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

فعالية مضخة Na-K ATPase عند النساء المصابات بما قبل الارتعاج اثناء الولادة

أطروحة

مقدمة الى مجلس كلية العلوم/ جامعة بابل

كجزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم/ الكيمياء

تقدمت بها

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بكلوريوس علوم - كيمياء/ جامعة بابل 2012

ماجستير علوم - كيمياء حياتية/ جامعة بابل 2016

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2022 م

الخلاصة

مرض ماقبل الارتعاج هي اضطراب خاص بالحمل البشري يتميز بظهور ارتفاع ضغط الدم المرتبط بالبروتين والذي يظهر بعد الأسبوع العشرين من الحمل لدى امرأة كانت ذات ضغط طبيعي سابقًا ويختفي تمامًا بحلول الأسبوع السادس بعد الولادة. حيث يعتبر السبب الرئيسي للاعتلال والوفاة أثناء الحمل، إذ إنه مرض متعدد الأنظمة ولكن أسبابه غير معروفة جيداً. ترتبط مقدمات الارتعاج بتشوهات الانظمة، بما في ذلك اضطرابات النقل الأيوني في خطوط الخلايا الوليدية والأمومية والمشيمة.

الهدف الرئيسي من هذا البحث هو دراسة Na-K ATPase من المعلمات البيوكيميائية ، ثم الدراسة الجينية لـ Na-K ATPase عن طريق اختيار نوعين من الأشكال الإسوية لجين ألفا وتقدير تعبير Na-K ATPase المشيمي في مرضى مقدمات الارتعاج مقارنة مع حالات الحمل الطبيعية عن طريق الكيمياء المناعية ، وكذلك المعلمات النسيجية الأخرى.

كانت هذه الدراسة عبارة عن دراسة حالة-ضابطة شملت 130 امرأة مشاركة تتراوح أعمارهن بين 16-40 عامًا. وكان الدم وأنسجة المشيمة من بين العينات المستخدمة. وقسمت عينات الدم إلى ثلاث مجموعات: السيطرة السلبية (30)، والمراقبة الإيجابية (55)، ومجموعة المرضى (45). بينما قسمت عينات المشيمة إلى مجموعتين: المجموعة الضابطة (50) والمريضة (40).

اشتملت الدراسة الحالية على ثلاثة أجزاء: الجزء الأول دراسة الكيمياء الحيوية لعينات الدم. تم جمع عينات الدم في أنبوب EDTA كمضاد للتخثر وطردها لعزل خلايا الدم الحمراء عن البلازما. تم استخدام طريقة معدلة لتحديد النشاط النوعي لـ Na-K ATPase في أغشية خلايا الدم الحمراء. بينما تم استخدام طريقة جديدة لتحديد نسبة المتغيرات باطنية النمو في بلازما الدم.

تضمن الجزء الثاني الدراسة الجينية لإنزيم Na-K ATPase المشيمي عن طريق اختيار جينين ألفا 1 و الفا 2 (*ATP1A1*، rs10924081) ، و (*ATP1A2*, rs373796693). تم اختيار المتغيرات الجينية التي سيتم دراستها في هذا المشروع بعناية وفقًا للنتائج الحديثة في هذا المجال ، وباستخدام العديد من قواعد البيانات المتخصصة. تم تصميم التنميط الجيني وتحسينه لجين ألفا 1 بواسطة طريقة تعدد أشكال طول جزء تقييد تفاعل البوليميراز (PCR-RFLP). بينما تم تصميم التنميط الجيني للجين Alpha 2 عن طريق تفاعل البلمرة المتسلسل - طريقة تعدد أشكال التشكل الفردي (PCR-SSCP) ثم التسلسل.

تضمن الجزء الثالث من الدراسة الحالية الفحص النسيجي لنسيج المشيمة مع التعبير الكيميائي المناعي لـ Na-K ATPase في جميع العينات.

أظهرت نتائج الاختبار الكيميائي الحيوي أن متوسطً النشاط النوعي لـ Na-K ATPase في مرضى مقدمات الارتعاج كان أقل بكثير مقارنة بمجموعة التحكم. بينما أظهرت نتائج نسبة التثبيط للمتغيرات باطنية النمو زيادة بشكل ملحوظ في مرضى مقدمات الارتعاج مقارنة بمجموعة الأصحاء. كما وجدت هذه الدراسة ارتباط عكسي بين نشاط الإنزيم النوعي ونسبة تثبيط المتغيرات باطنية النمو.

وفيما يتعلق بنتائج الاختبار الجيني أظهرت عدم وجود ارتباط أليلي أو وراثي معنوي مسجل بين مجموعة المرضى ومجموعة الأصحاء للجينين اللذين تمت دراستهما. بينما لاحظت بيانات التسلسل للجين (*ATP1A2*, rs373796693) وجود طفرة حذف TCCT في العينات التي تم فحصها.

وفيما يتعلق بنتائج القياس النسيجي لأنسجة المشيمة للحمل الطبيعي مقارنة مع مقدمات الارتعاج أظهرت أن العقد المخلوية والتليف اللحمي والشعيرات الدموية في الزغابات الطرفية أعلى بشكل ملحوظ في مرضى مقدمات الارتعاج مقارنة مع مجموعة الأصحاء.

بينما لم تظهر نتائج النخر الليفي واحتشاء المشيمة والتكلسات أي دلالة في الدراسة الحالية بين مرضى مقدمات الارتعاج مقارنة مع مجموعة الاصحاء.

أخيرًا، أظهرت نتائج التحليل النسيجي المناعي لأنزيم Na-K ATP المشيمي لمجموعة الاصحاء ومرضى مقدمات الارتعاج أعلى نسبة مئوية بشكل ملحوظ للأرومة الغاذية الخلوية عند <50٪ و> 75٪ في مرضى مقدمات الارتعاج مقارنة بمجموعة الاصحاء. كذلك، أظهرت النتائج وجود عقدة مخلوية أعلى بشكل ملحوظ عند> 50٪ في مرضى مقدمات الارتعاج مقارنة مع مجموعة الاصحاء. وكذلك أظهرت النتائج أن الخلايا الساقطية تظهر بشكل ملحوظ بنسبة 30-50٪ في مرضى مقدمات الارتعاج مقارنة بمجموعة الاصحاء.

بينما لم تظهر نتائج الخلايا البطانية الشريانية الحلزونية والخلايا البطانية الشريانية الزغبية أي أهمية في الدراسة الحالية بين مرضى مقدمات الارتعاج مقارنة مع مجموعة الاصحاء.

تشير نتائج هذه الدراسة إلى ما يلي: ترتبط مقدمات الارتعاج بانخفاض ملحوظ في نشاط مضخة الصوديوم في غشاء كريات الدم الحمراء مقارنة بالحمل الطبيعي.

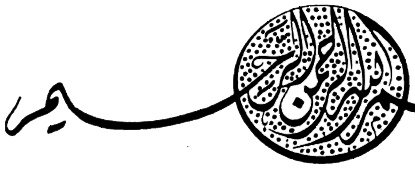
أظهرت نتائج نسبة تثبيط المتغيرات باطنية النمو ارتفاعًا في المرضى الذين يعانون من ماقبل الارتعاج مقارنة بالحمل الطبيعي، وهذا يرتبط جيدًا بانخفاض نشاط الإنزيم في هذه المجموعة.

لم يكن هناك تغيير معنوي في تعدد الأشكال لكل من الجينات المدروسة (*ATP1A1*، rs10924081 ، و *ATP1A2*، rs373796693) للمرضى ومجموعات المراقبة.

اما نتائج بيانات التسلسل للجين (*ATP1A2* , rs373796693) لاحظت أن هناك طفرة حذف TCCT في العينات التي تم فحصها.

أظهرت التغيرات في قياس انسجة المشيمة اختلافًا معنويًا للمعلمات التالية: العقد المخلوية ، والتليف اللحمي ، وعدد الشعيرات الدموية في الزغابات الطرفية ، بينما لم يكن هناك فرق كبير للمعلمات التالية: تكلس المشيمة ، نخر الفيبرينويد واحتشاء المشيمة. قد تُعزى هذه التغييرات إلى النضج المبكر لنسيج المشيمة في مرضى مقدمات التشنج.

اخيرا, أظهر التعبير المناعي النسيجي ل Na-K ATPase المشيمي اختلافًا كبيرًا في التعبير عن الأرومة الغاذية الخلوية في أنسجة المشيمة السابقة للتشنج، بينما لم تظهر المكونات الخلوية الأخرى (العقد المخلوية والخلايا البطانية الشريانية الحلزونية والخلايا البطانية الشريانية الزغبية) أي فرق يذكر.



﴿وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا﴾

صدق الله العظيم

سورة الأسراء (الآية ٨٥)

Acknowledgment

As I reach the end of my work, I would like to thank GOD (Allah) for the virtues of this blessing for implanting the soul of endurance and faith in myself to complete this study.

My heartfelt thanks and appreciation go to my supervisor, **Prof. Dr. Oda Mizil Yasser** for his scientific and valuable guidance, moral support, and encouragement during the study.

My special thanks and great gratitude to my supervisor, **Assist. Prof. Dr. Mazin Jaafar Mousa** for his experience, moral support, extension encouragement, and helped me throughout my work.

I would like to thank the Dean of the College of Science, the University of Babylon, and the head and staff members of the Department of Chemistry.

Also, I'd like to thank all mothers who participated in this study and the staff of both maternity and children hospital and Hilla teaching hospital, especially the staff of the hematology laboratory and operating theater.

I would like to express my immense gratitude to all Professors, **Prof. Dr. Mahmoud Hussain Hadwan**, **Prof. Dr. Hussein Oleiwi Al-Dahmoshi**, **Assist. Prof. Dr. Hayder Abdul Amir Makki** and **Lecturer. Dr. Hayder Obayes Hashim** for the unlimited help and providing the facilities to carry out this work.

Heartfelt thanks go to **Ms. Leqaa Abd Al-Khudar** for her support during the work.

Acknowledgment

I cannot find any words to express my sincere appreciation and gratitude to my family, especially my mother for her endless support, encouragement, and surrounded me with care. Special gratitude with love to my family for the kind support.

Thanks also go to my friends, who have encouraged and supported me through the times of study, celebrated with me through the good, and who was brilliant and understanding when I needed them to be; I take this opportunity to thank you, especially **Dr. Eman Jassim Mohamed**.

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Dedication

To

My angel in life

Who her prayer the secret of my success and her tenderness with a surgical balm

My Precious Mother

To

Whom my name is crowned by his name

Whom he granted me his kindness and righteousness

My Dear Father

To

Who are closer to my soul!

Whom they taught me that a life without love and cooperation is not worth anything

My Dear Brother and Sisters

To All those from whom I have received advice and support

I dedicate the harvest of my humble effort

List of Abbreviations

Abbreviation	Description
ADH	Antidiuretic Hormone
AFR	Africans
ALL	All Phase
AMR	Americans
APAs	Aldosterone-Producing Adenomas
BLAST	Basic Local Alignment Search Tool
BP	Blood Pressure
bp	Base Pair
CTS	Cardiotonic Steroids
DAB	3,3′-Diaminobenzidine
DPX	Dibutylphthalate Polystyrene Xylene
EAS	East Asians
EDLF	Endogenous Digitalis Like-Factor
EDTA	Ethylene Diamine Tetra Acetic Acid
EO	Endogenous Ouabain
ESRD	End-Stage Renal Disease
EUR	Europeans
GC	Gas Chromatograph
HELLP	Hemolysis, Elevated Liver Enzymes and Low Platelets
HRP	Horseradish Peroxidase
IHC	Immunohistochemically
IL	Interleukins
IUGR	Intrauterine Growth Retardation
MBG	Marinobufagenin
MDCE-5C	Digital Camera Scopelmage 9.0

Na_2-EDTA	Na_2- Ethylene Diamine Tetra Acetic Acid
NCBI	National Center for Biotechnology Information
NK	Natural Killer Cells
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction –Restriction Fragment Length Polymorphism
PCR-SSCP	Polymerase Chain Reaction-Single Strand Conformation Polymorphism
PE	Pre-Eclampsia
Pi	Inorganic Phosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PlGF	Placental Growth Factor
PTKs	Protein Tyrosine Kinases
r	Correlation Coefficient
RBCs	Red Blood Cells
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error for Mean
sFLT-1	Soluble FMS-Like Tyrosine Kinase 1
SNPs	Single Nucleotide Polymorphisms
SP	Sodium Pump
SSCP	Single-Strand Conformational Polymorphism
TE	Tris-EDTA Buffer
TEMED	Tetramethyl Ethylenediamine
TNF-α	Tumor Necrosis Factor- α
VEGFR-1	Vascular endothelial growth factor receptor 1
β-subunit	The Beta Subunit

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Certification of the Examination Committee

We are the examiner committee, certify that we have read the thesis entitled "**Na-K ATPase Activity in Preeclamptic Women in Labour**" presented by the student "**Hawraa Saad Hammood Jawad**" and examined her in its contents and in our opinion, it is accepted as a thesis for the degree of Doctor of Philosophy in Science- Chemistry/ Biochemistry with **Excellent** degree.

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CHAPTER ONE

INTRODUCTION

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1: Introduction

1-1: General Introduction

Every year, more than 200 million women become pregnant. Most pregnancies end with a live baby being born to a healthy mother. Unfortunately, for others, childbirth is not a joyous event but a time of pain, fear, suffering, and even death (WHO, 1998; Agunwa *et al*., 2017).

Risks of birth are deadly for half a million women every year. The causes of these deaths are essentially the same throughout the world. The common causes of maternal death are hemorrhage, unsafe abortion, obstructed labor, hypertensive disorders, and sepsis. It is estimated that 65,000 (12%) are due to hypertensive pregnancy disorders (Sitaula *et al*., 2021).

For many years, it was thought a toxin released from a pregnant woman's uterus caused the disorder, which was dubbed toxemia of pregnancy. All attempts to determine which toxin is producing have failed. As a result, the term "pregnancy toxemia" is no longer used because it is inaccurate. Therefore, the term preeclamptic is used to represent hypertensive disorders in pregnancy and may also refer to proteinuria (Hacker *et al*., 2010; Kenny and Mysers, 2017).

Preeclamptic is the most popular disorder that affects first pregnancies. The clinical manifestations are highly variable but hypertension and proteinuria are usually seen (Burton *et al*., 2019). Preeclamptic is a multisystem illness, but its aetiology is poorly understood (Staff, 2019). Preeclamptic has linked to a variety of system abnormalities, including ion transport disorders in neonatal, maternal, and placental cell lines, such as elevated sodium and calcium, which are often described in cases of essential hypertension (Adair *et al*., 2009).

A common hypothesis to explain the abnormalities in preeclamptic has been existing placenta-derived endogenous digitalis-like factors in plasma. These factors

are like cardiotonic steroids, inhibit the sodium-potassium adenosine triphosphatase enzyme (Na-K ATPase) transport complex, which functions as the sodium pump (SP). Inhibition of this enzyme has various effects that could lead to an increase in vascular resistance and causes some of the manifestations of preeclamptic (Adair *et al*., 2009; Ding, 2018).

The Na-K ATPase is an essential membrane protein that exists in most higher eukaryotes and the plasma membrane of cells (Dash *et al*., 2018). At comfort, it consumes 20-30% of ATP production to transport Na^+ out of and K^+ into the cell. Na-K ATPase is the largest protein complex in the family of P-type cation pumps. The minimum functional unit is a heterodimer of the α- and β-subunits and may also express with a small ion transport organizer of the FXYD family (Jorgensen *et al*., 2003; Clausen *et al*., 2017).

The sodium pump is distinguished by a complex molecular heterogeneity caused by the expression and different association of numerous isoforms of both its α- and β-subunits (Bejček *et al*., 2021). Individual genes of four α (α1, α2, α3, and α4) subunit isoforms and at least three β (β1, β2, and β3) subunit isoforms of Na-K ATPase have been identified in mammalian cells (Vasić *et al*., 2008).

Genetic polymorphism of Na-K ATPase has been widely studied in different diseases because ionic gradients are critical for any organ, disruption of Na-K ATPase activity has been implicated in numerous pathophysiological conditions such as hypertension, cancer (Durlacher *et al*., 2015), diabetes (Vague *et al*., 2004), and heart failure (Schwinger *et al*., 2003). The Na-K pump has been suggested as a possible chemotherapy target though the evidence is still lacking for its activity (Mijatovic *et al*., 2012). Until this date, there is no available data linking gene polymorphism of Na-K ATPase with preeclamptic in both maternal and fetal tissues.

In addition, careful analysis of genetic variation has important implications in many areas of biomedical research, including the identification of infectious agents, the diagnosis of infections, and the discovery of disease-causing mutations that are unknown or recognize (Gasser *et al*., 2007).

PCR–restriction fragment length polymorphism (PCR-RFLP) and Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) are two independent methods employed after amplification genotyping of DNA variations. Both techniques are applied in wide areas of checking applications to define single nucleotide polymorphisms (SNPs) (Hashim and Al-Shuhaib, 2019).

The PCR-SSCP identifies a possibly causal unknown SNP that could not be found by the PCR-RFLP. However, because no difficult processes are necessary to execute PCR-RFLP, it is widely employed in a variety of applications. On the other hand, PCR-RFLP is easier to process in terms of time and handover experience, the detection of a particular unknown SNP by PCR-SSCP has further chances (Konstantinos *et al*., 2008).

Finally, immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) using antigen-antibody interactions, with the position of antibody binding determined by direct antibody labeling or secondary labeling (De Matos *et al*., 2010). Although histological analysis of haematoxylin and eosin stained tissue sections remains the foundation of head and neck surgical pathology, immunohistochemistry has emerged as a valuable tool in the pathologist's armamentarium (Kabiraj *et al*., 2015).

1-2: Hypertensive Disorder During Pregnancy

Hypertensive disorders affect pregnancies and have been classified into four types, reflecting potential variation in etiology and pregnancy outcome (Brown *et al*., 2018).

1-2-1: Types of Hypertensive Disorder During Pregnancy

1- Chronic hypertension.

2- Gestational hypertension.

3- Preeclamptic.

4- Preeclamptic superimposed on chronic Hypertension.

1. Chronic Hypertension

Chronic hypertension is diagnosed when hypertension is verified before pregnancy or before 20 weeks gestation (blood pressure >140 mmHg systolic or >90 mmHg diastolic) or when post-partum high blood pressure does not improve (Braunthal and Brateanu, 2019). Women with chronic hypertension must be closely monitored throughout pregnancy since they are at a higher risk of adverse outcomes such as superimposed pre-eclampsia, placental abruption, fetal growth restriction, early delivery, and stillbirth (Donovan, 2012).

2. Gestational Hypertension

Gestational hypertension is defined when blood pressure (BP) ≥140/90 mm Hg on ≥2 occasions at least six hours apart, after 20 weeks gestation, in the absence of proteinuria, <300 mg in a 24-hour urine collection. Blood pressure resumption to normal by 6 weeks postpartum (Poon *et al*., 2021).

3. Preeclamptic (PE)

A pregnancy-specific disorder that is a multisystem illness emergence by hypertension ≥140/90 mm Hg on ≥2 occasions at least 6 hours apart, and

proteinuria ≥300 mg in a 24-hour urine collection, after 20 weeks gestation. The convulsive form of preeclamptic is eclampsia affects 0.1% of all pregnancies (Brown *et al*., 2018).

4. Preeclamptic Superimposed on Chronic Hypertension

Women with chronic hypertension may then develop pre-eclampsia. This is diagnosed when there is a new onset of proteinuria, sudden worsening of either hypertension or proteinuria, or development of other signs and symptoms of PE after 20 weeks of gestation (Braunthal and Brateanu, 2019).

1-3: Preeclamptic (PE)

Preeclamptic (PE) is a disorder of human pregnancy diagnosed by proteinuria more than 300 mg/day and hypertension (Bartal *et al*., 2020). It is a serious disorder that may lead to mother and fetal morbidity and death. The condition starts after 20 weeks of pregnancy (Seely and Ecker, 2014).

The incidence of preeclamptic is 2% to 10% of pregnancies depending on the definition used and population studied (Hung and Burton, 2006). Preeclamptic is classified as mild or severe based on the severity of hypertension, the amount of proteinuria, and the extent to which other organ systems are impacted (Hacker *et al*., 2015; Fox *et al*., 2019).

In severe, illness there may be haemolytic anemia, thrombocytopenia, hepatocellular dysfunction, peripheral edema, diplopia, or respiratory distress due to pulmonary edema. It generally develops during the third trimester (Akter and Khanum, 2021).

