NA)	
	D/D	2 (6.7%)	8 (26.7%)	**8.62 (1.60-46.38)**	
Dominant	I/I	28 (93.3%)	13 (43.3%)	1.00	<0.0001*
	I/D-D/D	2 (6.7%)	17 (56.7%)	**18.31 (3.67-91.23)**	
Recessive	I/I-I/D	28 (93.3%)	22 (73.3%)	1.00	0.08*
	D/D	2 (6.7%)	8 (26.7%)	5.09 (0.98-26.43)	
Overdominant	I/I-D/D	30 (100%)	21 (70%)	1.00	0.002*
	I/D	0 (0%)	9 (30%)	NA (0.00-NA)	

*Fisher Exact Test

As in the table (3-15), There was no significant association of genotypes in diabetic neuropathy (DN) and healthy individuals under different models of inheritances

Table (3-15): The association of rs373796693 genotypes with diabetic neuropathy (T2DN) vs. control in various models of inheritance.

Model	Genotype	Control	T2DN	OR (95% CI)	P-value
Codominant	I/I	28 (93.3%)	22 (73.3%)	1.00	0.031
	I/D	0 (0%)	4 (13.3%)	NA (0.00-NA)	
	D/D	2 (6.7%)	4 (13.3%)	2.55 (0.43-15.20)	
Dominant	I/I	28 (93.3%)	22 (73.3%)	1.00	0.08*
	I/D-D/D	2 (6.7%)	8 (26.7%)	5.09 (0.98-26.43)	
Recessive	I/I-I/D	28 (93.3%)	26 (86.7%)	1.00	0.671*
	D/D	2 (6.7%)	4 (13.3%)	2.15 (0.36-12.76)	
Overdominant	I/I-D/D	30 (100%)	26 (86.7%)	1.00	0.112*
	I/D	0 (0%)	4 (13.3%)	NA (0.00-NA)	

*Fisher Exact Test.

3.4.3 Correlation between Genotypes and phenotypes:

3.4.3.1 Correlation of rs10924081 genotype and Na-K ATPase activity and C-peptide:

As the ATP1A1 gene encodes for the α 1 catalytic isoform, which was exclusively expressed in RBCs and is preponderant in nerve, we studied an RFLP to investigate whether this polymorphism could explain Na-K ATPase variations. Table 3-16 showed the correlation between the genotype distribution of rs10924081 ATP1A1 gene with Na-K ATPase and C-peptide. Individuals that carrier genotypes AA, AG, and GG showed no significant differences in the activity of erythrocyte Na-K ATPase and levels of plasma C-peptide.

Table (3-16): The correlation between rs10924081 genotypes with Na-K ATPase activity and C-peptide.

Dependent Variable	(I)SNP	(J)SNP	Mean difference(I-J)	SE	P-value
Na-K ATPase activity	AG	AA	20.57205	82.05457	0.802
		GG	91.89744	179.11645	0.609
	AA	AG	-20.57205	82.05457	0.802
		GG	71.32538	163.66014	0.664
	GG	AG	-91.89744	179.11645	0.609
		AA	-71.32538	163.66014	0.664
C-peptide	AG	AA	-.24809	.34655	0.475
		GG	-.92864	.75649	0.222
	AA	AG	.24809	.34655	0.475
		GG	-.68055	.69121	0.327
	GG	AG	.92864	.75649	0.222
		AA	.68055	.69121	0.327

3.4.3.2 Correlation between rs373796693 genotypes combined with Na-K ATPase activity and C-peptide:

In Table (3-17), The results showed, Erythrocyte Na-K ATPase activity in heterozygous patients (ID) was significantly lowered when compared with homozygous patients (mean difference=-174.9867, p = 0.049). In patients with homozygous II and DD, there was no significant correlation between the gene polymorphism and Na-K ATPase activity. This indicates that patients with allele I, when present as a heterozygous could be more responsive to a reduction in the activity of Na-K ATPase.

On the other hand, no association between plasma C-peptide levels and the rs373796693 SNP as shown in table 3-17.

Table (3-17): The correlation between rs373796693 genotypes and Na-K ATPase activity and C-peptide.