Recommendations for the prevention of PE have included: low-dose of aspirin during pregnancy which is safe, calcium supplementation with a low dosage in regions with poor consumption, and treating previous hypertension with medicines (Souza *et al*., 2014; Wertaschnigg *et al*., 2019).

1-3-1: Diagnostic Signs of Preeclamptic

Although PE is considered a syndrome and a multisystem disorder, but women with PE are usually asymptomatic when the disease is first manifested. Hypertension and proteinuria are the two signs most easily detected (Kallela *et al*., 2016). So, preeclamptic is diagnosed as following

1. **Hypertension**

   In pre-eclampsia, hypertension is defined by an absolute blood pressure elevation of 140/90 mmHg or a relative increase in systolic or diastolic blood pressure of 30 mmHg or 15 mmHg. Typically, the rise in blood pressure is abrupt and begins during the third trimester (Kattah and Garovic, 2013).

2. **Proteinuria**

   Proteinuria is defined as a urine dipstick reading of 1+ or more than 300 mg in 24 hours. The development of proteinuria is the distinguishing feature between preeclamptic and gestational hypertension (Stefańska *et al*., 2020).

3. **Edema**

   is usually generalized involving the face and hands, but this sign is non-specific and observed in many normotensive women (Roberts *et al*., 2003).

1-3-2: Risk Factors of Preeclamptic

Risk factors associated with preeclampticare include:

- **First Pregnancies**

  Preeclamptic is far more likely during the first pregnancy than in subsequent ones (Brouwers *et al*., 2018).

- **Multiple Pregnancies**

  The risk is increased if a woman is carrying two or more fetal (Hernández-Díaz *et al*., 2009).

- **Pregnancy Gap**

  If the second pregnancy happens at least ten years after the first, the risk of preeclamptic is derated (Cormick *et al*., 2016).

- **New Paternity**

  Second pregnancies have a decreased risk of preeclamptic than first pregnancies (Seeho *et al*., 2016), but not if the mother has a new partner for the second pregnancy. One explanation for this is that repeated maternal exposure and adaptation to specific antigens from the same partner reduces the risk (Skjærven *et al*., 2002).

- **Family History**

  A woman whose mother or sister had preeclamptic is more likely to develop it (Bezerra *et al*., 2010).

- **Maternal Age**

  Women over 40 and teenagers >20 are more likely to evolve preeclamptic compared with women of other ages (Londero *et al*., 2019; Bouzaglou *et al*., 2020).

- **Certain Conditions and Illnesses**

  Women with diabetes, high blood pressure, migraines, and kidney disease are more likely to develop preeclamptic (Weissgerber and Mudd, 2015).

- **Obesity**

  Obesewomen are more likelyto develop preeclamptic (Mrema *et al*., 2018).

1-3-3: Aetiology and Pathogenesis of Preeclamptic

Although the exact cause of preeclamptic is unclear, except that the pathogenesis is thought to occur in two stages as shown in Figure 1-1. The first stage starts in the placenta is a pre-clinical condition characterized by abnormal

trophoblastic vascular of uterine arteries, resulting in placental hypoxia. While the second stage is characterized by an abnormal maternal endothelial response, resulting in hypertension, proteinuria, and edema (Borzychowski *et al*., 2006; Shanmugalingam *et al*., 2018).

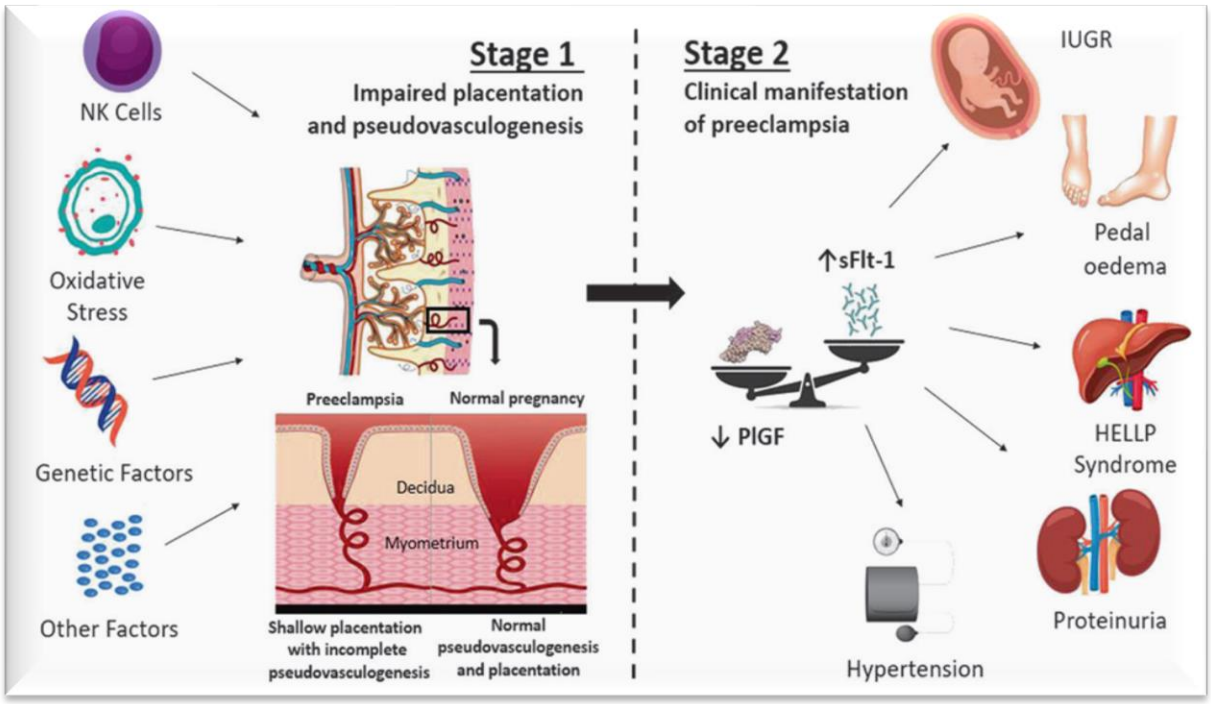


Figure 1-1: Elucidation of Two-Stage Pathophysiology of Preeclamptic (Shanmugalingam *et al*., 2018)

Whereas:

HELLP: Hemolysis, Elevated Liver Enzymes and Low Platelets, **IUGR:** Intrauterine Growth Retardation, **NK:** Natural Killer Cells, **PlGF:** Placental Growth Factor, **sFlt-1**: Soluble Fms-Like Tyrosine Kinase-1

So that, factors that have been likely to play a role in the etiology of preeclamptic are included:

1-3-3-1: Abnormal Trophoblastic Invasion

The extravillous trophoblast proliferates from an anchoring villous during normal placental implantation, as shown in Figure 1-2. In the inner part of the myometrium, the cytotrophoblast invades the uterine spiral arteries. Invasion of the spiral arteries is associated with tunica media degeneration and fibrinoid material

replacement, resulting in loss of resistance and marked dilatation of the spiral artery, as well as increased intervillous blood flow (Carter *et al*., 2015).

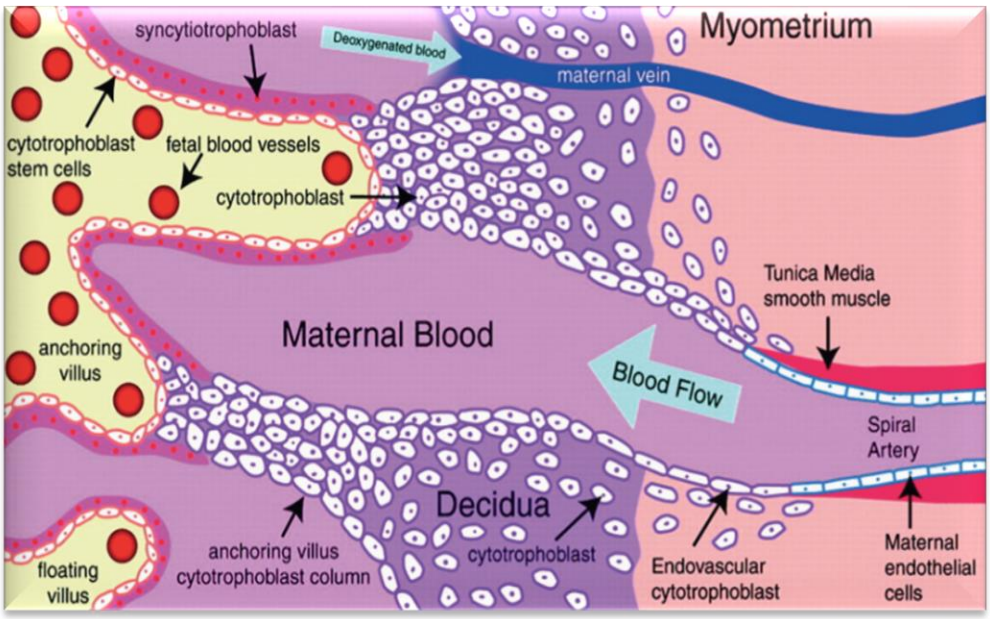


Figure 1-2: Normal Placental Implantation (Karumanchi *et al*., 2005)

In pre-eclamptic patients, the placenta suffers from defective implantation, as shown in Figure 1-3.

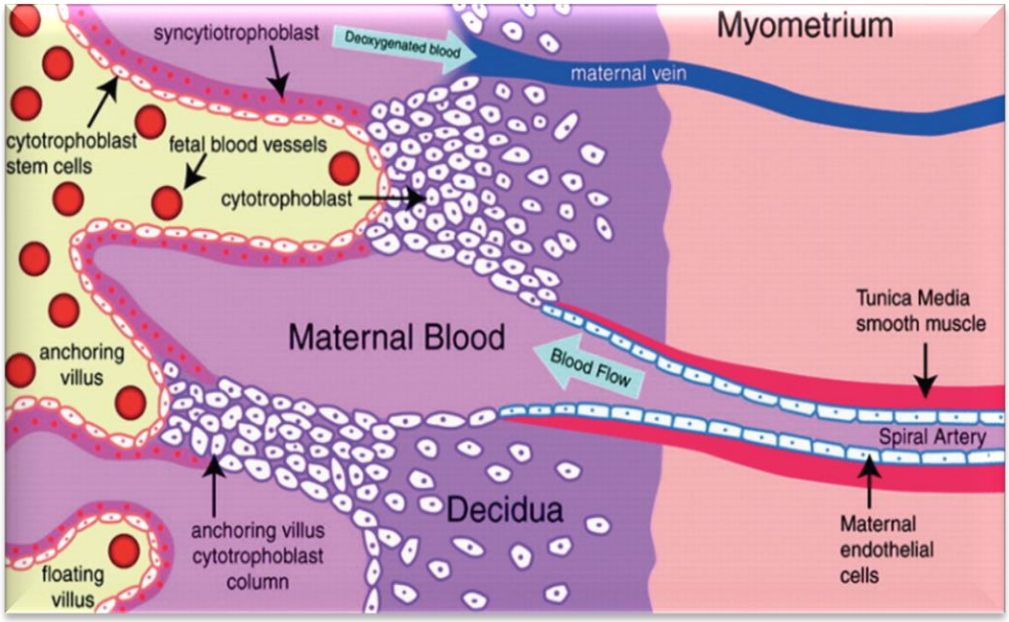


Figure 1-3: Placenta Implantation in Preeclamptic (Karumanchi *et al*., 2005)

The disease process is composed of two phases. The first phase is characterized by patchy trophoblast invasion so that the spiral arteries retain their muscular walls

which will prevent the development of high-flow, low- impedance uteroplacental circulation (Roberts and Escudero, 2012).

The pre-eclamptic placenta grows high resistance, resulting in reduced flow of blood and inefficient perfusion. These will result in placental ischemia and hypoxia. This most likely results in the formation of reactive oxygen species. When the body's normal endogenous antioxidants are depleted, oxidative stress occurs. This is most likely central to the clinical syndrome of preeclamptic (Testa *et al*., 1988).

Activation of the vascular endothelium all throughout the body will occur because of oxidative stress or other vasoactive substances produced by the placenta. All mother organs are impacted by general vascular endothelial dysfunction in the second stage of preeclamptic (Kenny and Myers, 2017).

1-3-3-2: Inflammatory Factors

Endothelial cell dysfunction may be caused by an overly activated state of leukocytes in the maternal circulation, according to one theory (Faas *et al*., 2000).

Briefly, cytokines like tumor necrosis factor- α (TNF-α) and interleukins (IL) may contribute to the oxidative stress associated with PE (Kalantar *et al*., 2013). This is distinguished by reactive oxygen species and free radicals, which result in the formation of self-propagating lipid peroxides (Shahidi and Zhong, 2010).

These, in turn, produce highly toxic radicals that cause endothelial cell injury, alter nitric oxide production, and disrupt prostaglandin balance. Other effects of oxidative stress include the production of lipid-laden macrophage foam cells, which is seen in atherosclerosis; activation of macrovascular coagulation, which is expressed by thrombocytopenia; and increased capillary permeability, which is manifested by edema and proteinuria (Manten *et al*., 2005; El-Sayed, 2017).

1-3-3-3: Immunological Changes

There is maternal immune tolerance to parentally derived placental and fetal antigens. PE syndrome is caused by a loss of tolerance or a disruption in its regulation (Kenny and Kell, 2018). There are numerous inferential data that point to an immune-mediated disorder. For example, the risk of PE is significantly increased when the formation of blocking antibodies to placental antigenic sites is hampered. In this case, the first pregnancy would be riskier (Labarrere, 1988).

Some women have elevated serum levels of antiangiogenic factors. One of these factors, soluble FMS-like tyrosine kinase 1 (sFLT-1) (Bdolah *et al*., 2006). sFLT-1 is an antiangiogenic factor expressed as an alternatively spliced VEGFR-1 variant that lacks both the transmembrane and cytoplasmic domains. sFlt-1 linked to VEGF and PlGF, inhibiting angiogenesis via VEGFR (Maynard *et al*., 2003).

1-3-3-4: Genetic Factors

For daughters of preeclamptic mothers, the risk of PE is 20% to 40%; for sisters of preeclamptic women, the risk is 11 to 37%; and for twin pregnancies, the risk is 22 to 47 percent (Nilsson *et al*., 2004).

1-3-3-5: Nutritional Factors

In general, a diet high in antioxidant-rich fruits and vegetables is associated with lower blood pressure (Aune, 2019). The incidence of PE was most doubled in women whose daily ascorbic acid consumption was less than 85 mg (Zhang *et al*., 2002).

1-4: ATPase Enzyme

ATPases (AAA^+) linked with different cellular activities are found in all known organisms and play a role in most significant cellular functions (Shorter and Houry, 2018). AAA^+ proteins can exist in most subcellular compartments of eukaryotic cells, as well as in archaea, bacteria, and viruses (Snider *et al*., 2008).

These AAA^+ proteins typically assemble into hexameric ring complexes that are required in energy-dependent macromolecule remodeling (Iyer *et al*., 2004). AAA^+ superfamily members have a highly conserved ATPase module of 200-250 amino acids that includes a core domain. Adenosine triphosphates (ATPase) are enzymes that degrade ATP's gamma phosphate to produce inorganic phosphate (Pi) and ADP (Bartolommei *et al*., 2013). This breakdown and liberation Pi generates energy, which enzymes using to perform chemical reactions that consume energy. This process is necessary to all of life's kingdoms (Kaundal *et al*., 2020).

AAA^+ proteins are involved in a wide variety of different functions ATPase acts as molecular motors that employ ATP hydrolysis energy to power different reactions such as protein trafficking and assembly, pumping of ions, cellular metabolism, muscle movement, motility of cells, and replication and transcription (Baker and Sauer, 2012; Rule *et al*., 2016). Some ATPases are transmembrane proteins that transport solutes across membranes, while others are cytoplasmic and may be correlated with a biological membrane such as the plasma membrane or that of organelles (Hanson and Whiteheart, 2005).

1-4-1: Classification of ATPase Family

ATPases are classified according to their function (ATP synthesis and/or hydrolysis), or structure (F-, V-, and A-ATPases contain rotary motors), and the type of ions they transport (Neupane *et al*., 2019). All rotary ATPases have two motors, R1 and RO, that are joined with one another, with one motor capable of driving the other (Colina-Tenorio *et al*., 2018). This is performed by joining each motor to central and peripheral stalks. The soluble R1 motors have three nucleotide-binding positions for ATP turnover, whereas the RO motors are membrane bound and translocate protons or other cations (Stewart *et al*., 2014).

1. **F-ATPases (F1FO-ATPases)**

   F-type ATPases are membrane-anchored rotary enzymes that link H^+ or Na^+ ion translocation across the membrane to synthesis or hydrolysis of ATP (Dibrova *et al*., 2010). F-type ATPases are exist in bacteria, mitochondria, and chloroplasts (Mulkidjanian *et al*., 2009).

2. **V-ATPases (V1VO-ATPases)**

   Essentially exist in eukaryotic vacuoles, catalyzing ATP hydrolysis to transport solutes and minimize pH in organelles such as the proton pump of the lysosome (Dibrova *et al*., 2010).

3. **A-ATPases (A1AO-ATPases)**

   A-ATPases exist in archaea and some extremophilic bacteria. They are organized similar to V-ATPases, but function as F-ATPases essentially such as ATP synthases (Gloger *et al*., 2015).

4. **P-ATPases (E1-E2-ATPases)**

   P-ATPases found in bacteria, fungi, and in eukaryotic plasma membranes and organelles, and functions to transport a diversity of various ions across membranes (Bramkamp *et al*., 2007).

1-4-2: Types of ATPase in Red Blood Cell Membrane

Three types of ATPase are found in the membrane of red blood cells. The comparison of the three types are shown in Table 1-1 (Kherd *et al*., 2017).

Table 1-1: Comparison among ATPase Families in Red Blood Cell Membrane (Lodish and Zipursky, 2000)

Type	P- Family	F- Family	V- Family
Substances Transported	H^+, Na^+, K^+, Ca^{2+}	H^+ only	H^+ only
Location of Specific Pumps	Na-K Pump plasma membrane of higher eukaryotes	Bacterial plasma membranes	Vacuolar membranes in plants, yeast, other fungi
	H-K Pump plasma membrane of gastric chief cells	Inner mitochondrial membrane	Endosomal and lysosomal membrane in animal cells
	Ca^{2+} pump plasma membrane of	Thylakoid membrane of chloroplast	Plasma membrane of certain acid-secreting animal cells

	eukaryotic cells		(e.g, some kidney tubule cells)
Structural and Functional Characteristics	Large catalytic α-subunits become phosphorylated through solute transport. β-subunits arrange transport	Multiple transmembrane and cytosolic subunits regularly perform to synthesize ATP on β cytosolic subunits powered by the passage of H^+ down an electrochemical gradient.	Multiple transmembrane and cytosolic subunits use the energy generated by ATP hydrolysis on a regular basis to pump H^+ ions from the cytosol to organelle lumens, acidifying them.

1-4-3: P-ATPase Family

P-type ATPases are a wide family of enzymes that are required for all forms of life, from archaebacteria to the far more sophisticated higher eukaryotes (Pedersen *et al*., 2012). In general, P-type ATPases are integral membrane proteins present in a wide range of membrane types, including plasma and cellular organelle membranes, where they transport cations, heavy metal ions, and lipids, producing and sustaining important chemical potential gradients across these membranes (Kaplan, 2002; Hossain and Clarke, 2019).

Three ATPase can be found in different amounts in the erythrocyte membranes (Hossain and Clarke, 2019). The Na-K ATPase was the first member of the family to be discovered (Skou and Hoffman, 1998). It helps preserve the electrochemical potential gradients for Na^+ and K^+ across the plasma membrane of animal cells and produces the foundation for electrical excitation in neurons and muscle cells (Bers *et al*., 2003). In plants and fungi, an equally significant and similar role to the Na-K ATPase is played by the plasma membrane H^+-ATPase (Serrano *et al*., 1986).

Other significant members of the family include Ca^{2+}-ATPases of the sarcoplasmic reticulum, plasma membrane, and secretory pathway, where they play essential roles in muscle role and Ca^{2+} signaling and are evenly crucial for animal viability (Vandecaetsbeek *et al*., 2011). The same is true for the gastric H^+, K^+-ATPase, which is responsible for stomach acidification, and the heavy metal

ATPases, which are required for trace metal homeostasis and detoxification in both prokaryotes and eukaryotes (Bublitz *et al*., 2010).

In this study, focus on the Na-K ATPase and their key roles in the plasma membrane of eukaryotes. This special subclass of P-type ATPase has developed independently to energize the plasma membrane by actively transporting Na^+ or K^+(Morth *et al*., 2011).

1-5: Sodium-Potassium ATPase (Na-K ATPase)

The Na-K ATPase is an important membrane protein present in the plasma membrane and exists in most higher eukaryotes cell (Blanco and Mercer, 1998; Shenoy *et al*., 2019). Skou first discovered it in 1957 as a membrane-bound protein (Skou, 1957).

This enzyme is made up of three types of subunits: the catalytic α subunit, and the regulatory β subunit regulating conformational stability and activity of the α subunit. In association with the αβ heterodimer. A third small polypeptide, the γ subunit, has been found. It does not appear to be required for the Na-K ATPase to be functional and may play a regulatory role (Floyd *et al*., 2017; Sun *et al*., 2020).

All three subunits, transport 3 Na^+ ions out the cell and 2 K^+ ions into the cell and thus protect the transmembrane potential balanced as shown in Figure 1-4 (Gürel *et al*., 2004; Pivovarov *et al*., 2019).

Most live cells have a high intracellular K^+ concentration and a low intracellular Na^+ concentration. In contrast, the outside of the cell contains a high concentration of extracellular Na^+ and a low concentration of extracellular K^+ (Cheng *et al*., 2013). As a result, a concentration gradient for intracellular K^+ and Na^+ loss and gain were discovered. This gradient is controlled by the activity

of various ionic channels and transporters, most notably the Na-K ATPase (Suhail, 2010).

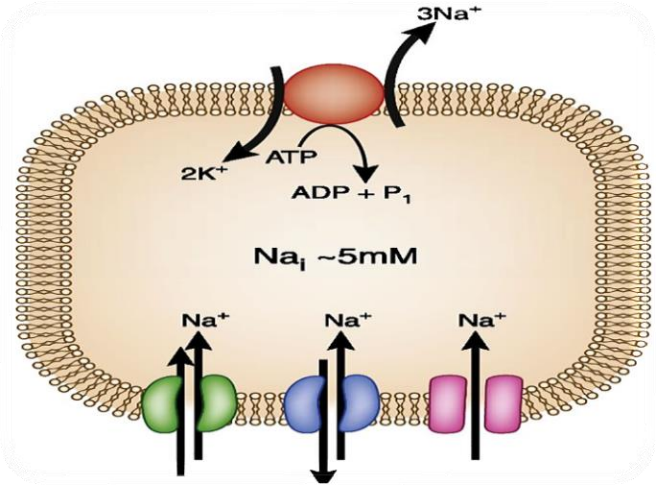


Figure 1-4: Movement of Sodium and Potassium Ions During One Cycle of the Na-K ATPase Pump

The enzyme can transport Na^+ and K^+ through the membrane in two ways. There is a pocket or door in the enzyme which is open to the extracellular fluid. Alternatively, the cytoplasm has an open pocket or door. The enzyme transports Na^+ and K^+ through the membrane by switching between the two states (Kumar and Clark, 2005; Omar *et al*., 2017).

1-5-1: Function of Sodium-Potassium ATPase

The basic function of the Na-K ATPase is to generate and maintain electrochemical sodium ion and potassium ion gradients across the cell membrane (Cassimeris *et al*., 2011).

The Na-K ATPase enzyme is responsible for maintaining the low intracellular Na^+ and high intracellular K^+ concentrations required for proper cellular functioning. This enzyme's activity happens in many steps and is dependent on ATP hydrolysis (Kaplan, 2002). After the connection of ATP to the enzyme, three Na^+ ions from the cytoplasm associate with the active site of Na-K ATPase. Phosphorylation of the Na-K ATPase (at aspartate residue) results in its conformational change. Because of this change, three bound Na^+ ions are released

out of the cell. Thereafter, two extracellular K^+ ions join along with the dephosphorylation method and are transported into the cell (Shinoda *et al*., 2009; Obradovic *et al*., 2013).

Na-K ATPase also serves as a specific target for ouabain, digitalis, and endogenous cardiac glycosides. They have an indirect influence on cardiac contractility (Bagrov *et al*., 2009). Cardiac glycosides inhibit its activity by connecting to the extracellular portion of Na-K ATPase and raise the concentration of Na^+ (Paula *et al*., 2005). These actions precede an increase in intracellular calcium ion concentration which in turn, increases heart contraction (Chakraborti and Dhalla, 2016).

1-5-2: Structure of Sodium-Potassium ATPase

Na-K ATPase is made up of three subunits: α, β and γ, with each subunit having a number of isoforms that enable functional adaptability across different cell types, illustrating the various roles and responses provided by Na-K ATPase activation across cell types (Kinoshita *et al*., 2014; Cui and Xie, 2017). However, the (gamma) subunit is not found in all cells, and the other subunits are necessary for Na-K ATPase to function (Nepal *et al*., 2021).

1-5-2-1: The Alpha Subunit

The α-subunit is the enzyme's catalytic subunit. It possesses ten transmembrane helices and a molecular weight of about 110 kDa (Lobato-álvarez *et al*., 2016). Both the C- and N-termini exist on the cytoplasmic side. The α-subunit is tightly linked with the β-subunit in a 1:1 stoichiometry (Zacherl, 2014).

In humans, four distinct isoforms (α1, α2, α3, and α4) have been found in cells, each of which is expressed in a tissue-specific and cell-specific way (Schwinger *et al*., 2003).

1. The Alpha 1 (α1) Isoform

The α1 has been proven to be expressed in all tissues studied thus far (Shamraj and Lingrel, 1994; Li and Langhans, 2015). The α1 subunit is encoded by the gene *ATP1A1*. The *ATP1A1* promoter has a high GC content, which is a feature shared by all housekeeping genes. It contains a possible TATA box (Shull *et al*.,1990). The nucleotide sequence from about 100 bp to the transcription start position is significantly preserved between the human and rat *ATP1A1* genes (Kobayashi *et al*., 1997).

ATP1A1 mutations are unlikely to be harmful, however somatic mutations in *ATP1A1* can lead to disturbed hormone balance in a subset of aldosterone-producing adenomas (APAs) in the adrenal gland (Beuschlein *et al*., 2013). Adrenal excess of aldosterone causes hypertension in up to 5% of hypertensive patients, and patients are often treated if adrenal adenomas are detected and removed (Azizan *et al*., 2013).

The usual signal route is that adrenal cells depolarize and open voltage-gated calcium channels in response to the peptide hormone angiotensin II and extracellular potassium, and the increase in cytoplasmic calcium levels increases aldosterone synthase production (Hattangady *et al*., 2012; Spät *et al*., 2016).

if the downstream signals are directly produced by mutations in the systems that typically govern membrane potential and calcium levels, including a potassium channel, the reliance on external stimuli can be avoided (Choi *et al*., 2011).

ATP1A1 mutations convert the Na-K ATPases into ion channels, allowing sodium or protons (depending on the mutation) to enter the cell. Potassium is not transferred, thus physiological potassium levels are insignificant (Azizan *et al*., 2013). The consequence of converting a pump into a channel is far more severe

than simply turning the pump off. Only locations where mutations cause the pump to become a cation channel have been documented in APAs, (Zennaro *et al*., 2015) but many of the disease-causing mutations in *ATP1A2* and *ATP1A3* seem to impact pump function (Rossini and Bigiani, 2011).

2. The Alpha 2 (α2) Isoform

The α2 isoform is found in skeletal muscle, adipocytes, brain, heart (Moseley *et al*., 2003; Wang *et al*., 2017), and placenta (Esplin *et al*., 2003). The α2-subunit is encoded by the gene *ATP1A2*. The promoter area of human *ATP1A2* contains a potential TATA box (Shull *et al*., 1989).

Mutations in *ATP1A2* have been reported to cause hemiplegic migraine and epilepsy in an autosomal dominant manner, occasionally co-occurring in families. It has also been linked to a unique type of migraine known as alternating hemiplegia of children (Swoboda *et al*., 2004; Castro *et al*., 2008).

The effects of *ATP1A2* mutations are thought to be caused by one of two mechanisms. The first hypothesis is that the mutations cause an increase in extracellular potassium, which can lead to impaired potassium ion clearance and therefore induce CSD (Gritz and Radcliffe, 2013). The second hypothesis is since the distribution of *ATP1A2* is co-localized with the Na^+, Ca^{2+} exchanger, the mutations to *ATP1A2* would cause intracellular sodium to increase, which increases intracellular calcium levels through the Na/Ca^{2+} exchanger, similar to FHM1, resulting in glutamate release and a decrease in glutamate clearance which can also lead to CSD (Gritz, 2015). Both hypotheses result in making the brain more susceptible to CSD and therefore migraines with aura.

3. The Alpha 3 (α3) Isoform

The α3 isoform is exist in nerves, brain, and heart tissues (Zahler *et al*., 1996; Henriksen *et al*., 2013). The α3-subunit is encoded by the gene *ATP1A3*. The

human *ATP1A3* promoter has large GC content, but no classical TATA box (Li and Langhans, 2015).

Autosomal dominant mutations in *ATP1A3* were beginning appeared to produce rapid-onset dystonia parkinsonism (Aguiar *et al*., 2004). Later, two more neurological disorders, alternating hemiplegia of childhood, were discovered (Heinzen *et al*., 2012), and CAPOS (cerebellar ataxia, areflexia, optic atrophy, and sensorineural hearing damage) (Demos *et al*., 2014).

Although the three syndromes were first classified as phenotypically distinct, it has since become obvious that many patients do not strictly fall into one group or the other, but may have symptoms that lie on a continuous continuum as well as symptoms that are specific to individual mutations (Paciorkowski *et al*., 2015).

4. The Alpha 4 (α4) Isoform

The α4 isoform has been existing in the testis (Blanco *et al*., 2000; Syeda *et al*., 2018), and present in human and mouse skeletal muscle. The α4 subunit is encoded by the gene *ATP1A4* (Keryanov and Gardner, 2002). The main role of α4 isoform is in the sperm motility (Woo *et al*., 2000; Lestar *et al*., 2018).

ATP1A4 deficiency reduces sperm motility, depolarizes the membrane potential, and increases intracellular sodium, whereas *ATP1A4* overexpression increases sperm motility (Jimenez *et al*., 2011).

At the biophysical level, when compared to the other isoforms in a cell free system, *ATP1A4* has a low potassium for ouabain and sodium and a regular for potassium (Clausen *et al*., 2017).

While, in a biological system, *ATP1A4* is less sensitive to changes in voltage, extracellular sodium, and temperature than *ATP1A4* (Clausen *et al*., 2016).

1-5-2-2: The Beta Subunit

The major role of the Na-K ATPase β-subunit is to act as a chaperone for the α-subunit and to coordinate its distribution to the plasma membrane (Geering, 2008; Kryvenko *et al*., 2021). The β-subunit has a molecular weight of about 34 kDa but due to glycosylation, it shows on SDS-PAGE as a diffuse band moving between 50-70 kDa (Zacherl, 2014).

The β-subunit of consists of one transmembrane segment and a rather small N-terminal cytoplasmic domain in comparison to its C-terminal extracellular glycosylated region (Hasler *et al*., 1998; Donnet *et al*., 2001). The β subunit, three different isoforms have been identified: β1, β2 and β3. All isoforms correlate promiscuously to produce a functional pump (Blanco and Mercer, 1998; Rotoli *et al*., 2017).

1. The Beta 1 (β1) Isoform

The β1 has a generalized expression in almost all tissues and cells (Derfoul *et al*., 1998). The β1-subunit is encoded by the gene *ATP1B1* (Shi *et al*., 2016). Beta 1 may respond to oxidative stress by glutathionylation a cysteine in the center of its transmembrane helix, which is not seen in the other betas types (Rasmussen *et al*., 2010). In vitro study have revealed that the E1 state of the enzyme promotes glutathionylation of cysteine more than the E2 state (Garcia *et al*., 2015).

2. The Beta 2 (β2) Isoform

The β2 isoform is exist in skeletal muscle (Lavoie *et al*., 1997) pineal gland (Shyjan *et al*., 1990), and nervous tissues (Peng *et al*., 1997). The β2-subunit is encoded by the gene *ATP1B2* (Wang *et al*., 2011). Functionally, beta 2 has the most powerful effects on the kinetic characteristics of the pump, decreasing the

apparent potassium affinity and increasing the extracellular sodium affinity compared to beta1 and 3 (Larsen *et al*., 2014).

3. The Beta 3 (β3) Isoform

The β3-isoform is found in many tissues, including the kidney, lung, stomach, colon, spleen, and liver (Langhans *et a*l., 2016). The β3-subunit is encoded by the gene *ATP1B3*. The human *ATP1B3* promoter includes a TATA-like sequence (Malik *et al*., 1998). *ATP1B3* is found on chromosome 3 in the q22-23 region. Na-K ATPase is involved in normal cellular activities, but it also plays an important role in carcinogenesis (Lu *et al*., 2016). The expression of the β3 subunit of Na-K ATPase was higher in human gastric cancer tissues than in normal matched tissues (Li *et al*., 2017).

1-5-2-3: The Gamma Subunit

FXYD proteins are a family of tiny regulatory proteins. FXYD proteins feature a single trans-membrane segment, as well as an extracellular N terminus and cytoplasmic C terminus (Yap *et al*., 2021).

FXYD protein is a hydrophobic polypeptide with a molecular weight ranging from 8 to 14 kDa. This family has seven members and is expressed in different tissues (Chakraborti and Dhalla, 2016).

The FXYD proteins are also found in different tissues. FXYD1 (phospholemman) is expressed in the heart and skeletal muscle; FXYD2 (γ-subunit) is expressed in the kidney; FXYD3 (MAT-8) in the stomach and colon; FXYD4 (CHIF) in the kidney and colon; FXYD5 (dysadherin) in the intestine, lung, and kidney; and FXYD6 and FXYD7 in the brain (Béguin *et al*., 2001; Geering, 2006).

FXYD2 considers γ-subunit of Na-K-ATPase and FXYD2 gene may be found on chromosome 11q23 (Sweadner *et al*., 2000; Zhou *et al*., 2021). It encodes two

splice variants, FXYD2a and FXYD2b, which were identified using mass spectrometry and vary solely in their most NH_2-terminal amino acids (Küster *et al*., 2000). FXYD2 is found mostly in the kidney (Mayan *et al*., 2018).

FXYD2 was the first FXYD protein shown to be linked with Na-K-ATPase and to have a functional influence on its transport characteristics (Geering, 2006). FXYD2 may have many simultaneous and separate impacts on Na-K ATPase activity, according to evidence from various experimental techniques. In both the presence and absence of extracellular Na^+, FXYD2 was demonstrated to enhance the apparent K^+ affinity of Na-K-ATPase at high negative membrane potentials (Béguin *et al*., 2001; Meyer *et al*., 2020).

Furthermore, it has been observed that FXYD2 enhances the K^+ antagonism of intracellular Na^+ binding, indicating that FXYD2 has an additional effect on intrinsic K^+ binding at cytoplasmic locations (Pu *et al*., 2001). Finally, FXYD2 reduces the Na^+ activation of Na/K pump currents, as well as the Na^+ and K^+ activation of Na-K ATPase activity (Arystarkhova *et al*., 1999).

1-5-3: Regulation of Sodium-Potassium ATPase

Na-K ATPase, a regulatable enzyme, is to be regulated in both the short and long term depending on the stimulus (Therien and Blostein, 2000). Short-term control often entails either modifying the enzyme's kinetic characteristics or altering the trafficking of Na-K ATPase from the cytoplasm to the plasma membrane (Suhail, 2010).

Long-term control of Na-K ATPase, on the other hand, has a broad effect on Na-K ATPase production and degradation. A substantial amount of research indicates that Na-K ATPase is controlled by a variety of internal and extrinsic variables (Sznajder *et al*., 2002).

Extrinsic factors that influence Na-K ATPase activity include inflammatory mediators, hormones, neurotransmitters, and cardiotonic steroids (Hernández-R., 1992). Furthermore, Na-K ATPase is inherently controlled by phosphorylation and dephosphorylation events by kinases and phosphatases, which are driven by exogenous stimuli and intracellular second messengers (Mohan *et al*., 2019).

The phosphorylation-mediated regulation of Na-K ATPase is essentially achieved by protein kinase C (PKC), protein kinase A (PKA), and protein tyrosine kinases (PTKs) (Beguin *et al*., 1994).

These protein kinases influence the activity of Na-K ATPase either directly or indirectly. The direct regulation of Na-K ATPase activity is through phosphorylation of its subunits. In contrast, indirect regulation of Na-K ATPase involves the signaling of numerous pathways that alter the transcription factor associated with controlling Na-K ATPase gene expression (Suhail, 2010; Nepa, 2019).

1-6: Endogenous Digitalis

Endogenous cardiotonic steroids (CTS), also called digitalis-like factors (Paczula *et al*., 2016). Cardiotonic steroids are steroid hormones that circulate in the bloodstream, and they are eliminated in the urine. CTS is thought to have crucial roles in health and illness (Bagrov *et al*., 2009).

Endogenous cardiotonic steroids are linked to increased salt consumption and higher blood pressure, and they are partially to blame for target organ damage in arterial hypertension (Schoner and Scheiner-Bobis, 2008; Paczula *et al*., 2019). CTS act primarily through their ability to inhibit the ubiquitous transport enzyme sodium-potassium adenosine triphosphatase (Buckalew, 2015).

The synthesis of cardiotonic steroids from cholesterol occurs in the glomerular and fascicular layer of the adrenal glands (Murrell *et al*., 2005), but it has been discovered that CTS can also be liberally produced by the placenta (Hilton *et al*., 1996), heart (D'Urso *et al*., 2004), and hypothalamus. The biosynthesis of cardiotonic steroids is unclear and appears to be complicated (Słabiak-Błaż and Piecha, 2021).

1-6-1: Types of Endogenous Digitalis

They are classified into two groups by their chemical structure: cardenolides derived from plant and bufadienolides essentially derived from animal source (Tang *et al*., 2016).

1-6-1-1: Endogenous Cardenolides

Cardenolides are steroids with distinct chemical properties, that have a five-membered unsaturated lactone ring linked to the steroid nucleus at position 17 β (Luckner and Diettrich, 1988). Also, cardenolides contain different group combinations such as hydroxyl, sulfate, or carbohydrate (El-Mallakh *et al*., 2019).

1. Endogenous Ouabain

The endogenous ouabain (EO) is a steroid hormone secreted by the adrenal gland with cardiotonic effects. The cardiac glycosides are a class of drugs derived from the leaves of the *digitalis purpurea* and in other plants with appositive inotropic effect on the heart (Simonini *et al*., 2018). The chemical structure of ouabain is shown in Figure 1-5.

For a long time, they were successfully utilized as primary therapy for congestive heart failure and arrhythmias. This hormone raises to the picomolar range in the plasma of hypertensive humans (Manunta *et al*., 1999; Pavlovic, 2014), after acute physical exercise, and in pregnancy (Simonini *et al*., 2018).

EO levels are also higher in patients with kidney failure (Manunta *et al*., 2011; Iatrino *et al*., 2019), myocardial infarction (Goto *et al*., 1996), and congestive heart failure (Blaustein and Bova, 1991).

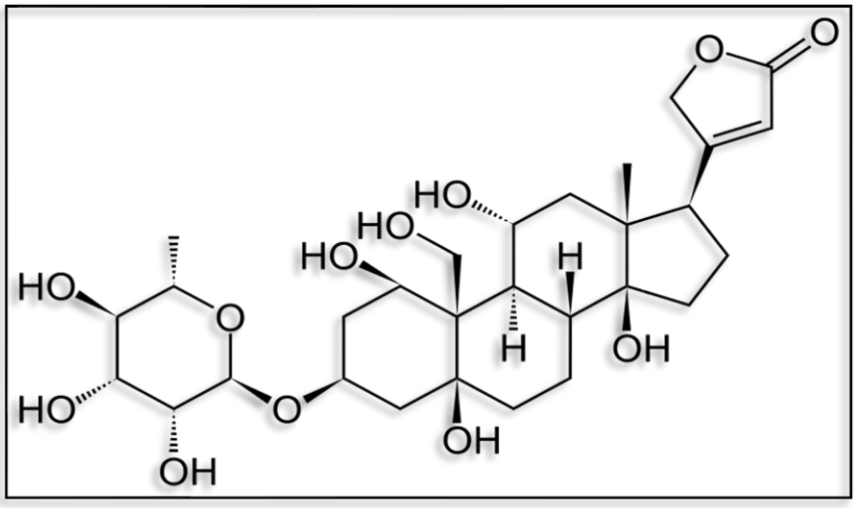


Figure 1-5: Chemical Structure of Ouabain (Bagrov and Shapiro, 2008)

EO modifies cardiac function and modulates cellular proliferation and differentiation in the heart, in addition to its hypertensive effects (Aydemir-Koksoy *et al*., 2001), kidney, and vascular smooth muscle (Dvela-Levitt *et al*., 2015).