Dependent Variable	(I)SNP	(J)SNP	Mean difference(I-J)	SE	P-value
Na-K ATPase activity	ID	II	-174.98657*	87.44924	0.049*
		DD	-104.35165	110.57001	0.348
	II	ID	174.98657*	87.44924	0.049
		DD	70.63492	84.82078	0.407
	DD	ID	104.35165	110.57001	0.348
		II	-70.63492	84.82078	0.407
C-peptide	ID	II	.03970	.33684	0.906
		DD	.26015	.42590	0.543
	II	ID	-.03970	.33684	0.906
		DD	.22045	.32672	0.502
	DD	ID	-.26015	.42590	0.543
		II	-.22045	.32672	0.502

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5. Conclusions and Recommendations

5.1 Conclusions:

1. Diabetes duration correlates well with the incidence of diabetic neuropathy, probably due to disagreement of intracellular metabolic pathways and ionic balance.
2. There is reduced specific activity of Na-K ATPase in red blood cell ghosts in type 2 diabetic neuropathy patients compared with patients type 2 diabetes mellitus without neuropathy and normal control individuals.
3. Endogenous digitalis reflected by the inhibition percentage of Na-K ATPase correlates positively with diabetic neuropathy.
4. Plasma C-peptide concentration is significantly lowered in patients with type 2 diabetic neuropathy as compared with control and type 2 diabetes mellitus without neuropathy.
5. Reduction of erythrocyte Na-K ATPase activity in patients of T2DN was a significantly association with endogenous digitalis, HbA1C, and age of individuals, while, there was no significant association plasma C-peptide, BMI and duration of diabetes.
6. There was no significant association of allelic and genotypic of ATP1A1 rs10924081 with diabetic neuropathy when compared with diabetic without neuropathy and control subjects.
7. For ATP1A2 rs373796693 : patients who carry the I allele could be a risk to develop diabetic neuropathy, whereas patients who carry the D allele could be protective.
8. Patients who carry the ID, DD genotype of ATP1A2 rs373796693 have protected for diabetic neuropathy. The protective allele is inherited as a dominant protective allele.
9. ATP1A1 rs10924081 genes have not associated with erythrocyte Na-K ATPase and plasma C-peptide.

10. patients who have the ID genotype ATP1A2 rs373796693 showed a significantly lower Na-K ATPase activity in RBC ghosts when compared with other genotypes, while, no significant association between plasma C-peptide and any genotype of both studied genes was noted.

5.2 Recommendations:

1. Increasing the sample size may contribute in further clarifying the association of genetic polymorphism in diabetic neuropathy.
2. Studying the association of genetic components of other protein moieties of Na-K ATPase enzyme (α3, α4, β1, β2 and FXYD genes) with diabetic neuropathy.
3. Studying the level of individual cardiotonic steroids in patients with diabetic neuropathy in order to isolate the source of major inhibitory activity, and could developed a new medication for this condition.

4. Discussion:

4.1 Study the association of diabetes duration, age and BMI with diabetic neuropathy.

Diabetic neuropathy DN is a serious consequence of diabetes that has several risk factors[217]. The duration of illness was a substantial risk factor for the development of diabetic neuropathy. In this study, the duration of diabetes was found to be positively related to diabetic neuropathy with a p-value <0.05) as shown in Table (3-1). Our finding is supported by Rivillis et al. [218], and Ashok et al. [219]. Long-term diabetes is linked to increased glycosylation end products generation, metabolic disturbances, endothelial damage, and oxidative products[220,221].

Polyneuropathy is less common in patients with DM for less than 5 years and more common in those with DM for more than 15 years, according to Oguejiofor, et al. (2010) [222]. Another research demonstrates that 15.2% of Type 2 diabetes patients had impaired nerve conduction velocity when they were diagnosed, while only 2.3% of diabetic patients have symptoms of polyneuropathy[223]. The majority of diabetic neuropathy patients in the current study were ranged from 40 to 80 years.

As shown in the Table 3-1, there was a significant association between age and diabetic neuropathy by p<0.01. In diabetics, age is a known risk factor for the development of neuropathy, and several studies have established a continuous increase in the incidence of neuropathy as time progresses[224]. Because aging produces a gradual decline in neurological functioning, even in the absence of diabetes, the symptoms must be interpreted differently [225,226].

On the other hand, Delcourt et al. discovered that DN was no longer associated with age [227], They found a significant association between peripheral neuropathy and diabetes duration in younger individuals but none in

older ones. Duration of diabetes has taken into consideration, older individuals may be less likely to acquire particular problems than younger patients, as demonstrated in retinopathy and neuropathy[227].

In current study, no significant association between body mass index BMI and diabetic neuropathy as shown in the table (3-1). Robinson, et al. reported a similar conclusion (1992) [228]. whereas, Cohen, et al. (1998), recounted bodyweight as an independent risk factor for diabetic peripheral neuropathy [229].