Finally, it has the ability to increase myogenic tone while decreasing renal blood flow (Manunta *et al*., 2011). The major site of ouabain activity is thought to be the alpha-subunit of Na-K ATPase. Ouabain has a high affinity for inhibiting Na-K ATPase, primarily binding to isoforms 2 and 3 in vascular and brain tissues, respectively. This inhibition increases cytoplasmic Na^+ concentration, which decreases the activity of the Na^+/Ca^{2+} exchanger and, as a result, increases the amount of Ca^{2+} available to stimulate contraction in tissues such as the heart. As a result, there is a positive inotropic effect (Blaustein, 1993; Hamlyn *et al*., 2003).

2. Endogenous Digoxin

Digoxin is derived from the foxglove plant Digitalis purpurea. It is a cardiotonic glycoside that belongs to the digitalis class, (Grubb and Mentz, 2020).

The chemical formula of digoxin is $C_{41}H_{64}O_{14}$ (Sharma and Purkait, 2012) and the chemical structure of digoxin is shown in Figure 1-6.

Digoxin is useful in people with systolic heart failure, also known as heart failure with a low ejection fraction (ejection fraction less than 40%). However, there is no advantage in terms of mortality reduction (Virgadamo *et al*., 2015). It is utilized for rate control in atrial fibrillation or atrial flutter when traditional treatments have not attained the heart rate goal (Callahan, 2012).

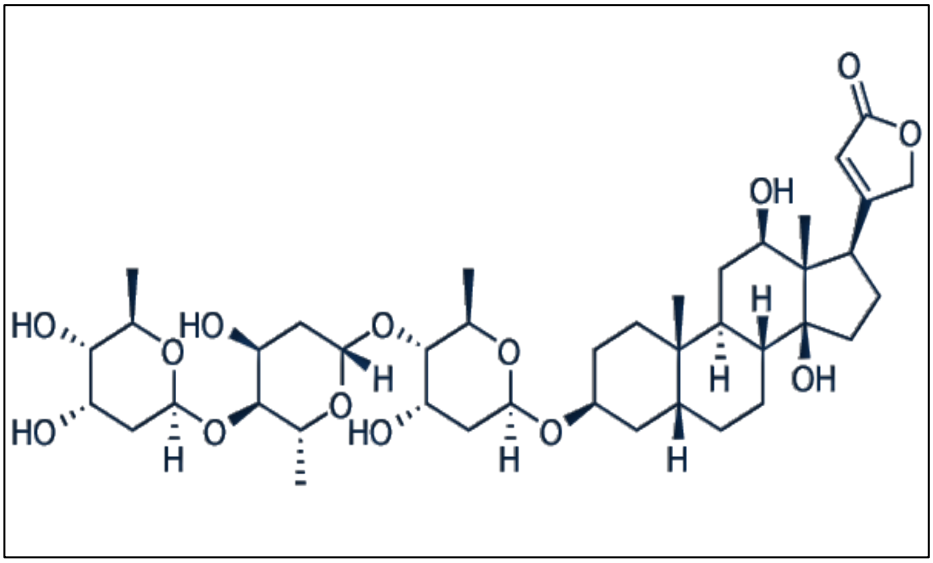


Figure 1-6: Chemical Structure of Digoxin (Bagrov and Shapiro, 2008)

Digoxin has shown modest success in treating fetal supraventricular tachyarrhythmia (Oudijk *et al*., 2004). The lowest effective dose should be given to the mother as digoxin might cause uterine contractions and result in abortion (Mutlu *et al*., 2019).

1-6-1-2: Endogenous Bufadienolides

Bufadienolides contain that have a six-membered unsaturated lactone ring linked to the steroid nucleus at position 17 β and doubly unsaturated six-membered lactone ring (Botha, 2016).

1. Endogenous Marinobufagenin

Marinobufagenin (MBG) is an endogenous cardiotonic steroid (CTS), all of which are inhibitors of the sodium-potassium adenosine triphosphatase (Na-K ATPase), also called digitalis-like factors. By chemical structure, MBG belongs to bufadienolides (Paczula *et al*., 2016).

MBG, which was first discovered in toads, may be found in high amounts in the skin of amphibians and the structure of marinobufagenin as shown in Figure 1-7 where it is hypothesized to be integral to water and electrolyte homeostasis. MBG concentrations respond appropriately to changes in environmental salinity whereas in humans, raised plasma concentrations of bufadienolides are connected with excessive salt and fluid accumulation (Bagrov *et al*., 2009).

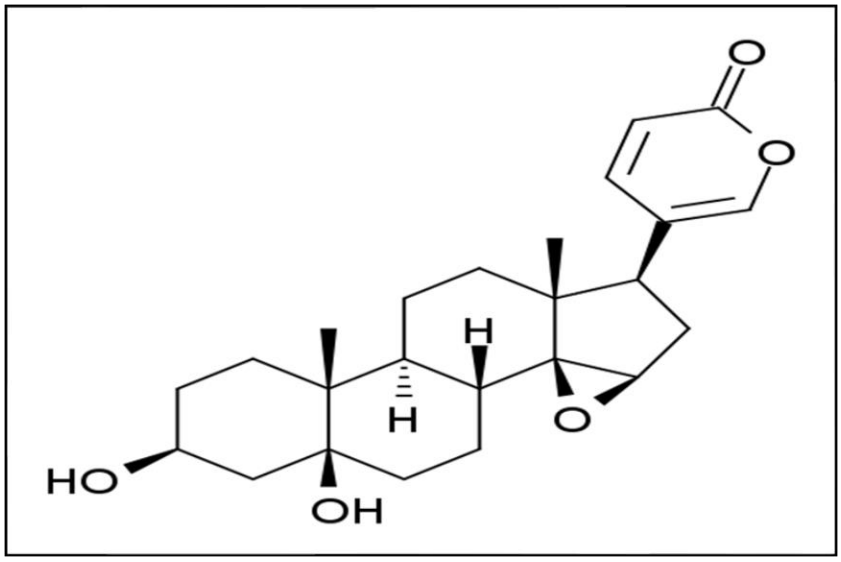


Figure 1-7: Chemical Structure of Marinobufagenin (Bagrov and Shapiro, 2008)

MBG plasma concentrations are raised by sodium loading and in turn raise natriuresis by a pressure induced mechanism via vasoconstriction and by direct effects on the renal tubule. In line with this notion, raised concentrations of MBG were reported for a variety of clinical conditions correlated with body fluid volume expansion, such as congestive heart failure (CHF), end-stage renal disease (ESRD), hypertension, renal ischemia, and preeclampsia (Haller *et al*., 2014).

1-6-2: Role of Endogenous Digitalis in Preeclamptic

Pregnancy is associated with plasma volume expansion as a result of fluid retention and salt retention in the kidneys (Aguree and Gernand, 2019). So, the role of cardiotonic steroids in pregnancy and pregnancy-related diseases is logical to investigate. Graves *et al*. were the first to show increased circulating levels of cardiotonic steroids during pregnancy and hypothesize that cardiotonic steroids play a role in preeclamptic pathogenesis (Graves, 1987).

The role of cardiotonic steroids in pregnancy is still unknown (Bagrov *et al*., 2009). In experimental animals, the role of cardiotonic steroids in preeclamptic has been studied further during days 14-20 of pregnancy. In Sprague-Dawley rats pregnant, salt supplementation with drinking water containing 1.8% sodium chloride was linked to an increase in plasma levels of marinobufagenin (but not ouabain), an increase in blood pressure, proteinuria, and a decrease in fetal weight, and size (Bagrov and Shapiro, 2008).

1-7: Aims of the Study

The aims of this study are as follows:

1. To detect the specific activity of Na-K ATPase in red blood cell ghosts in preeclamptic patients compared with normal pregnancies.
2. To evaluate the percentage inhibition of endogenous digitalis in preeclamptic patients compared with normal pregnancies.
3. To evaluate the association of gene polymorphism of placental Na-K ATPase with preeclamptic and its severity.
4. To estimate the expression of placental Na-K ATPase in preeclamptic patients compared with normal pregnancies by immunohistochemistry, as well as other histological parameters.

2-1: Patients and Study Settings

2-1-1: Study Settings

This study was carried out on female patients attended to Babylon Teaching Maternity and Pediatric and Hilla Teaching Hospital in Babylon governorate in the city of Hilla. All samples were collected during the period of 1th of February 2019 till 20th February 2020. On other hand, the practical side of the study was performed at the advanced Biotechnology laboratory in the College of the Pharmacy, University of Babylon.

2-1-2: Study Design

The type of study is a case-control study to compare between the control group and the patient group.

2-1-3: Study Population

This study included 130 samples from blood samples and 90 samples of placenta tissue, and the age of women participants ranged from 16 - 40 years. Samples were divided into the following groups:

1. **Blood Samples**

   A. Positive Control Group: Taken from normal pregnancies (55 Samples).
   B. Negative Control Group:Taken from non-pregnant women (30 Samples).
   C. Patient Group: Taken from patients with preeclamptic (45 Samples).

The following tests were accomplished on blood samples

1. Specific activity of Na-K ATPase on the red blood cell membrane.
2. Estimation of the percentage inhibition of endogenous digitalis.

2. **Placenta Samples**

   A. Control Group: Taken from normal pregnancies (50 Samples).

B. Patient Group: Taken from patients with preeclamptic (40 Samples).

The following tests were assessed in placental tissue

1. Histologic examination to assess the degree of perivascular fibrosis.
2. Immunohistochemical staining to evaluate the expression of Na-K ATPase on placental tissue.
3. Cytogenetic analysis to demonstrate genetic polymorphism of the Na-K ATPase gene in placental tissue (Alpha Isoform).

2-1-4: Ethical Issues

Ethical approval was obtained from the following authorities:

1. Approval by the scientific committee of the college of science (university of Babylon, Iraq) and the department of chemistry in the same college.
2. Approval of the scientific committee of the maternity hospital and children hospital in Babylon governorate, Hilla city.
3. Approval of the scientific committee of Hilla teaching hospital in Babylon governorate, Hilla city.
4. To gain verbal acceptance from participate women, the goals of this study were explained to all participants in the current study.

2-1-5: Collection of Data

The inclusion and exclusion criteria for this study were as follows:

1. Inclusion Criteria

The socio-demographic characteristics for the selection of the study individuals included patients suffering from preeclamptic. Other parameters recorded in patient questionnaire are maternal age, systolic pressure, diastolic pressure,

albumin in urine, edema, gestational age (weeks), type of delivery, and the weight of baby. The questionnaire form was shown in appendix A.

2. Exclusion Criteria

Any subject suffered from the following:

- Age over 40.
- Smoking.
- Pre-existing hypertension.
- Renal disease.
- Pre-existing diabetes or gestational diabetes.

2-2: Materials

The commercially available laboratory kits and the chemicals used in this study were shown in the Table 2-1.

Table 2-1: The Standard Kits and the Chemicals Used in This Study

No.	Materials	Symbol	Company Supplied
1.	Acetic Acid	CH_3COOH	Sigma-Aldrich, USA
2.	Acrylamide	C_3H_5NO	Himedia, India
3.	Adenosine Triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	Merck, Germany
4.	Agarose	/	Himedia, India
5.	Ammonium Persulfate	$(NH_4)_2S_2O_8$	Sigma-Aldrich, USA
6.	Bis Acrylamide	$C_7H_{10}N_2O_2$	Himedia, India
7.	Boric Acid	BH_3O_3	Thomas Baker, India
8.	Bovine Serum Albumin	/	Sigma-Aldrich, USA
9.	Bromophenol Blue	$C_{19}H_{10}Br_4O_5S$	Himedia, India
10.	Ethanol	C_2H_5OH	Fluka
11.	Ethidium Bromide	$C_{21}H_{20}BrN_3$	Himedia, India

No.	Materials	Symbol	Company Supplied
12.	Ethylene Diamine Tetra Acetic Acid (EDTA)	$C_{10}H_{14}N_2O_8 .2H_2O$	Himedia, India
13.	Formaldehyde 37%	CH_2O	Chemanol, KSA
14.	Glycerol	$C_3H_8O_3$	Sigma-Aldrich, USA
15.	Immune Histochemistry/ Kit	/	Bio SB, USA
16.	Ladder 100 Bp	/	Biolab, England
17.	Ladder 50 Bp	/	Biolab, England
18.	Magnesium Dichloride	$MgCl_2$	Sigma-Aldrich
19.	Marker Anti Na-K ATPase	/	Abcam, USA
20.	Master Mix	/	Cyntol, Russia
21.	Methanol	CH_3OH	Hayman, England
22.	Na_2- Ethylene Diamine Tetra Acetic Acid (Na_2-EDTA)	$C_{10}H_{14}N_2Na_2O_8 .2H_2O$	Himedia, India
23.	Normal Saline	NaCl 0.9%	Sachana, India
24.	PCR Master kit	/	Promega, USA
25.	Phosphorus Kit	/	Linear, Spanish
26.	Polyacrylamide	$(C_3H_5NO)_n$	Himedia, India
27.	Potassium Chloride	KCl	Sigma-Aldrich
28.	Primer	/	Bioneer, Korea
29.	Proteinase K	/	Promega, USA
30.	Restriction Enzyme-Tru91 sib	/	Cyntol, Russian
31.	Silver Nitrate	$AgNO_3$	BDH, England
32.	Sodium Acetate	CH_3COONa	Sigma-Aldrich
33.	Sodium Chloride	NaCl	Sigma-Aldrich
34.	Sodium Dodecyl Sulfate (SDS)	$NaC_{12}H_{25}SO_4$	Sigma-Aldrich
35.	Sodium Hydroxide	NaOH	Sigma-Aldrich
36.	Tetramethyl Ethylenediamine (TEMED)	$C_6H_{16}N_2$	Bio Pure, Korea
37.	Total Protein Kit	/	Biomaghreb

No.	Materials	Symbol	Company Supplied
38.	Tris – HCl	$C_4H_{11}NO_3$. HCl	Himedia, India
39.	Xylene	C_8H_{10}	BDH, England

2-3: Instruments and Equipment

The devices and tools used in this study were shown in the Table 2-2.

Table 2-2: The Device and Tools Used in This Study

No.	Devices and Tools	Company Supplied
1.	Autoclave	Prestige Medical, England
2.	Centrifuge	Hettich, Germany
3.	Different Size Mechanical Pipettes	Biolab, Germany
4.	Disposable Cup	China
5.	Disposable Syringe (3 mL)	China
6.	Disposable Test Tube (10 mL)	Meheco, China
7.	EDTA Tube	AFCO, Jordan
8.	Electronic Balance	Sartorius, Germany
9.	Eppendorf (1.5 mL)	Meheco, China
10.	Horizontal Gel Electrophoresis	ATTA, Japan
11.	Incubator	Memer Edelsta, Germany
12.	Khan Tube	Meheco, China
13.	Magnetic Stirrer with Hot plate	Grant, England
14.	Micro-Centrifuge	Hettich, Germany
15.	Microscope	Olympus, Japan
16.	Microtome	Germany
17.	Microwave Oven	Memmert UNB, Germany
18.	Nano- drop	Bio-drop, England
19.	pH Meter	Cirson, EU
20.	Rotating Mixer	Benchmark Scientific

No.	Devices and Tools	Company Supplied
21.	Spectrophotometer	Aple, Japan
22.	Thermocycler (PCR)	Biometra, Germany
23.	Ultra-Sonic Cleaner	Kodo, Korea
24.	UV. Trans Illuminator	E-Graph/ Japan
25.	Vertical Gel Electrophoresis	Cleaver, England
26.	Vortex	Digisystem, Taiwan
27.	Water Bath	Memmert UNB, Germany

2-4: Software and Databases

Many software and databases were used in this study. The most used software and databases were listed in the Table 2-3.

Table 2-3: The Software and Databases Used in This Study

No.	Software or Database	Produced Company or Website
1.	ChemDraw	PerkinElmer
2.	dbSNP	https://www.ncbi.nlm.nih.gov/projects/SNP/
3.	Excel 2016	Microsoft®
4.	GraphPad®	http://graphpad.com/quickcalcs/contingency1/
5.	MedCalc Statistical Software	MedCalc Software Ltd
6.	Mendeley Reference Management	http://Mendeley.com
7.	NCBI-Primer BLAST	https://www.ncbi.nlm.nih.gov/tools/primerblast
8.	SNPStats®	http://bioinfo.iconcologia.net/en/SNPStats_web
9.	SPSS Statistics Version 26	IBM® SPSS®
10.	WatCut Online Software	http://watcut.uwaterloo.ca/template

2-5: Collection of Samples

All the samples were collected from individual lived in Babylon governorate, and the samples of blood and placenta tissue collected into the following methods:

2-5-1: Collection of Blood Samples

Venous blood samples were drawn from all subjects by using a disposable syringe (3 mL) in the sitting position with a tourniquet and collected into the EDTA tube and mixed gently. Blood was centrifuged at 3000 Xg for approximately 10 minutes.

After centrifuge, the plasma was transferred to a plain tube and stored at -20˚C until the analysis of endogenous digitalis. Whereas immediately, the red blood cell concentrate was used for the measurement of specific activity of Na-K ATPase.

2-5-2: Collection of Placental Samples

The placenta samples were collected from all pregnant subjects after delivery by using a disposable cup. A small piece of placenta tissue was placed in a cup and preserved in normal saline until delivered to the laboratory. Placental samples were sonicated by ultrasonic cleaner for 15 min at room temperature for obtaining cell suspension for DNA extraction.

After sonication, 10 mL from the normal saline supernatant was transferred to a plain tube. The supernatant was centrifuged for 10 min at 3000 Xg then, the supernatant was discarded, and the precipitate stored at -20 C for genetic study. Whereas the remaining placental tissue was preserved in 10% formal-saline for histological and immunohistochemically study.

2-6: Biochemical Tests

2-6-1: Determination Specific Activity of Na-K ATPase

2-6-1-1: Principle

The specific enzyme activity in RBCs membranes (ghosts) was determined as inorganic phosphate produced through enzymatic hydrolysis of ATP and expressed in milligrams of phosphate per gram of protein released through 30 minutes of incubation (Pi), according to a modification of the method described by (Kaššák *et al*., 2006).

2-6-1-2: Preparation of ATPase Reagent

The reagent of ATPase was prepared by dissolving of the following materials

1. Tris – HCl (100 mM, prepared by weighting 1.2 g).
2. $MgCl_2$ (10 mM, prepared by weighting 0.095 g).
3. KCl (15 mM, prepared by weighting 0.11 g).
4. NaCl (85 mM, prepared by weighting 0.4 g).
5. Na_2-EDTA (1 mM, prepared by weighting 0.036 g).
6. ATP (2 mM, prepared by weighting 0.1 g).

All materials dissolved in 100 mL of distilled water then adjusted the pH of the reagent at 7.4 by adding sodium hydroxide.

2-6-1-3: Preparation of Red Blood Cell Ghosts

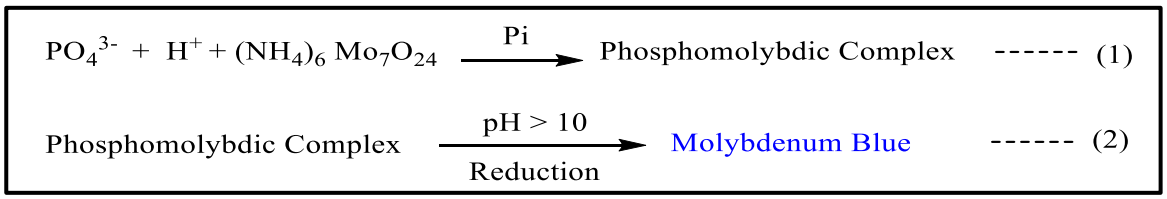
1. Fresh blood was collected in EDTA tubes as an anticoagulant and centrifuged at 3000 Xg for 10 min.
2. RBCs were obtained by taking 40 µl of red cell sediment after centrifugation.
3. To 1 ml of pharmaceutically available normal saline, the 40 µL red cell sediment was added and washed three times with subsequent centrifugation and decanting the residual normal saline supernatant.