4.2 Sample parameters:

4.2.1 Age:

Age of diabetic neuropathy (DN) patients was higher significantly as compared with diabetes mellitus without neuropathy T2DM and healthy subjects with a $p < 0.05$ as shown in the Table 3-2. This agrees with findings acquired by Flynn et al., (1995)[230], the age-related occurrence of diabetic neuropathy peaked at 40-49 years and increased with age. Alder, et al., for example, have found similar observations (1997)[231].

Peripheral neuropathies are a widespread illness among the elderly. Axonal injury or demyelination, as well as damage to large or small fibers, can induce peripheral neuropathy. In the United States in 1999–2000, a simple test for decreased sensation at the foot revealed that 28 % of individuals aged 70–79 years and 35 % of those over 80 years had peripheral neuropathy[232].

4.2.2 Body mass Index(BMI):

As realized in the Table 3-2, no significant differences between diabetes with and without neuropathy and healthy individuals ($p=0.444$). According to a study by Popescu, , et al. (2016)[232]. Another research by Oh Tae Jung, et

al.(2019) discovered that BMI was greater in those with DPN than in people without DPN[233].

4.2.3 Glycated haemoglobin (HbA1C):

Glycated haemoglobin measurement has introduced additional dimensions to glycaemia evaluation. This test may be used to calculate the average glycaemia across months [234]. HbA1C test has been offered by Keontg and Gabbay, along with their co-workers, as a diabetes control marker. HbA1C is the outcome of post-transcriptional glycosylation of haemoglobin at the amino-terminal valine of the beta chain. This is a gradual, irreversible chemical process that proceeds throughout the RBC's life, with the amount of HbA1C produced being mostly determined by the current plasma glucose levels[235].

In our study, The levels of HbA1C were lower significantly in patients with DN when compared with T2DM with $p<0.05$. The lower results suggest that Haemoglobin A1C levels in diabetic patients have a close relationship, there is also an indirect proportion between groups 1 and 2. The possible explanation is that patients with diabetic neuropathy (group 1) have been subjected to strict control of blood glucose by their treating physicians as the first-line regimen, even then, group 2 could be less able to monitor glucose levels directly, as shown in Table 3-2.

Raised HbA1C levels indicates in diabetic neuropathy, which is demonstrating that hyperglycemia or an equivalent metabolic abnormality is a key component in the development of neuropathy. The HbA1C elevation induces a shift to the left in the oxygen dissociation curve, resulting in tissue hypoxia, and this is one of the explanations for the etiology of diabetic neuropathy and other microvascular complications [236]. The degree of hyperglycemia and abnormal glycaemic haemoglobin levels have a substantial

impact on the results of the sensory and motor nerve conduction study (NCS) tests. This might be related to the fact that abnormal HbA1c levels are linked to neuromuscular twitches and fibre densities [217].

4.2.4 Erythrocyte Na-K ATPase activity:

The chemical energy released by the hydrolysis of one molecule of ATP is used by Na-K ATPase to transport three Na^+ ions out of and two K^+ ions into the cell. This generates an electrochemical gradient across the cell membrane, which is necessary for secondary active transport of nutrients and metabolites, as well as the excitability of neurons and muscle cells when combined with K^+ channels [237]. Diabetes mellitus causes a decrease in erythrocyte membrane Na-K ATPase activity, according to studies done so far in humans and animals. That further causes hemodynamic dysfunction, rheological abnormalities induced by decreased erythrocyte deformability, and increased fluidity, along with consequences including nephropathy, neuropathy, cardiovascular diseases, and microangiopathy [238-240].

The present study has revealed that erythrocyte Na-K ATPase activity decreased significantly in patients with DN when compared with T2DM and control individuals $p<0.05$ as shown in Table 3-2. Except for a few research on nerve biopsies, the great majority of human investigations have been conducted on erythrocytes[241]. Diabetes causes a reduction in enzyme activity in nearly all organs, as well as in erythrocytes, according to the study by Vague .P., et al. [242]. Diabetes neuropathy exhibited lower Na-K ATPase activity than diabetics without neuropathy among the two diabetic groups ($P< 0. 01$). These results are consistent with Koc et al.[243] and Zhang et al.[244]. This enzymatic dysfunction is possibly joined with the relative insulinopenia of hyperglycaemic IDDM patients [245]. In other study, Humayoun et al.[246] detected no significant differences in Na-K ATPase activity comparing type 2

diabetes patients and healthy controls. Disturbances of the membrane lipid organization can illuminate the dropping in Na-K ATPase activity[247]. Alternatively, a disorder of the metabolism of the essential fatty acid, linoleic acid, leads to a depletion of y-linoleic acid disturbing the fatty acid composition of the membrane phospholipids. These two mechanism bring about a misplacement of the Na-K ATPase in its phospholipid sleeve, which can shift its activity. Lower levels of Na-K ATPase can also be attributed to two factors[242]. Aldose reductase and sorbitol dehydrogenase enzymes shifted the glucose amount that reached the polyol pathway and converted it to sorbitol and fructose. Moreover, NADPH is required for the aldose reductase enzyme. The quantity of nitric oxide synthase is reduced when NADPH is reduced, therefore nitric oxide levels are reduced, diminishing blood flow [248].

NADPH deficiency also lowers Myo-inositol levels. Furthermore, sorbitol and fructose build up in the nerves, lowering Myo-inositol levels. Finally, reduced Na-K ATPase activity is caused by decreasing Myo-inositol levels [243]. The oxidative stress induced by DM might also be a factor in the decrease in Na-K ATPase activity. Increased oxidative stress promotes lipid peroxidation in the cell membrane, which lowers the activity of the Na-K ATPase[249]. Increased oxidative stress causes sodium ions to accumulate in axons, preventing depolarization of the neural membrane and resulting in a reduction in nerve conduction velocity [243,244]. Thus, the molecular cause of microvascular damage in diabetes might be a decrease in erythrocytes Na-K ATPase activity. If this is true, erythrocyte Na-K ATPase activity might be a useful measure for predicting the risk of diabetes complications in a patient, as well as a target for protective drugs. Rather than being the direct source of diabetic damage, Na-K ATPase activity decrease might occur in conjunction with other effects of diabetes on cells[250].

4.2.5 Endogenous digitalis (Na-K ATPase inhibitory):

Endogenous digitalis (Na-K ATPase inhibitory): are specific inhibitors of Na-K ATPase [250]. Na-K ATPase inhibitors are a class of steroids that act as regulators of the ubiquitous enzyme Na-K ATPase. They include a series of composites that bind to the extracellular segment of the Na-K ATPase and prevent it from working. The amino acids needed for Na-K inhibitor attachments are found in the extracellular regions of the alpha-subunit between helices one/two and helices seven/eight. They've been discovered in virtually all tissues, including plasma, although the adrenal, hypothesis and hypothalamus contain the greatest amounts[251]. Endogenous digitalis have been linked to the etiology of NaCl-sensitive hypertension, diabetes mellitus (DM), preeclampsia (PE) [176], and renal failure[252]. Inhibition of Na-K ATPase (NKA) is associated with increased levels of marinobufagenin (MBG), an endogenous bufadienolides in experimental rats and humans with type 1 and type 2 diabetes [253].

In our study, the results of endogenous digitalis were a higher significant in patients of DN when compared with T2DM and healthy subjects ($p< 0.05$) as shown in Table (3-2). This agrees with a study had been conducted by Bagrov, et al. (2005) in patients with insulin-dependent diabetes and healthy North African subjects compared with healthy Caucasian subjects[188].

Patients and experimental animals with diabetes mellitus have been reported to have elevated levels of cardiotonic steroids as well as impaired Na-K ATPase activity[254,255]. More recent research indicates that rats with type 1 diabetes possess higher urinary levels of marinobufagenin and more profound inhibition of the Na-K ATPase than rats with type 2 diabetes. Several researchers have found an increase in endogenous digitalis in individuals with diabetes mellitus, regardless of the fact that compounds are known as

hypertension and natriuretic-promoting agents. Wasada and his colleague have reported that plasma endogenous digitalis were linked with the severity of insulin resistance (as measured by the euglycemic hyperinsulinemia clamp) but not with blood pressure rise in NIDDM patients[256].

Endogenous digitalis increased in individuals with NIDDM and gestational diabetes mellitus. As a result of the association between endogenous digitalis and insulin secretion. These results were independent of hypertension, and endogenous digitalis did not correlate with blood pressure[257]. Although the increase in endogenous digitalis in NIDDM patients is most likely secondary, the data suggest that these chemicals may regulate insulin sensitivity and/or insulin secretion, especially since both of these processes are Ca^{2+} dependent[258-260].

4.2.6 plasma C-peptide:

C-peptide is a short peptide that connects two proinsulin chains and is dissociated before insulin is released. It is released into the blood in an equimolar quantity to insulin by pancreatic beta-cells. Recent data suggests that it performs various physiologic functions in addition to its structural role. In varied pathological situations, C-peptide affects intracellular signaling pathways and might be a novel therapeutic target for a variety of diseases, including diabetes consequences[261].