4. The washed RBCs obtained by the above procedure were subjected to lysis by the addition of deionized distilled water by adding 500 µL DDW. and centrifuge at 3000 Xg for 10 min.

2-6-1-4: Determination of Inorganic Phosphorus

A. Principle

Inorganic phosphate reacts with molybdic acid forming a phosphomolybdic complex as shown in equations 1 and 2. It produced a reduction in alkaline medium originates a blue molybdenum colour which intensity was proportional to the quantity of phosphorus existing in samples (Burtis *et al*., 2012; Koronkiewicz *et al*., 2018).



B. Reagents

The components of the inorganic phosphorus kit were illustrated in the Table 2-4.

Table 2-4: The Composition of Inorganic Phosphorus Kit

Reagents	Composition	Concentration
R1 (Molybdate Reagent)	Ammonium Molybdate Sulphuric Acid	7 mmol/L 0.8 mmol/L
R2 (Reducing Solution)	Hydroxylamine	0.64 mol/L
R3 (Color Developer)	Sodium Hydroxide	3 mol/L
CAL (Standard)	Chloride Phosphorus Standard	100 mg/L 5 mg/dL

C. Preparation of Working Reagent

The working reagent was prepared by mixing equal volumes of R1 with R2, and this reagent was stable for 8 hours.

D. Procedure

Three tubes were prepared, and the procedure carried out as in the following Table 2-5.

Table 2-5: Procedure Used for Determination of Inorganic Phosphorus

Tubes	Blank	Sample	Standard
Working Reagent	1 mL	1 mL	1 mL
CAL (Standard)	/	/	50 µL
Sample	/	50 µL	/
All tubes were mixed and let to stand for 10 min. at room temperature			
R3 Developer	0.5 mL	0.5 mL	0.5 mL
All tubes were mixed, and the absorbance was reading at 740 nm against the blank			

E. Calculation

The results were calculated as follows:

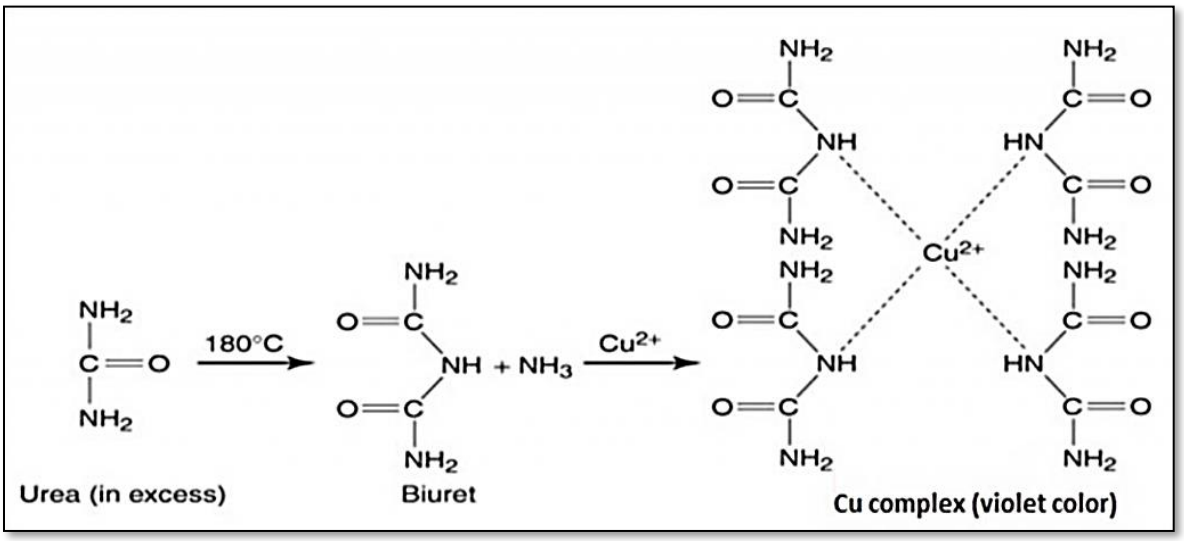
$$\text{Concentration of Inorganic Phosphorus (mg/L)} = \frac{\text{Abs. Sample}}{\text{Abs. Standard}} * \text{Concentration of Standard}$$

Concentration of Standard = 100 mg/L

2-6-1-5: Determination of Total Protein by Biuret Method

A. Principle

The Biuret approach focused on the complexation of cupric ions in the protein's peptide bonds to functional groups. To form a Cu^{2+}-protein complex and produce a violet-colored chelate product as shown in Scheme 2-1 that was measured at 540 nm by absorption spectrophotometrically, two peptide bonds or longer were required (Burtis *et al*., 2012).



Scheme 2-1: Biuret Reagent Reacts with an Alkaline Solution of $CuSO_4$ to Form a Violet Chelate Compound (Keppy and Allen, 2000)

B. Reagents

The components of the total protein kit were illustrated in the Table 2-6.

Table 2-6: The Composition of Total Protein Kit

Reagents	Composition	Concentration
R1 (Alkaline Reagent)	Potassium-Sodium Tartrate Sodium Hydroxide Potassium Iodide	12 mmol/L 0.6 mmol/L 30 mmol/L
R2 (Coloring Reagent)	Cooper Sulfate (Nocive)	0.6 mol/L
R3 (Standard)	Bovine Albumin	50 g/L 5g/Dl

C. Preparation of Working Reagent

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

D. Procedure

Three tubes were prepared, and the procedure carried out as in the following Table 2-7.

Table 2-7: Procedure Used for Determination of Total Protein

Tubes	Blank	Sample	Standard
Working Reagent	1 mL	1 mL	1 mL
Reagent 3 (Standard)	/	/	20 µL
Sample	/	20 µL	/
All tubes were mixed and incubated for 2 min. at room temperature then the absorbance was reading at 540 nm against the blank			

E. Calculation

The result was calculated as follows:

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

Concentration of Standard = 50 g/L

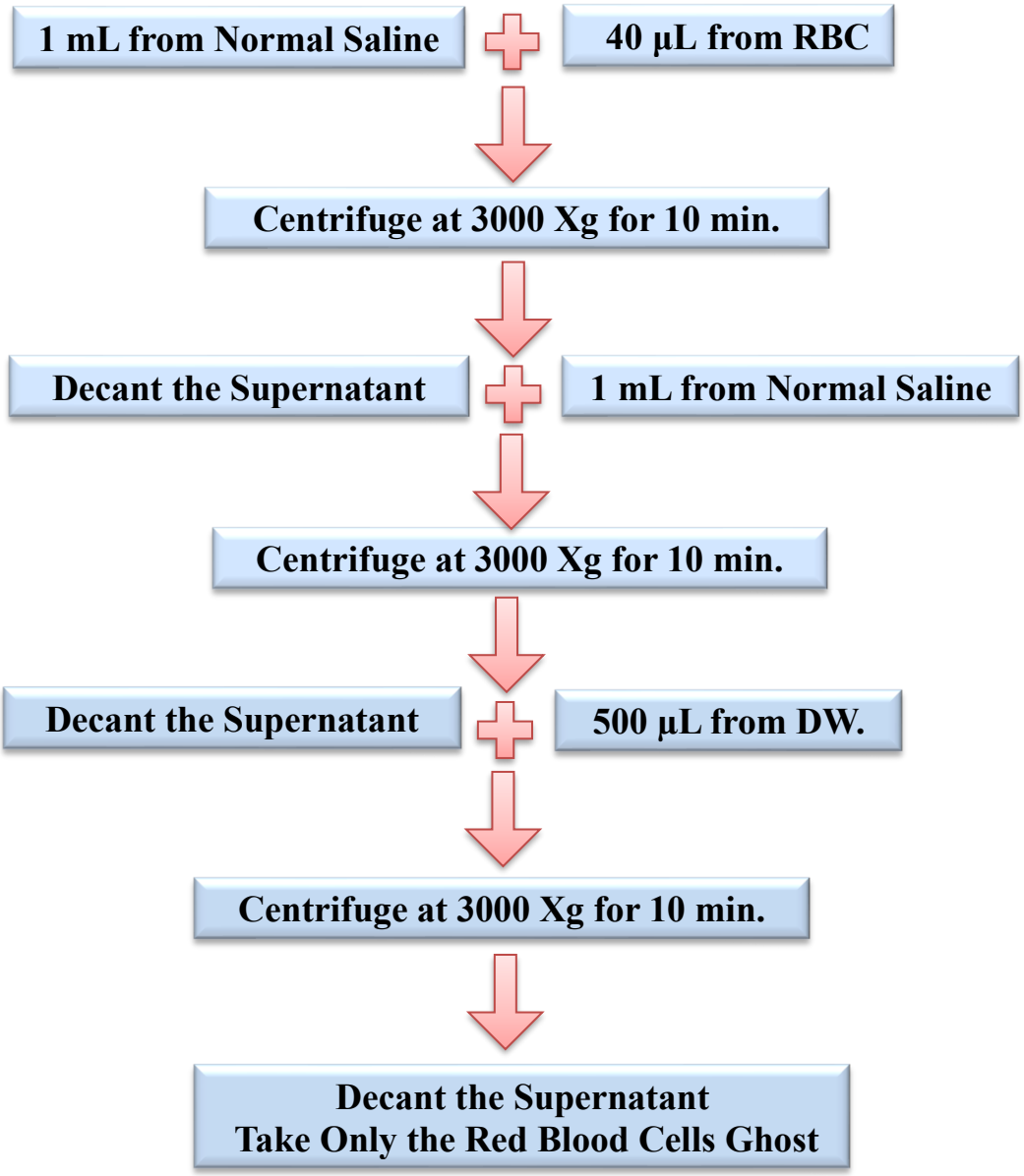
2-6-1-6: Measurements of Specific Activity of Na-K ATPase

The modified method for determining specific activity of Na-K ATPase illustrated below and a brief procedure was shown in Scheme 2-2:

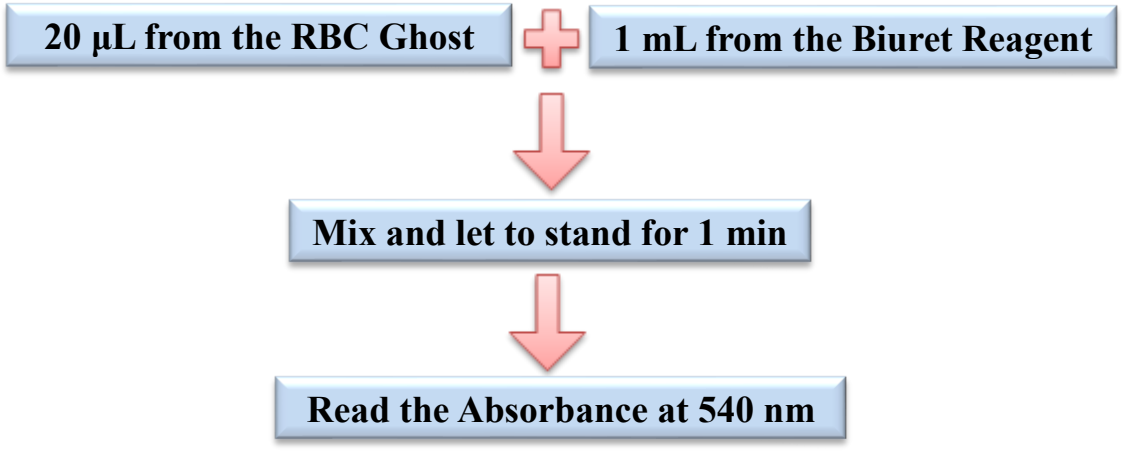
1- Ten microliters of red blood cell ghosts were added to 500 mL of ATPase reagent prepared in section B and incubated for 30 min. exactly.
2- After incubation, the samples were centrifuged at 3000 Xg for 10 min. then pulled 50 µL from the supernatant to determine inorganic phosphates.
3- The inorganic phosphates were determined spectrophotometrically according to the method illustrated in section D.
4- For standardization, protein concentration in red blood cell ghosts was estimated according to the standard biuret method illustrated in section E.
5- Specific enzyme activity was expressed as the inorganic phosphorus to protein concentration as follows:

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

1- Preparation of Red Blood Cell Ghosts



2- Determination the Concentration of Total Protein



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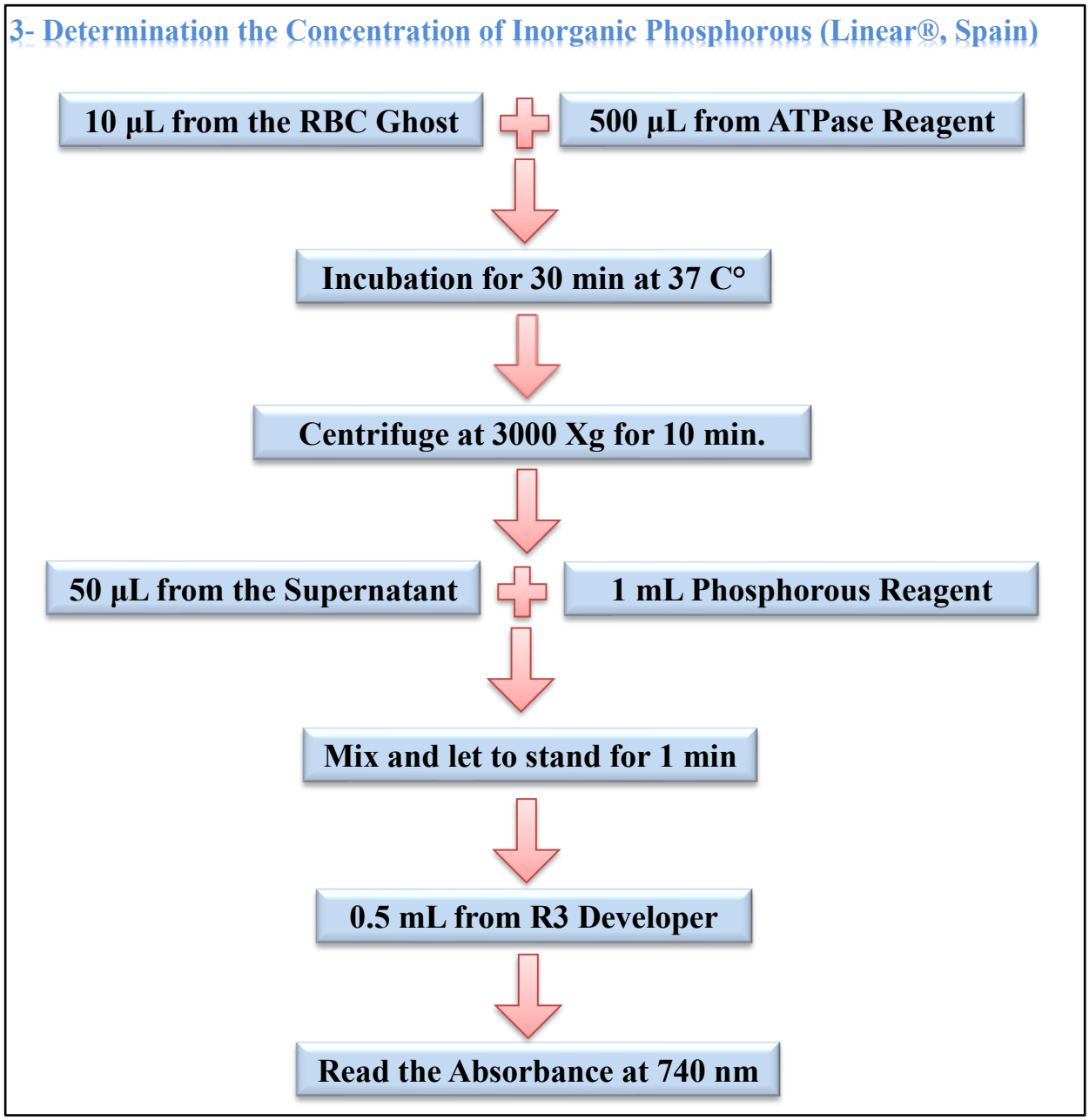


Scheme 2-2: Brief Procedure for Determining Specific Activity of Na-K ATPase

2-6-2: Evaluation the Inhibition Percentage of Endogenous Digitalis

2-6-2-1: Principle

Endogenous digitalis work primarily by inhibiting the Na –K ATPase transport enzyme, which is found throughout the body. A portion of the Na-K ATPase does not appear to actively "pump" sodium or potassium, but it is closely linked to other important signaling pathways (Graves *et al*., 1989; Paczula *et al*., 2016).

2-6-2-2: Evaluating the Inhibition Percentage of Endogenous Digitalis

The novel method for estimation of the percentage inhibition of endogenous digitalis illustrated below and a brief procedure was shown in Scheme 2-3:

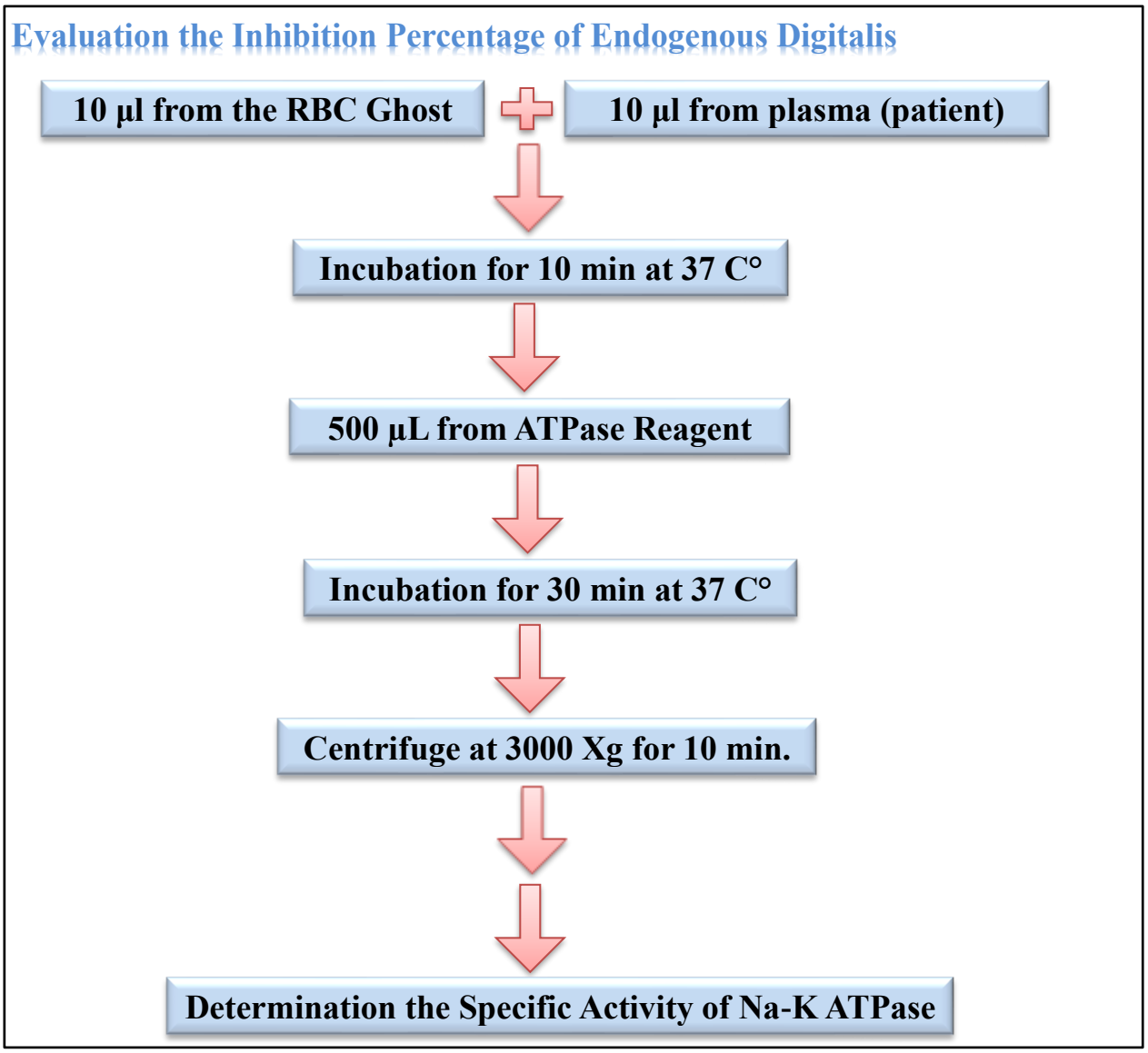
1. Red blood cell membrane ghosts were prepared as mentioned above in section 2-6-1 C.
2. The evaluation of the specific activity of Na-K ATPase in the red blood cells was accomplished.
3. In another tube, 10 µl plasma (patient) and 10 µl ghosts (negative control), were mixed and then incubated in a water bath for 10 minutes.
4. After the incubation, 500 µL of ATPase reagent as prepared above was added and incubated for 30 min. exactly at 37 C˚.
5. After the incubation period over, Na-K ATPase activity was determined as described above in section 2-6-1-6.
6. The Percentage of inhibition for endogenous digitals was expressed as follows:

$$\% \text{ Inhibition of Endogenous Digitals} = \frac{\text{Specific Activity N. C.- Specific Activity P.}}{\text{Specific Activity of N. C.}} * 100\%$$

Where:

N.C.: Negative Control

P.: Patient



Scheme 2-3: Brief Procedure for Evaluating the Inhibition Percentage for Endogenous Digitalis

2-7: Genetic Parts

2-7-1: Markers Selection

The present study had selected the Na-K ATPase alpha gene polymorphisms depending on reviewing the recent literatures. Also, the two isoforms of the Alpha gene were selected because they were more polymorphic. In addition, it was covering the target.

1. ***ATP1A1*, rs10924081.**
2. ***ATP1A2*, rs373796693.**

2-7-2: Design Procedure for the Gene *ATP1A1* (rs10924081)

The genotyping approach for the gene *ATP1A1* (rs10924081) was chosen based on (Hashim and Al-Shuhaib, 2019). While the PCR-RFLP design was done according to the procedure of (Hashim *et al*., 2015). The sequence of the amplified region of *ATP1A1* was shown in Figure 2-1.

AGTCTTCTACTTTGCCCTTGACACAGGCAGCTTGTCCCCAGTCCTGAGACCT/TAAGCATTT
TCTTACGAATTCTACGGTAATGAAAGGTGCTATAAATGTT/TAATCTCTCACTTGGTATTT
TCAGATAAGTAACCAGAAGTAGGTAGTTGGGAATAGCATTGGCTTTGAGACTTTTCAGTA
GGCCAGGG

Figure 2-1: Sequence of Amplified Region That Used to *ATP1A1* (rs10924081) PCR-REFLP Genotype

2-7-2-1: Primer Design for the Gene *ATP1A1*

The primer was designed using the NCBI-primer BLAST online software (https://www.ncbi.nlm.nih.gov/tools/primerblast). The primers pair were selected according to the demand criteria such as product length, the similarity of melting temperature, primers length, and specificity. The primer sequence and their properties are listed in Table 2-8.

Table 2-8: Sequences of the Forward and Reverse Primers for the Gene *ATP1A1*

Primer Pairs	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self-5' Complementarity	Self-3' Complementarity
Forward Primer	AGTCTTCTA CTTTGCCCT TGACA	23	38	60	59.61	43.48	3.00	3.00
Reverse Primer	CCCTGGCC TACTGAAA AGTC	20	226	207	57.88	55.00	4.00	1.00
Product Length	189							

2-7-2-2: Restriction Enzyme Selection for the Gene *ATP1A1*

The selection of the suitable restriction enzyme was performed by the aid of WatCut online software (http://watcut.uwaterloo.ca/template) as shown in Figure 2-2, selected the restriction enzyme according to several criteria such as the lesser primer mutations needed, the distance of mutation from the variant, compatibility of the produced primers, cost, and availability.

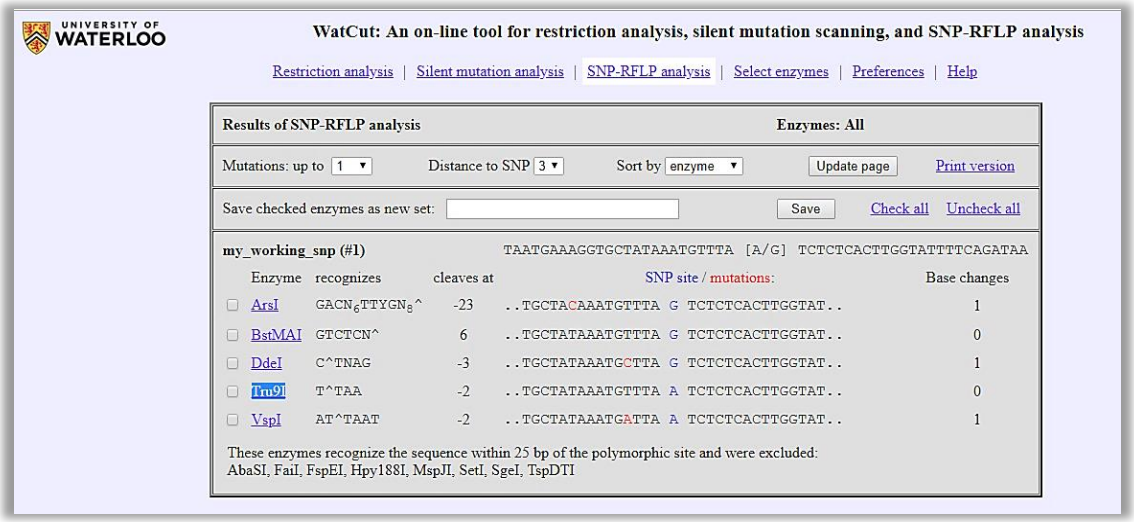


Figure 2-2: Result's Interface of Watcut Software

2-7-3: Design Procedure for the Gene *ATP1A2* (rs373796693)

The genotyping approach for the gene *ATP1A2* (rs373796693) was chosen based on (Hashim and Al-shuhaib, 2019). While the PCR-SSCP design was done according to the procedure of (Hashim *et al*., 2015). The selected region contained two exons. Also, the chosen region has significant variations and a high frequency, as indicated in Table 2-9 and Figure 2-3.

Table 2-9: The Important Variants of Region Selected in the *ATP1A2* Gene

Variant I/D	Chr: Bp	Alleles	Global MAF	Class	Clin. Sig.	Consequence
rs114284892	1:160120873	C/A/G/T	< 0.001	SNP		Intron Variant
rs146329414	1:160121093	G/A	0.007	SNP		Non-Coding Transcript Exon Variant
rs2854246	1:160120884	T/C	< 0.001	SNP		Intron Variant

Variant I/D	Chr: Bp	Alleles	Global MAF	Class	Clin. Sig.	Consequence
rs201111192	1:160121186	C/T	< 0.001	SNP		Splice Region 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
rs575480986	1: Between 160121096 and 160121097	-/C	< 0.001	Insertion		Non-Coding Transcript Exon Variant
rs373796693	1:160120895-160120898	TCCT/-/CCTT	0.311	"Sequence alteration"	Benign	"Splice region variant"
rs61734527	1:160121203	G/A	0.007	SNP	Benign	Synonymous Variant
rs144106169	1:160121226	G/A/T	< 0.001	SNP	Uncertain Significance	Missense Variant

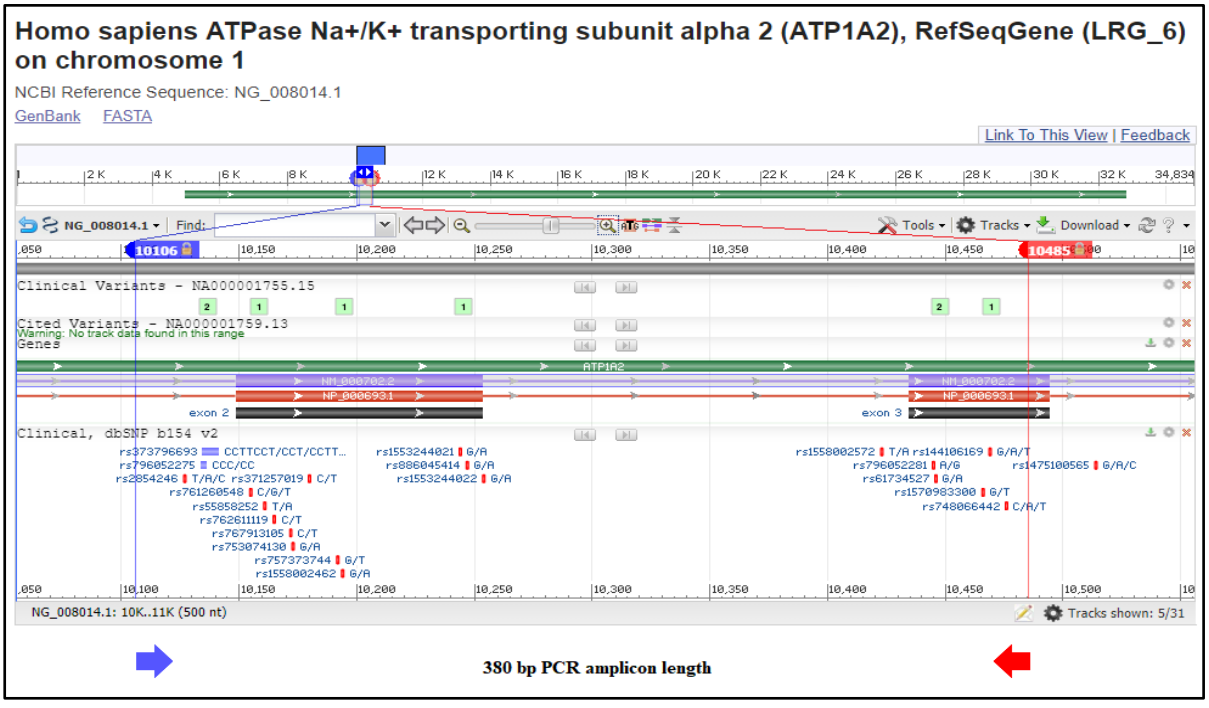


Figure 2-3: The Exact Position of Retrieved 380 bp Amplicon that Partially Covered a Portion of Intron 1 of *ATP1A2* Gene within Chromosome No. 1 (Genbank Acc. No. NG_008014.1).

Refer to the Starting Point of Amplicon

Refer to Endpoint of Amplicon

2-7-3-1: Primers Design for the Gene *ATP1A2*

The primer was designed using the NCBI-primer BLAST online software (https://www.ncbi.nlm.nih.gov/tools/primerblast). The primers pair were selected according to the demand criteria such as product length, the similarity of melting temperature, primers length, and specificity. The primer sequence and their properties are listed in Table 2-10.

Table 2-10: Sequences of the Forward and Reverse Primers for the Gene *ATP1A2*

Primer Pairs	Sequence (5'->3')	Length	Start	Stop	Tm	GC%	Self-5' Complementarity	Self-3' Complementarity
Forward Primer	CCCCTCT CTTCCCT GACTCT	20	16012 0863	16012 0882	59.66	60.00	3.00	1.00
Reverse Primer	GTCCACT TGGTATT TGCGGC	20	16012 1242	16012 1223	59.83	55.00	4.00	3.00
Product Length	380							

2-7-4: DNA Extraction Protocol

Genomic DNA was isolated from placental tissue using a slightly modified version of the method described by (Hashim and Al-Shuhaib, 2020). The DNA was stored at −20°C until it was processed. This method included many steps illustrated as below:

2-7-4-1: Preparation of Solution

A. Washing Solution

The washing solution contained 10% methanol and 90% Tris buffer. Tris buffer prepared by weighing 0.31 gm from Tris- HCl in 80 ml DDW. then adjusting the pH to 7.5 and completed the volume to 100 ml by DDW.

B. Ethylene Diamine Tetra Acetic Acid (EDTA)

To prepare 2 mM from EDTA, 0.03 gm from EDTA dissolved in 40 ml of DDW., then adjusting the pH to 8 and completed the volume to 50 ml by DDW.

C. Lysis Buffer

This buffer was prepared by adding 0.03 g from Tris-HCl, 0.06 g from EDTA, and 0.2 g from SDS to 70 ml D.W. then pH was adjusted to 8 and completed the volume to 100 ml by D.W.

D. Sodium Acetate

To prepare 3 M from sodium acetate, 4.9 g from sodium acetate dissolved in 15 ml of DDW. then adjusting the pH to 4.5 and completed the volume to 20 ml by DDW.

E. Tris-EDTA Buffer (TE):

This buffer was prepared by adding 0.157 g from Tris-HCl and 0.029 g from EDTA to 80 ml D.W. The pH was adjusted to 8 and completed the volume to 100 ml by D.W., then autoclaved at 12 C˚ for 15 minutes, and stored at 4 C˚ until being used.

2-7-4-2: Cells Washing

1. The frozen placenta tissue was placed in a vacutainer tube was thawed at room temperature and mixed gently.
2. Then, only 500 µl of the placenta tissue was transferred to an eppendorf tube of 1.5 ml capacity.
3. Up to 1 ml of washing buffer was added to the placenta tissue, mixed, and incubated at ambient temperature for 10 min in a rotating mixer.
4. The mixture was centrifuged at 10000 g for 2 min.
5. After discarding the supernatant, 1 ml of washing buffer was added again, and the tube was inverted several times to wash the pellet then centrifuged at the same speed for 10 seconds.
6. The supernatant was discarded, and the pellet was gently suspended with 1 ml of washing buffer by forth and back pipetting with a wide orifice tip, and centrifuged for 1 min.

7. The previous step was repeated once, twice, or once a yellowish-white precipitate of tissue.

2-7-4-3: Cells Lysis

1. The pellet was suspended with 200 µl of cell suspension buffer and mixed several times to remove cellular aggregations.
2. Then, 200 µl of cell lysis buffer was added to the generated homogenate, mixed, and then left at room temperature for 5 min.

2-7-4-4: Proteins Precipitation

1. Proteins were denatured by mixing of cellular lysate with 100 µl of protein precipitation buffer (sodium acetate) then vortexes for 20 sec.
2. Afterward, the suspension was centrifuged at 10000 Xg for 10 min.
3. The supernatant containing DNA was transferred to a new 1.5 ml centrifuge tube.

2-7-4-5: DNA Precipitation and Washing

1. Up to 1 ml of absolute ethanol was added to the supernatant and inverted until optimally DNA threads appeared.
2. Subsequently, centrifugation at 10000 Xg for 1 min was performed.
3. The supernatant was discarded and 1 ml of DNA washing buffer was added and mixedwell with the pelleted DNA and left for 1 min to re-suspend DNA.
4. The suspension was centrifuged at 10000 Xg for 1 min and the supernatant was discarded.

2-7-4-6: DNA Recovery

1. After dehydration at room temperature, the pelleted DNA was mixed with 100 µl of DNA elution buffer (TE).
2. The mixture was incubated for 15 min at 65°C in a water bath to speed up recovery.

2-7-5: Estimation of DNA Concentration and Purity

The DNA quantity and quality were measured using nano-drop device as shown in Figure 2-4, employing the scanning ability of diode array from 200-320 nm. The absorbance profile was then processed and analyzed to determine the DNA concentration and purity by calculating the 260/280 and 260/230 ratios.

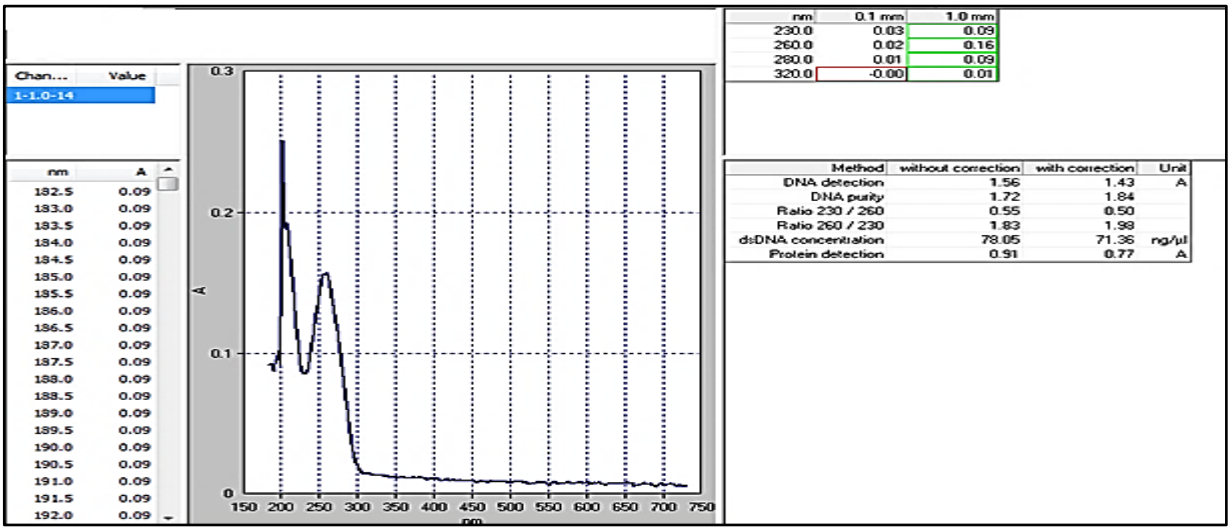


Figure 2-4: Measurement of DNA Concentration and Purity by Using Scan Drop Device

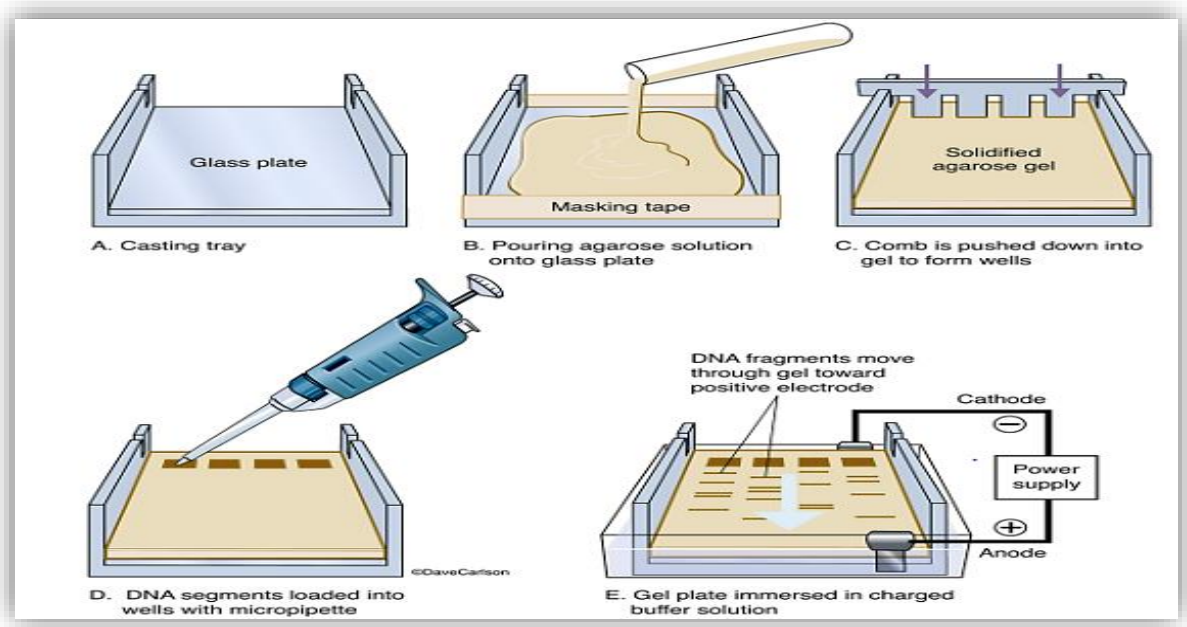
In order to estimate the purity of DNA a 260/280 ratio was used; the DNA was considered pure if it was between 1.7-2.0. Similarly, A ratio of 260/230 also should be ranged from 1.8-2.2 for a pure DNA sample otherwise contamination with RNA and protein was occur. The protocol included measuring light absorption by 2 µl of TE as blank followed by 2 µl of the sample (Khare *et al*., 2014).

2-7-6: Extracted DNA Molecular Weight and Integrity Estimation

The agarose gel electrophoresis technique was using to determine the molecular weight and the integrity of extracted DNA. The extracted DNA was colorless, so gel loading dye purple was used with DNA to ease the loading step of the electrophoresis procedure.

2-7-6-1: Principle of Gel Electrophoresis

Gel electrophoresis is a method used to distinguish DNA fragments by their size. DNA samples are loaded into wells at one end of a gel, and an electric current is applied to pull them through the gel as shown in Scheme 2-4. DNA fragments are negatively charged, so they travel towards the positive electrode. As all DNA fragments have the same amount of charge per mass, small fragments travel through the gel more rapidly than large ones (Ylmaz *et al*., 2012).