As shown in Table 3-2, Plasma C-peptide levels were found to be significantly lower in DN compared to T2DM and healthy individuals $p<0.05$. Parallel to other analyses by Qiao et al.(2017) [262], they established fasting C-peptide in the clinical diabetic peripheral neuropathy DPN group was significantly lower than the non-DPN group. Diabetes duration increased, the islet function diminished gradually, this led to reduced C-peptide and insulin levels, and the prevalence of DPN increased [262]. However, Sari et al.

(2005)[263] demonstrated that C-peptide did not correlate with sensorial neuropathy.

4.3 Study the correlations of Erythrocyte Na-K ATPase activity with other parameters in our study:

As shown in Table 3-3, the correlation between erythrocyte Na-K ATPase activity and Na-K ATPase inhibitory (endogenous digitalis) was a significantly negative (r=-0.167, and p= 0.042). Inverse relationship between these variables due to insulin lack and a deficient endothelial nitric oxide bioavailability. In diabetic animals, the activity and amount of Na-K ATPase subunits in the plasma membrane are decreased. Furthermore, the elevated plasma levels of endogenous ouabain in such animals may be reducing the Na-K ATPase activity, which is already low[264].

As a result, the Na^+ gradient is reduced, and Na gradient-dependent activities, particularly Na^+/Ca^{2+} exchange, are inhibited, increasing the total amount of intracellular stored calcium (Ca^{2+}). As a consequence, endogenous digitalis has the potential to influence Ca^{2+}dependent activities, as well as cell response to diverse stimuli. Although endogenous digitalis were best known for their role in promoting hypertension and natriuresis, several clinical studies have shown that they are also elevated in patients with IDDM [256,265], NIDDM, and pregnant women with GDM [266]. Furthermore, in diabetic patients, some researchers have reported volume expansion, reduced Na-K ATPase activity, increased Ca^{2+}, and decreased Mg^{2+} concentrations[257,258,267,268]. These findings raise the question of whether endogenous digitalis is linked to diabetes.

The correlation between Erythrocyte Na-K ATPase activity and plasma C-Peptide was not significant positively (r=.032, p=0.694) in our research. The C-peptide signal route was revealed by Othomo et al.(1996) [269], who used Na-K ATPase as a target protein. C-peptide stimulates G-proteins and Ca^{2+}

dependent signaling pathways, according to their findings. After 24 hours, intensive insulin treatment administered by an artificial pancreas restores Na/K ATPase activity in erythrocyte membranes [270]. Insulin, in fact, enhances the sodium affinity of Na-K ATPase in diabetic rats' skeletal muscle and adipocytes [271]. Insulin's impact on Na-K ATPase, as mediated by the insulin receptor, appears to follow the same signaling route as G-protein and calcium modulin phosphatase [271,272]. As a result, both C-peptide and insulin appear to increase Na-K ATPase activity[271,272].

Agreeing to our evidence, the correlation between Na-K ATPase activity and HbA1C levels and age of areas of study was a higher significant negatively ($r=-0.380$, $p<0.01$, $r=-0.495$, $p<0.01$, respectively). Poor metabolic regulation is a major cause of diabetes complications[273,274]. Therefore, it's possible to realize a correlation between low Na-K ATPase enzyme levels and metabolic control parameters. However, in another study, no significant correlation between age and erythrocyte Na-K ATPase activity was found in diabetes patients and healthy controls ($r= -3.95 \times l0^{-3}$ and $r= -5.7 \times 10^{-2}$, individually). The activity of the Na-K ATPase was shown to be strongly associated with HbA1. As a result, individuals who appeared to have better metabolic control had decreased Na-K ATPase activity[275].

Further, follow a line of investigation suggests that diabetes-induced Na-K ATPase dysfunction may have a role in the etiology of human diabetic neuropathy and that it is unrelated to excellent metabolic management[273,276]. It was formerly thought that erythrocyte Na-K ATPase levels were linked to poor glycaemic management. There is increasing evidence that suggest an inverse correlation between Na-K ATPase enzyme activity and increasing age. In the aging process, oxidative degradation of membrane lipids, cytosolic glycolytic enzyme depletion, and low intracellular K^+ concentration all contribute to decreased enzyme function[277].

But even so, there was no correlation between erythrocyte Na-K ATPase activity with BMI and disease prevalence for our research groups (r=-0.009 ,p=0.910 and r=-0.138,p=0.134). In contrast, the duration of diabetes and enzyme activity was insignificantly negative (r=-0.138, p=0.134). The findings show that erythrocyte Na-K ATPase enzyme activity is reduced in type 2 diabetes, and that this drop is connected to the duration of the disease[278]. We believe that variables other than BMI and diabetes duration are contribute to the patient's group's low enzyme levels.