Scheme 2-4: Agarose Gel Electrophoresis Method

2-7-6-2: Procedure of Agarose Gel Electrophoresis

The electrophoresis was carried out according to (Sambrook and Russell, 2001) as describe below in brief :

1. The gel (1%) was prepared by dissolving 0.5 gram of agarose in 50 ml of 0.5X TBE buffer formed from [Tris base pH=6.8 (0.0445 M), borate (0.0445 M) and EDTA (0.001M)] and heated by microwave oven for 2 minutes.
2. The agarose homogenized then cooled to 55 C° by water bath.
3. A 50 µl of ethidium bromide stock (1mg/ml) solution was added to the gel and mixed by swirling.

4. The gel then poured to the gel try and let to polymerize for 30 minutes.
5. The polymerized gel then transferred to the electrophoresis devise and submerged with 0.5 TBE running buffer.

2-7-6-3: Loading the Samples on Gel Electrophoresis Instrument

1. Five microliters of extracted DNA were mixed with 1 µl of loading buffer and loaded carefully by mechanical pipet to the gel wells.
2. The electrophoresis was carried out by setting the device on 100 volts and 50 mA for 15 minutes.
3. The gel then imaged, and the image analyzed by CS analyzer® software to determine the extracted DNA molecular weight.

2-7-7: Polymerase Chain Reaction Amplification

2-7-7-1: Principle of Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technology used for quick and easy amplifying DNA sequences, which is based on the principle of enzymatic replication of nucleic acids (Staněk, 2013).

2-7-7-2: Reconstituting and Diluting Primers

The nuclease-free H_2O was added to each primer to obtain a master stock that would be used again to obtain a working stock. The following steps were followed for reconstituting and diluting the primers

1. The tubes were spin down before opening the caps.
2. The desired amount of free nuclease water was added according to the guidelines of manufacturer to obtain a 100 Pico moles/µl (Master Stock).
3. The tubes were vortex properly for re-suspend the primers evenly.
4. A volume of 10 µl of the each primers stock was transferred to a 1.5 ml eppendorf tube that contains 180 µl of sterile nuclease-free water (working stock of primer mix).

5. The master stock was stored at -20 ˚C.
6. The working stock was stored at -20 ˚C
7. The working stock was thawed on ice and vortexes before using in PCR and then stored at -20 ˚C.

2-7-7-3: Optimization of Polymerase Chain Reaction Conditions

The Na-K ATPase alpha gene in the current study was amplified by PCR amplification performed in a programmable thermal cycler gradient PCR system. The amplification of each target region was optimized by gradient PCR, and the best efficient and specific annealing temperature that produce the most efficient, specific product was chosen for further PCR amplification procedure, the latter was performed according to add the components for amplification of each SNP as in Table 2-11 and the starting conditions for PCR were listed in Table 2-12. After optimization, the best thermo-cycling conditions for all SNPs were listed in Table 2-13 for gene *ATP1A1* and Table 2-14 for gene *ATP1A2*.

Table 2-11: The PCR Ingredients Concentration for Each Optimization Process

No.	Components	Concentration	Volume
1.	DNA	30-40 ng/µl	2 µl
2.	Master Mix	2.5X	8 µl
3.	Forward Primer	100 Pico moles/µl	1 µl
4.	Reverse Primer	100 Pico moles/µl	1 µl
5.	$MgCl_2$	25 mM	0.5 µl
6.	Nuclease Free Water	/	7.5 µl
7.	Final Reaction Volume	/	20 µl

Table 2-12: The Starting Thermo-Cycling Condition for PCR Products for SNPs Studied

No.		Stage	Temperature	Incubation Time	Cycle Number
1		Initial Denaturation	94 C°	5 min	1
2	I	Denaturation	94 C°	30 sec	35
	II	Annealing	55-66 C°	30 sec	
	III	Polymerization	72 C°	30 sec	
3		Final Polymerization	72 C°	5 min	1

Table 2-13: Optimized Thermo-Cycling Condition for Gene *ATP1A1* (PCR-REFLP)

No.		Stage	Temperature	Incubation Time	Cycle Number
1		Initial Denaturation	94 C°	5 min	1
2	I	Denaturation	94 C°	30 sec	35
	II	Annealing	60 C°	30 sec	
	III	Polymerization	72 C°	30 sec	
3		Final Polymerization	72 C°	5 min	1

Then the amplification products were separated by electrophoresis through 2.5% agarose gel stained with ethidium bromide. The PCR product length was 189 b.

Table 2-14: Optimized Thermo-Cycling Condition for Gene *ATP1A2* (PCR-SSCP)

No.		Stage	Temperature	Incubation Time	Cycle Number
1		Initial Denaturation	94 C°	5 min	1
2	I	Denaturation	94 C°	30 sec	35
	II	Annealing	63 C°	30 sec	
	III	Polymerization	72 C°	30 sec	
3		Final Polymerization	72 C°	5 min	1

Then the amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide. The PCR product length was 380 b.

2-7-8: Restriction Digestion for PCR-RFLP for Gene *ATP1A1*

The restriction reaction carried according to manufacture instructions by mixing the following

1. Five µl of PCR product.
2. Eight µl of water and half units of the selected restriction enzyme.
3. Restriction buffer (10X) and 1.5 µl (each restriction enzyme has its restriction buffer supplied by the manufacturer).
4. Bovine serum albumin 0.15 µl.
5. The reaction mixture was incubated at 37 Cº for 16 hours.
6. The restriction reaction product was resolved on 2.5% agarose electrophoresis.

2-7-9: Single-Strand Conformation Polymorphism for Gene *ATP1A2*

2-7-9-1: Principle of Single-Strand Conformation Polymorphism

Single-strand conformational polymorphism (SSCP) analysis is an easy and critical technique for mutation disclosure and genotyping. The precept of SSCP analysis is dependent on the fact that single-stranded DNA has a defined conformation. Altered modulation due to a single base variation in the sequence can reason single-stranded DNA to immigrate differently down nondenaturing electrophoresis conditions (Dong and Zhu, 2005).

2-7-9-2: Polyacrylamide - Bisacrylamide Stock Solution Preparation

Stock solution 30% w/v of 29.7: 0.3 of acrylamide – bisacrylamide was prepared by weighing 29.7 gm of acrylamide and 0.3 gm of bisacrylamide then dissolved in ultra-pure distill water and complete the volume to 100 ml. The stock solution is wrapped with aluminum foil and stored in 40 ˚C.

2-7-9-3: Preparation of Loading Dye

Dissolve 0.025 g of bromophenol blue and 0.025 g of xylene cyanol in 9.5 ml formamide then add 100 µl sodium hydroxide. Make up to 10 ml with deionized water.

2-7-9-4: Preparation and Separation of Sample for SSCP Electrophoresis

The PCR product length and the specificity of the PCR reaction for each SNPs were checked by polyacrylamide gel electrophoresis, the electrophoresis was carried out according to (Sambrook and Russell, 2001) briefly as the following

1. The two glasses of the electrophoreses device was washed and dried by alcohol, then reassembled to the device according to the manufacture directions.
2. The sixteen of polyacrylamide gel (10%) was prepared by mixed from a 3.3 ml of polyacrylamide stock solution (30%), 2 ml of 5X TBE buffer, 5 ml of double distilled water, 200 µl of ammonium persulfate (10%) and 20 µl of TEMED was added and mixed briefly by stiriling.
3. As quickly as possible the mixture was poured to the device by disposable pasteur pipette, and the wells forming comb was inserted gently to its location.
4. The gel was brood in 37°C for 45 minutes to polymerize.
5. After the polymerization, the tank of electrophoresis apparatus was filled in 1X TBE buffer, and the comb was taken away, then the wells were washed of polyacrylamide gel by the buffer.
6. A pre-run was carried out for 30 minutes under a constant current 50 mA and 100 V.
7. After the pre-run the eppendorf was contain 2.5 µl of the sample and 2.5 µl dye were put in water bath at 90°C for 10 minutes, and the tube directly was placed into ice, then the sample loaded in the well by mechanical pipet.

8. The electrophoresis run was carried out until the bromophenol blue dye reach to the tow third of the gel, under a constant current 50 mA and 100V for 6 hours.
9. The gel then stained by first solution (stain solution) was prepared from 10% ethanol, 0.5 % acetic acid and 0.2 gm silver nitrate, the second solution was used to development reaction, it prepared from 3 gm sodium hydroxide and 0.1% formaldehyde, the mixture were complete to 100 ml of water and then put in water bath at 60°C. and stop solution is the last solution was added to stop reaction , it was prepared from 10% ethanol and 0.5 % acetic acid.
10. The gel then imaged, and the image analyzed by CS analyzer software.

2-7-10: Sequencing for Gene *ATP1A2*

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.

2-7-10-1: DNA Sequence Data Analysis

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

2-7-10-2: Interpretation of Sequencing Data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed if with the respective sequences in the reference database

using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in SSCP-PCR amplicons as well as in its corresponding position within the referring genome.

2-7-10-3: Checking the Novelty of SNPs

The observed SNPs were submitted to the dbSNP database to check their originality. Each SNP was re-positioned according to its place in the reference genome subsequently, the determination of the presence of previous SNP

2-8: Histologic and Immunohistochemical Staining Test

2-8-1: Preparation of Histological Sections

Placental tissue samples were taken with a thickness of 2 mm. Tissue processing was done according to (Suvarna *et al*., 2019) briefly as the following protocol

1. **Fixation**

   The fixation step was done by using formal saline for four hours.

2. **Dehydration**

   The dehydration step was made by increasing concentrations of alcohols (changes of alcohol solutions in 70%, 80%, 90%, 100%, each change of concentration incubated for two hours).

3. **Clearing**

   The clearing was done by three changes of xylene, two hours for each.

4. **Paraffin Embedding**

   Paraffin embedding was done by two changes of melted paraffin on 65 ˚C each for two hours.

5. **Blocking**

   Paraffin blocks of tissue were made.

6. **Sectioning**

   Slides of 3-5 micrometers thick were made using a special instrument known as the microtome.

7. **Deparaffinization**

   The paraffin was removed from prepared slides by hot air oven for 2 hours at 90˚C.

8. **Clearing**

   The excess paraffin was removed from slides by using two changes of xylene each change for 15 min.

9. **Rehydration**

   The rehydration step was done by decreasing concentrations of alcohol (three changes, each of 2 min.), then running in tap water.

10. **Staining**

    a. Harris hematoxylin used to stain for 3 minutes, then rinsed for 1 minute with tap water.

    b. Staining with eosine for 10 sec. then, rinsed in running tap water.

11. **Dehydration**

    The dehydration step was made by increasing concentrations of alcohols, each for two minutes.

12. **Clearing in Xylene**

    The clearing was done by two changes of xylene, ten minutes for each.

13. **Mounting in mounting media (Canada balsam or DPX)**

    The slides at this step have been ready for examination.

2-8-2: Immunohistochemistry Staining Test

2-8-2-1: Principle of Immunohistochemistry Staining

This technique depends on the detection of the product of gene expression (protein) in cells using specific monoclonal antibodies, i.e., primary antibody for a specific epitope that binds to nuclear-targeted protein. The bound primary antibody

is then detected by a secondary antibody, which contains a specific label (in this context we used peroxidase-labeled polymer conjugated to anti-immunoglobulin). The substrate is DAB in chromogen solution. A positive reaction will result in a brown-colored precipitate at the antigen site in tested tissue (O'Hurley *et al*., 2014).

2-8-2-2: Immunohistochemistry Staining Protocol

The immunohistochemistry detection kit is a reliable and convenient tool to identify specified gene expression on tissues. In this study, the procedure of immunohistochemistry staining was carried out according to a kit from Bio SB, USA (Suvarna *et al*., 2019), and using Na-K ATPase antibody as a marker. This method includes many steps illustrated as following

1. The section with paraffin embedding tissue was cut by microtome at 4 microns.
2. The slides were put in an incubator at 60 ˚C for overnight.
3. The dewaxing was removed by xylene three times, each for 5 min.
4. The slides were dehydrated by ethanol alcohol, by using different concentrations of 100%, 90%, 70%, 50% each change of concentration incubated for 5 min.
5. The retrieval solution was put in the water bath and when the temperature was 65˚C the slides were put and when temperature 99˚C was left for 20 min.
6. The slides were removed from the retrieval solution and let cold then, washed with buffer solution three times.
7. The slides were made with peroxidase solution for 5 min. then, washed with buffer solution three times.
8. The primary antibody was added for 40 min. then, washed with buffer solution three times.
9. The secondary antibody was permitted to link for 15 min. then, washed with buffer solution three times.

10. The HRP was applied for 15 min. then, washed with buffer solution three times.
11. The DAB was added for 15 min. then, washed with buffer solution 3 times.
12. Counter stain was added on the slides (Harris's Hematoxylin was used to stain the nucleus, and Eosin Y was used to stain cytosol) for 1 min.
13. The slides were washed with D.W. and repeated steps of rehydration and clearing.
14. The cover slide was mounted, then examined by a microscope.

2-8-2-3: Evaluation of Immunohistochemistry Staining Results

Immunohistochemistry was given intensity and percentage scores, based on the intensity of positive staining and number of cells staining, respectively. A scale of (0-4) was used to measure relative intensity with (0) corresponding to no detectable IHC reaction and (1-4) equivalents to low, moderate, and high, respectively. Positive cells were counted in ten different fields at 1000 X for each sample and the average of positive cells of the ten fields was determined assigning cases to one of the following scores as below

- **Score 0 (Negative):** No stained cells.
- **Score 1 (+):** The positive cells (stained) represented less than 10% of total cells.
- **Score 2 (++):** The positive cells (stained) represented more than 10% - 30% of total cells.
- **Score 3 (+++):** The positive cells (stained) represented more than 30% - 50% of total cells.
- **Score 4 (++++):** The positive cells (stained) represented more than 50% of total cells.

2-8-2-4: Photography

Photography was done using digital camera Scopelmage 9.0 (MDCE-5C) with high effective and 24-bit true color or higher.

2-9: Statistical Analysis

The statistical analysis was carried as follow by using many software

1. The analysis of results was carried out using statistical package of the social sciences (SPSS®) software version 26 to get the variables as mean, standard error for mean (SEM), confidence interval, and T-test for comparison between two groups.
2. Pearson correlation coefficient (r) was used to determine correlation between some of the biochemical parameters in pregnancy and pre-eclampsia. A p-value of ≤ 0.05 was considered significant.
3. The phenotypic odds ratio calculated by the aid of Medcalc software version 20.
4. Phenotypic means and standard deviation were compared by student T-test by employing IBM®SPSS® software.
5. The genetic association parameters were carried out by the aid of SNPStats® online software (Solé *et al*., 2006). Except, that genotypes association which was carried out by Fisher's exact test by the aid of Quickcalcs software from GraphPad®.

3-1: Demographic Characteristics of the Study Groups

Preeclamptic is a multisystem and multifactorial illness that reason intrauterine growth restriction and vascular dysfunction in both the mother and the fetus (Al-Jameil *et al*., 2014). Although there is much ongoing research in the domain of preeclamptic the definitive reason for this condition is still unknown. Different reasons for this syndrome have been assuming, including abnormal formation of the placenta, chemical imbalances, genetic factors, and immunological mechanisms (Romero and Chaiworapongsa, 2013; Gathiram and Moodley, 2016).

In this study, preeclamptic has been specifically dealt with because although it is a well-established menace to the woman and to her fetus, they are also considered as the main contributors of maternal and perinatal morbidity (Witlin *et al*., 2000).

The demographic characteristics for the study groups, represented by mean ± SEM after adjustment for the matching criteria, maternal age. The present study showed no statistically significant difference in maternal age between normal pregnancy and preeclamptic. These results of maternal age are similar to the findings of other studies such as (Kumar *et al*., 2010; Shamsi *et al*., 2010; Fedorova *et al*., 2018).

The control group samples were randomly selected and clustered in a way that simulates the age distribution of the patient group. This maternal age matching helps to eliminate differences in the results of parameters that may arise because of the significant maternal age variation. The maternal age distribution for preeclamptic patients is representing in Figure 3-1. The mean and SEM in preeclamptic are 26.978 ± 0.862 respectively.

There is an acceptance that preeclamptic occurs more often in women under 20 or over 35 years of age (Valensise *et al*., 2008), but the present study shows that age

groups (20–30) were significantly the safe age against the occurrence of preeclamptic. While the risk is significant among the age group 35 years and more.

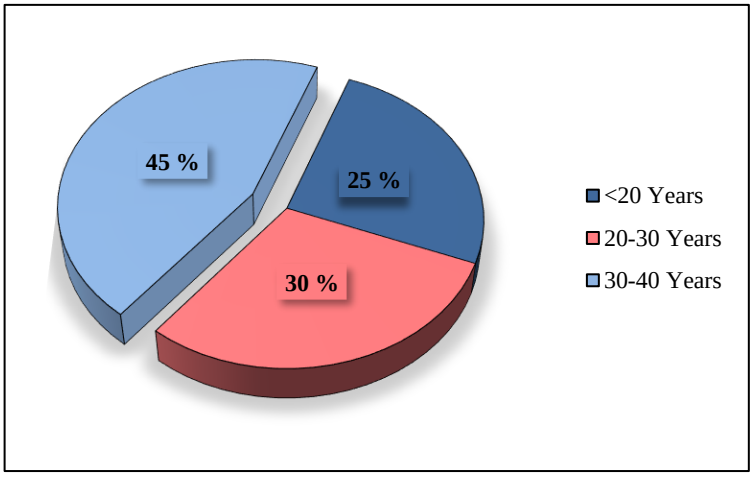


Figure 3-1: Maternal Age Distribution in Patients of Preeclamptic

As well, the mean and SEM values of the clinical characteristics of the control and patient groups are shown in Table 3-1.

Table 3-1: The Demographic Characteristics of Groups Studied

Variables	Control Mean ± SEM	Patient Mean ± SEM	P- Value
Albumin in Urine	0.225 ± 0.100	2.806 ± 0.242	<0.001*
Diastolic BP (mmHg)	77.105 ± 1.219	98.205 ± 1.936	<0.001*
Systolic BP (mmHg)	117.94 ±1.690	149.512 ± 1.708	<0.001*
Gestational Age (Weeks)	36.105± 0.321	34.846 ± 0.518	0.043*
Weight of Baby (gm)	3104.80 ± 74.41	2726.30 ± 144.04	0.025*

*P-value < 0.05 was significant

The results for mean systolic and mean diastolic pressures in preeclamptic were shown significantly higher than in normal pregnant women. As well, the mean of albumin in the urine in preeclamptic is significantly higher than that of normal pregnant women. However, these results are expected because of the criteria used for preeclamptic diagnosis.

Clinically, preeclamptic presents as hypertension, proteinuria, with or without edema during pregnancy (Albayrak *et al*., 2010). Even with an adequate electrolyte and water content during pre-eclampsia, these are mainly located in the interstitial with a resulting decrease in intravascular circulating volume. This reduction in intravascular circulation volume results in the activation of baroreceptors and liberation of antidiuretic hormone (ADH) water retention and natriuretic (Darkwa *et al*., 2017).

The other clinical features include gestational age. The present study shows that there is a very highly significant difference $P < 0.043$ in the mean weeks of gestation at labor which is lower in cases 34.846 weeks than in controls 36.105 weeks. Also, the induction rate and cesarean section are more in cases than in controls.

These differences are due to the fact that delivery is the definitive treatment of PE and should be considered regardless of gestational age if any of the following "ominous" features are present such as severe persistent hypertension > 160/110 mmHg, deteriorating renal function, the sign of cerebral edema that may precede fit, severe HELLP syndrome, the sign of hepatic involvement, IUGR and, hemoconcentration (James and Nelson-Piercy, 2004; Mammaro *et al*., 2009).

In addition, so the results of baby weight appeared significantly lower in pre-eclampsia, than in normal pregnant women. For live births, comparing the birth weight of cases newborns to that of controls newborns (without adjustment for the gestational age) reveals a highly significant risk $P= 0.025$ for cases to give birth to low weight babies birth. It is well known that mothers who have PE usually have smaller babies. This is part the result of preterm birth or shortened gestational duration because early delivery is a consequence of PE, and it is the only effective treatment. This has masked the fact that most infants born to mothers with PE are not small when compared with infants born to mothers without PE at the same gestational age (Xiong *et al*., 2000).

These outcomes show that preeclamptic is correlated with low fetal weight and premature birth (Logue *et al*., 2016).