4.4 Allelic and Genotypic Association of rs10924081 ATP1A1 With Diabetic Neuropathy

The significance of this protein is highlighted by a number of dominant illnesses caused by mutations in genes encoding for Na-K pump catalytic α-subunit isoforms. We evaluate the literature for diseases induced by missense mutations in ATP1A1, the gene that encodes the Na-K pump's α1 isoform, which is widely expressed. Primary aldosterone's with secondary hypertension, endocrine syndrome, Charcot-Marie-Tooth disease, peripheral neuropathy, complex spastic paraplegia, another neuromuscular disorder, and hypomagnesaemia with seizures and cognitive delay, a condition affecting the renal and central nervous systems, are among the various ailments[279].

The findings of recent experiments prompted researchers to explore ATP1A1 gene variants linked to Na-K ATPase activity; these polymorphisms might be used as potential indicators for diabetic neuropathy susceptibility. For example, the ATP1A1 gene is widely expressed in peripheral nerves and red blood cells, and polymorphisms in this gene have been associated to neuropathy in T1DM patients [280,281]. A single nucleotide polymorphism (SNP) in intron 1 of ATP1A1 was realized by restriction fragment length polymorphism (RFLP) using the Bgl II enzyme in Caucasian people with >10 years of T1DM[280,281]. In Chinese T2DM patients, a PstI SNP was discovered in

exon 2 of ATP1A1, whereas a Bgl II SNP was discovered in intron 1 of ATP1A1 in Turkish T2DM patients [282].

The *ATP1A1* encodes for the α1 subunit of the Na-K ATPase, a protein ion pump that is responsible for the active transport of Na^+ and K^+ across the plasma membrane[283]. The *ATP1A1* gene is located on chromosome 1, Many different types of cells have Na-K ATPases with the alpha-1 subunit (https://ghr.nlm.nih.gov/gene/ATP1A1). We selected the ATP1 A1 gene coding for the alpha 1 isoform as the applicant gene for susceptibility to diabetic neuropathy.

In our result, we identified a rs10924081 ATP1A1 SNP at chromosome 1 in intro 9 (corresponding to nucleotide 189 of the PCR result) by restriction fragment length polymorphism (RFLP) using *MSPI* digestion, which yielded 189 bp. If the G allele of rs10924081 is present, the PCR product split the 189 bp product into two polynucleotide chains with molecular weights of 52 bp and 137 bp. If the allele A of rs10924081 is present, the PCR product split the 189 bp product into three polynucleotide chains with molecular weights of 52 bp, 49 bp, and 88 bp, as observed in Fig.(3-2).

The results showed no significant allelic or genotypic association between patients of DN and T2DM in our research. This indicated, that subjects who are not carriers of an allele or genotype could be a risk factor for developing the disease. This finding agrees with several other studies carried out in different populations and disagreed with many other studies which found a significant relationship between ***ATP1A1*** and diabetic and diabetic neuropathy.

A shred of experimental evidence by Vague, et al. (1997). The first intron of the ATP1A1 gene, which codes for the ATPase alpha 1 isoform, has a restriction polymorphism. This isoform predominates in nerve tissue and is found only in red cells. The presence of the restricted allele is significantly

linked to DN among European diabetics, with a relative risk of 6.5. (95% CI 3.3-13)[281].

In other analysis, they employed PstI digestion to find a G94A SNP at nucleotide 27 in exon 2 (equivalent to nucleotide 94 of the PCR result), which yield 217 and 94 bp fragments in diabetes groups alone. The diabetic groups housed only the A (risk/restrictive) allele with the AA genotype, whereas the controls possessed only the G (protective) allele with the GG genotype[284]. T2DM patients with or without neuropathy (with AA genotypes) had a significantly different genotype dispersion than controls (with GG genotypes) ($P<0.01$). Because both diabetes groups had the same genotypic distribution, they found no indication of a relationship between the G94A SNP and diabetic neuropathy[284]. In DN, Zhang et al. observed a lower likelihood of the A allele and the AA genotype than in T2DM [244]. This implies that the specificity given by the ATP1-A1 polymorphism mutation makes the enzyme's activity more vulnerable to the harmful effects of diabetes. Insulinopenia appears to be more important than hyperglycemia in determining the impact of the ATP1-A1 polymorphism. Indeed, Na-K ATPase activity did not change in type 2 diabetes individuals with or without the restricted allele on oral therapy, but it did differ significantly in those treated with insulin[276].

4.5 Association of Allelic and genotypic of rs373796693 ATP1A2 with diabetic neuropathy :

The α2 subunit of a Na-K ATPase is encoded by the ATP1A2 gene on chromosome 1q23. This catalytic subunit binds Na^+, K^+, and ATP then uses ATP hydrolysis to exchange Na^+ ions out of the cell for K^+ ions in. Pumping Na^+ creates the strong Na^+ gradient required for glutamate and Ca^{2+} transfer. In new-borns, the gene is mostly expressed in neurons, whereas in adults, it is mostly expressed in glial cells (https://ghr.nlm.nih.gov/gene/ATP1A2). The modulation of potassium and glutamate reuptake from the synaptic cleft into the glial cell is one of this particular ATPase's essential functions in adults.

A high number of mutations in the ATP1A2 gene have been identified that are linked to pure Familial Hemiplegic Migraine FHM and FHM in conjunction with cerebellar ataxia, juvenile alternating hemiplegia, benign focal infantile convulsions, and various epilepsies[285]. Autosomal dominant inheritance is linked to pathogenic mutations in the ATP1A2 gene. On the other hand, penetrance varies, and clinical symptoms might be minor, moderate, or severe[286].

As shown in Fig (3-4), the ***rs373796693*** SNP realized for ATP1A2 in intron 2 of chromosome 1 (corresponding to nucleotide 380 of the PCR result) by Sanger sequencing. There was a significant allelic association of allele I between DN and T2DM (OR =2.857,95% CI=1.265-6.452) as shown in Table (3-10). In contrast, there was a significant allelic association between T2DM and control subjects for allele I (OR= 0.100, %95 CI=0.032-0.312), also a significant allelic association of allele I between DN and healthy individuals (OR=0.286, %95 CI=0.086-0.944) as shown in Table (3-11), (3-12) respectively. On the other hand, For allele D, there was a significant allelic association between DN and T2DM patients (OR=0.350, 95 % CI =0.155-0.790) as shown in Table (3-10). There was a significant association between the allelic association of allele D between T2DM and control individuals

(OR=10.000, %95 CI =3.208-31.169) as shown in Table (3-11). and a significant allelic association of allele D between DN and controls subjects (OR=3.500,%95 CI=1.059-11.568) as shown in Table 3-12. This indicated allele I could be the diabetes protective allele D could be the diabetes risk allele.

In addition, when studying the association of rs373796693 genotypes between control, T2DM, and DN under different models of inheritance as shown in Tables (3-13), (3-14), and (3-15), obtained the patient's carrier genotype ID could be more to develop diabetic neuropathy. There was found evidence of a correlation between the rs373796693 SNP and diabetic neuropathy since both diabetes groups had different genotypic distributions.

4.6 Correlation between Genotypes and phenotypes:

4.6.1 Correlation of rs10924081 genotype and Na-K ATPase activity and C-peptide:

The major goal of this study was to investigate at how Na-K ATPase activity varied depending on genetic (ATP1A1,ATP1A2 genotype). As the ATP1A1 gene encodes for the α 1 catalytic isoform, which is exclusively expressed in RBCs and is preponderant in nerve, The RFLP technique was used to investigate whether this polymorphism could explain Na-K ATPase variations. The genotype distribution of the rs10924081 ATP1A1 gene with Na-K ATPase and C-peptide is shown in Table 3-16. The activity of erythrocyte Na-K ATPase and plasma C-peptide levels were not significantly different in people with the genotypes AA, AG, and GG. This hypothesis is consistent with the findings of numerous prior investigations in different groups, but it contradicts the findings of other studies that found a significant connection between ATP1A1 and diabetes and diabetic neuropathy.

The association between ATP1A1 with diabetic neuropathy might be recorded by Vague, et al. (1997), observed Na-K ATPase activity in homozygous diabetic patients without the restriction site was higher than in

heterozygous diabetic patients with restriction site ($p < 0.01$). In contrast, among healthy subjects no correlation was observed between the gene polymorphism and Na-K ATPase activity with an average 400 nmol Pi· mg protein^{-1}·h^{-1} in subjects homozygous for the unrestricted allele and 393 nmol Pi· mg protein^{-1}·h^{-1} among heterozygous subjects[287].

Furthermore, Abosheasha, et al. (2019) reported a association between the genotype distribution of the ATP1A1 gene, Na K ATPase, and C peptide in their experiment. Subjects with the AA genotype had a significant decline in Na-K ATPase activity and C peptide levels ($P< 0.001$) when compared to those with the GG genotype[284].

Moreover to Na-k ATPase, they also established a link between this SNP and lower levels of C Peptide. Because this SNP can affect mRNA stability, gene expression level, and protein conformation, the occurrence of an identical SNP, that does not alter amino acids, doesn't dismiss the possibility that the mutation does not affect gene function [288]. In the agreement, their analyses, those published by Zhang et al.[244], demonstrated a strong link between the identical SNP and T2DM[284]. In an Asian population, Zhang et al.[244] found a correlation between the A allele and increased Na-K ATPase activity, implying that it might be a protective allele for diabetic neuropathy. This variation in the A allele relationship might be due to different ethnic groupings (Egyptian vs. Chinese). They identified a connection between this SNP and a decrease in C-Peptide levels, in addition to Na-k ATPase[244].

Na-K ATPase is statistically decreased when the restricted allele is present in diabetic patients. This suggests that the particularity conferred by the mutation associated with ATP1A1 polymorphism renders the enzyme’s function more susceptible to the deleterious effect of diabetes. More than hyperglycemia, insulinopenia seems to be determinant on the effect of ATP1A1 polymorphism[284].

The interaction of intron polymorphism with reduced product activity is most likely the result of linkage disequilibrium with a DNA mutation that affects ATP1A1 gene expression. This mutation may change the genes that code of mechanisms that determine gene expression or the coding sequences, affecting the enzyme's activity. It would result in decreased enzymatic activity as a result of either a reduction in the number of enzyme molecules or structural changes to the molecules[287], which is compensated when C-peptide levels are sufficient, but C-peptide deficiency alters Na-K ATPase, and the ATP1A1 polymorphism exacerbates the situation[276].

The presence of a variant allele of the ATP1A1 gene, which codes for the α1 isoform of the Na-K ATPase, is linked to lower Na-K ATPase activity in type 1 diabetic patients but not in control subjects[287]. Inferring that the presence of the variant allele makes ATPase activity more sensitive to the deleterious effects of diabetes, with this effect likely secondary to the lack of C-peptide. They also detected that in type 2 diabetics, ATPase activity is influenced by the existence of the variant allele as well as C-peptide levels[281]. Low Na-K ATPase activity in RBCs and sciatic nerves of diabetes patients appears to be linked to low C-peptide levels[281].

4.6.2 Correlation between rs373796693 genotypes combined with Na-K ATPase activity and C-peptide:

Due to metabolic changes in myo-inositol or free radical formation, almost all diabetic neuropathy patients have decreased Na-K ATPase activity, which causes sodium ions to pile up in the axons, preventing depolarization of the neural membrane and resulting in a decrease in nerve conduction velocity[243,244]. As a result, rather than a metabolic problem, gene mutation may be the cause of diabetic neuropathy (genetic susceptibility)[284].

This leads us to investigate the relationship between *ATP1A2* SNP, Na-K ATPase activity, and diabetic neuropathy. To achieve that, we start by searching

the Iraqi population for the previously revealed SNP. Indeed, in diabetic neuropathy and diabetes without neuropathy groups, the Sanger sequencing approach identified a region rs373796693 SNP in intron 2 of chromosome 1 (matching to nucleotide 380 of the PCR result).

The results exposed the I allele could be a risk allele for the pathogenesis of chronic neuropathy by investigating the correlation of rs373796693 between diabetes and diabetic neuropathy. As a consequence, only the I (risk/restrictive) allele was found in the diabetic neuropathy groups as heterozygous or homozygous ID, II genotypes, whereas the D allele could be protective was found in the DD genotype.

In genotype distribution, Patients with type 2 diabetic neuropathy and type 2 diabetes mellitus without neuropathy which has heterozygous ID genotypes had significantly reduced erythrocyte Na-K ATPase activity (p =0.049*). Intriguingly, this indicates an association between the I allele and the progression of not just diabetic Mellitus but also diabetic neuropathy in the current investigation. This leads that the I allele could be a risk issue for diabetic neuropathy patients in the reviewed Iraqi residents.

Because there is no association between plasma C-peptide levels and the rs373796693 SNP as shown in table 3-17, we suggest that C-peptide does not influence the rest of diabetic neuropathy.

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قسم الكيمياء

تقييم فعالية انزيم Na^+/K^+ ATPase في تعدد الاشكال المورثة للاعتلال العصبي لدى مرضى السكري في محافظة بابل.

اطروحة مقدمة إلى
كلية العلوم- جامعة بابل
وهي جزء من متطلبات نيل درجة دكتوراه فلسفة في العلوم/ كيمياء

من قبل
ايمان حميد حميدي ابراهيم

بكالوريوس علوم كيمياء (2006) جامعة بابل
ماجستيرعلوم كيمياء/كيمياء حياتية (2012) جامعة بابل

بإشراف

أ.د. عوده مزعل ياسر

أ.م.د. مازن جعفرموسى

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