3-2: Biochemical Tests Results

3-2-1: Determination Specific Activity of Na-K ATPase

The Na-K ATPase is a membrane enzyme that regulates membrane potential, cell permeability of Na^+, K^+, and Ca^{2+}, as well as excitatory neurotransmitters. It also plays a role in the cell cycle and differentiation. The primary need for cellular homeostasis and many activities is the balance of Na^+ and K^+ between the intracellular and extracellular compartments (Kherd *et al*., 2017). The study's motivation was the possibility of using Na-K ATPase as a biomarker to diagnose preeclamptic.

In this study, the specific activity of Na-K ATPase was determined using a modified method that included ATP as an enzymatic reaction substrate, $MgCl_2$ as a cofactor, and Na-EDTA to inhibit Ca^{+2} ATPase. The specific activity of Na-K ATPase in a preeclamptic patients were significantly lower than in a normal pregnant woman, as shown in Table 3-2.

Table 3-2: The Specific Activity of Na-K ATPase in Control Group Compared with Preeclamptic

Specific Activity of Na-K ATPase mg/g. min	Groups	Mean ± SEM	P-value
	Patient	0.239 ± 0.043	0.002*
	Control	0.399 ± 0.021	

*P- value < 0.05 was significant

This method depends on the simultaneous estimation of inorganic phosphate (Pi) and protein content in a reaction mixture utilized to test the specific activity of Na-K ATPase. The current procedure is more suitable, accurate, and faster than other methods for evaluation Na-K ATPase activity. It also eliminates the possibility

of errors in protein determination due to high lipid content in other classical methods (Sarkar, 2002).

The results of specific activity of Na-K ATPase showed a good agreement with the previous studies, for instance, Carreiras *et al*., showed the erythrocyte Na-K ATPase activity was lower in pre-eclamptic women compared with normotensive for samples of the maternal and umbilical cord (neonate) (Carreiras *et al*., 2001).

Also, according to the results of Kherd *et al*., show the mean of specific activity of Na - K ATPase in pregnant women is higher than in healthy women. This could be attributable to the proportional involvement of enzymes in the metabolite transport mechanism or to thermogenesis during pregnancy (Kherd *et al*., 2017).

As well, according to the findings of Fedorova *et al*., (2018) show the erythrocyte Na-K ATPase activity was lower in preeclamptic than normal pregnant (Fedorova *et al*., 2018).

While the sensitively and specialty for modified method to determine specific activity of Na-K ATPase was calculated by using Medcalce software and results shown in the Table 3-3 and Figure 3-2.

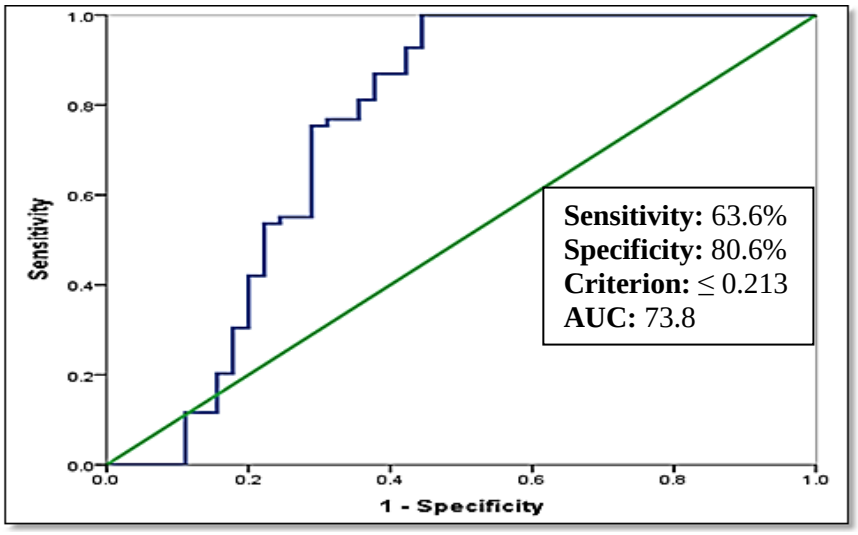


Figure 3-2: The ROC Curve for Modified Method of Determine Specific Activity of Na-K ATPase

Table 3-3: The Predicted Cut-off Value of Specific Activity of Na-K ATPase

Variables	Cut-off Value	Sensitivity	Specificity	+ PV	-PV	AUC
Specific Enzyme Activity mg/g. min	0.213	63.6%	80.6%	68.3%	77.1%	73.8

AUC: Area Under Curve

The results of this method have more specificity than sensitivity and the best cut off value as shown at 0.213 mg/g. min.

3-2-2: Evaluation the Inhibition Percentage of Endogenous Digitalis

Although endogenous digitalis like-factor (EDLF) is linked to the development of a variety of physical problems, its significance in preeclamptic is unknown (Peng *et al*., 2018). So, this study is investigating the association between endogenous digitalis and many clinical and biological pregnancy-related characteristics to evaluate endogenous digitalis as a novel and effective biomarker for preeclamptic diagnosis.

In this study, using a novel method to determine the percentage inhibition of endogenous digitalis by measuring specific enzyme activity in control and patients. The results of the inhibition percent of endogenous digitalis in patients with preeclamptic were significantly higher than normal pregnant women, as shown in Table 3-4. This method is the first procedure in Iraq used to determine the total inhibition percent of endogenous digitals than other methods which evaluate the concentration of types of endogenous digitals like ouabain and marinobufagenin.

Table 3-4: The Percent Inhibition of Endogenous Digitalis for Study Groups

Inhibition Percentage of Endogenous Digitalis	Groups	Mean ± SEM	95% Confidence Interval for Mean		P-value
			Lower	Upper	
	Patient	35.852 ± 2.692	14.402	21.526	<0.001*
	Control	17.964 ± 1.784	30.428	41.275	

While the sensitively and specificity for novel method was calculated by using Medcalce software and results shows in the Table 3-5 and Figure 3-3.

Table 3-5: The Predicted Cut-off Value of Inhibition Percentage of Endogenous Digitalis in Preeclamptic Patients

Variables	Cut-off Value	Sensitivity	Specificity	+ PV	-PV	AUC
Inhibition Percentage of Endogenous Digitals	31.65%	52.2%	85.1%	54.2%	83.8%	75.8

AUC: Area Under Curve

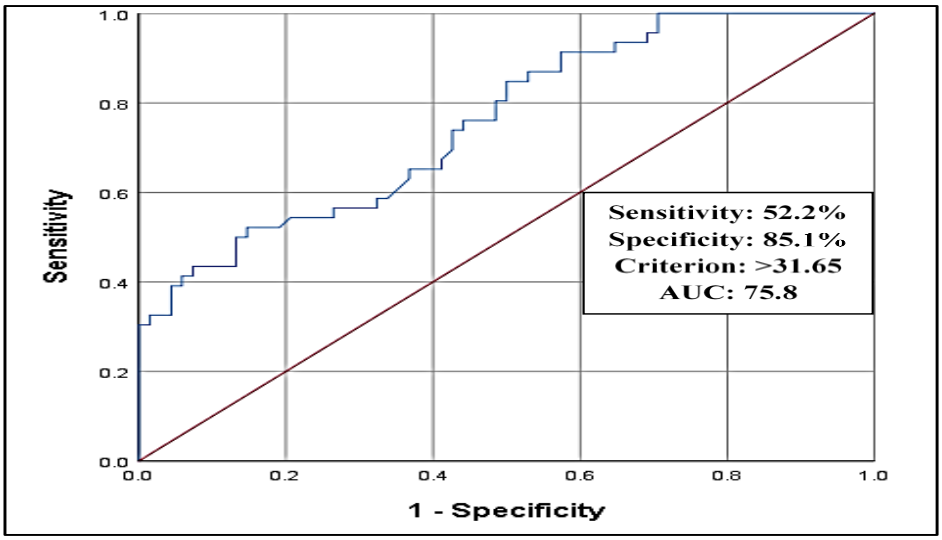


Figure 3-3: The ROC Curve for New Method to Evaluate the Inhibition Percentage of Endogenous Digitalis

The result of this method show more specificity than sensitivity and the best cut off value as shown at 31.65%.

3-2-3: Correlations between the Measured Biochemical Parameters

The result of the correlation between specific enzyme activity and criteria for digenesis preeclamptic are shown in Table 3-6 and this result shows an inverse relationship between enzyme activity, systolic pressure, and diastolic pressure. This finding is particularly remarkable since the more Na-K ATPase reduced, the more individual presented rises diastolic blood pressure, appeared by the significant negative correlation between these variables, which propose

a possible role for Na-K ATPase lowering in blood pressure increase (Malfatti *et al*., 2012).

While the results of the correlation between inhibition percent of endogenous digitalis and criteria for digenesis preeclamptic show a direct relationship between inhibition percent of endogenous digitalis, systolic pressure, diastolic pressure, and albumin in the urine.

Table 3-6: The Correlation among Specific Enzyme Activity, Inhibition Percent of Endogenous Digitalis and Criteria for Digenesis Preeclamptic

Variables	Systolic Pressure		Diastolic Pressure		Albumin in Urine	
	r	P-Value	r	P-Value	r	P-Value
Endogenous Digitalis	0.672**	<0.001*	0.588**	<0.001*	0.731**	<0.001*
Enzyme Activity	-0.290*	0.012	-0.313**	0.007	-0.163	0.206

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05

Additionally, the results of the correlation between specific enzyme activity of Na-K ATPase and inhibition percent of endogenous digitalis are shown an inverse relationship between specific enzyme activity and inhibition present of endogenous digitalis.

3-3: Genetic Results

3-3-1: Genetic Results of *ATP1A1* Gene Polymorphisms

ATP1A1 is found in human chromosome 1 area 1p2l cen and is practically expressed throughout the body, particularly in the kidneys and central nervous system (CNS) (Lin *et al*., 2021). On top of each kidney exist adrenal glands, which are hormone-producing (Hiller-Sturmhöfel and Bartke, 1998). The passage of sodium and potassium ions in the adrenal glands helps regulate the synthesis of the hormone aldosterone, which controls blood pressure by maintaining adequate salt and fluid levels in the body (Beuschlein *et al*., 2013).

Mutations in the *ATP1A1* gene impair the Na-K ATPase's normal function. The malfunctioning pumps permit sodium or hydrogen (H^+) ions to enter the cell. The abnormal flow of these ions raises aldosterone production (Stindl *et al*., 2015). So, this excess in aldosterone leads to high blood pressure and an increased risk of heart attack and stroke (Tsai *et al*., 2021).

In the present study, the genotyping for *ATP1A1* (rs10924081) gene was detected using PCR-RFLP; to find out the alleles frequencies are associated with preeclamptic disease and genotypes frequencies in control and preeclamptic under different models of inheritance.

3-3-1-1: Optimization of rs10924081 PCR-RFLP Genotyping

The starting thermo-cycling condition for the PCR product of the designed primers pair is shown in Figure 3-4. The results show the best annealing temperature are 60 ◦C which be used for *ATP1A1* gene polymorphism.

While the results of the optimized PCR product of the designed primers pair which be used in rs10924081 genotyping is 189 bp as shown in Figure 3-5.

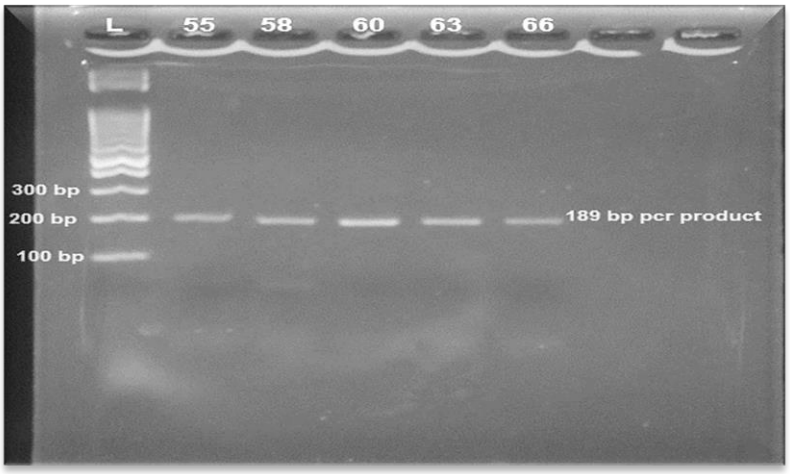


Figure 3-4: Starting Thermo-Cycling Condition for rs10924081, the Electrophoresis Carried Out on Agarose Gel (2.5%), Lane L = 100 bp, Other Lanes= 189 bp for PCR Product

Figure 3-5: Optimized for PCR Product for rs10924081, the Electrophoresis Carried Out on Agarose Gel (2.5%), Lane L = 100 bp, Other Lanes= 189 bp for PCR Product

The PCR product was digested with Tru91 restriction enzyme. This enzyme is cut the product of PCR in T/TAA. The Figure 3-6 shows the electrophoresis bands pattern of Tru91 digested PCR product.

In this study it will spilt the 189 bp in two polynucleotide chains with a molecular weight of 52 bp and 138 bp if the allele G presents in the PCR product. Otherwise when allele A found in PCR product the 189 bp spilt to three polynucleotide chains with molecular weight 88 bp, 52 bp and 49 bp.

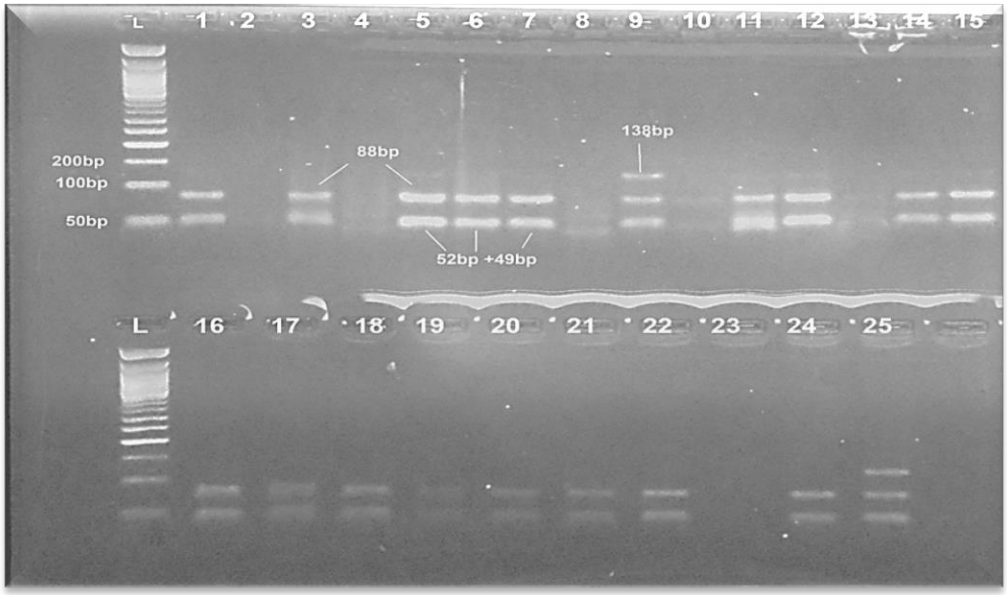


Figure 3-6: PCR RFLP Genotype for rs10924081 (Lane L: DNA Ladder ; Lanes 9 and 25 AG Genotype ; Lanes 2,4,8 and 23 Negative Lanes ; Other Lanes AA Genotype)

3-3-1-2: Allelic and Genotypic Association of rs10924081 with Pre-eclampsia

Alleles frequency for patients and control are listed in the Table 3-7. The results showed that there was no significant allele frequency difference between patients and control groups.

Table 3-7: Alleles Frequency and Allelic Association of rs10924081

Allele	Control		Case		OR (95% CI)	P-Value
	Count	Proportion	Count	Proportion		
A	92	0.92	67	0.96	1.942 (0.497-7.595)	0.528
G	8	0.08	3	0.04	0.515 (0.132-2.014)	

The findings of our study revealed that the allele frequencies of the Babylon province population are within the global range and do not exhibit extreme values, as shown in Figure 3-7.

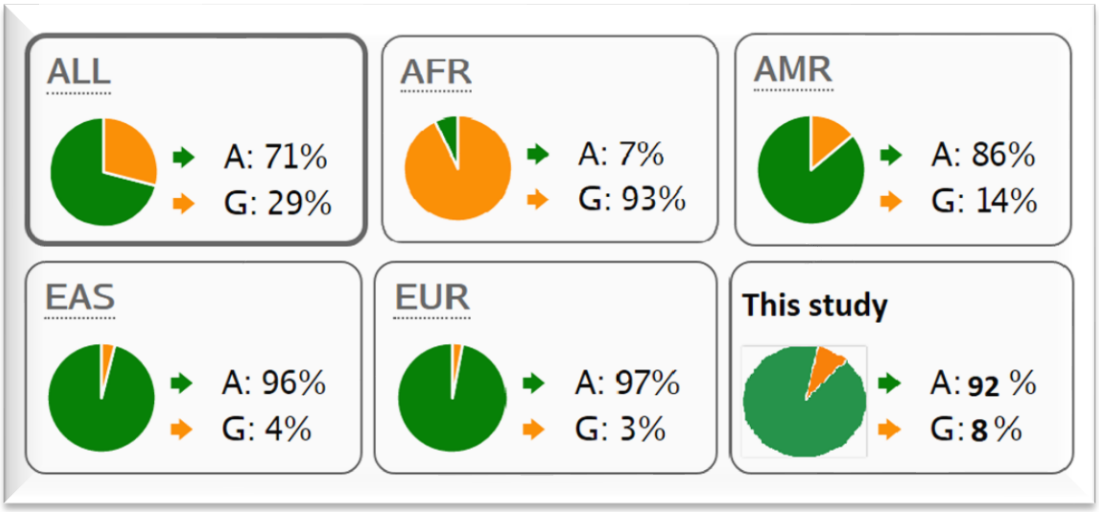


Figure 3-7: Alleles Frequency of rs10924081 in Babylon Province Population Compared to Several Populations. The Data and Figures Other Than Studied Population are Adapted From 1000 Genomes Project Phase (ALL= All Phase, AFR= Africans, AMR= Americans EAS= East Asians, EUR=Europeans)

Also, the results from Hardy-Weinberg equilibrium exact test revealed that the both control and patient groups genotype frequency flow the Hardy-Weinberg equilibrium as seen in the Table 3-8.

Table 3-8: Genotypes Frequency of rs10924081 and the Deviation from Hardy- Weinberg Equilibrium Represented by the Exact Test P-Value

Group	Genotype					P-value
	AA	AG	GG	A	G	
Control	42	8	0	92	8	1.00
Case	32	3	0	67	3	1.00

The association of each genotype with preeclampticwas further tested under different models of inheritance. The result seen that there was no significant association of AA (homozygote) and AG (heterozygote) genotype with pre-eclampsia, as seen in the Table 3-9.

Table 3-9: Association of rs10924081 Genotypes with Preeclamptic Under Different Models of Inheritance

Genotype	Control	Case	OR (95% CI)	P-Value
A/A	42 (84%)	32 (91.4%)	1.00	0.30
A/G	8 (16%)	3 (8.6%)	0.49 (0.12-2.00)	

3-3-1-3: Association of rs10924081 Allelic and Genotypic with Biochemical Results

The results of correlation among rs10924081 allelic and genotypic with enzyme activity and endogenous digitalis are shown in Table 3-10 and resalts appeared no association between rs10924081 allelic, enzyme activity, and endogenous digitalis.

Table 3-10: Association of rs10924081 Genotypes with Enzyme Activity and Endogenous Digitalis

Biochemical Test	Genotype	Mean ± SEM	Mean Difference	P-value
Enzyme Activity mg/g.min	A/A	0.245 ± 0.029	-0.113	0.126
	A/G	0.358 ± 0.064		
Endogenous Digitalis	A/A	22.613 ± 2.451	4.76	0.474
	A/G	17.853 ± 7.954		

3-3-2: Genetic Results of *ATP1A2* Gene Polymorphisms

Alpha-2 subunits are primarily located in nervous system cells termed glia, which protect and maintain nerve cells (neurons). During its action in glia, the protein plays a significant function in the normal role of neurons (Castro *et al*., 2008).

In this study, the genotyping for *ATP1A2* (rs373796693) gene was detected using PCR-SSCP then sequence; to find out which alleles frequencies are linked with preeclamptic disease and genotypes frequencies in control and preeclamptic under various forms of inheritance.

3-3-2-1: Optimization of rs373796693 PCR-SSCP Genotyping

The starting thermo-cycling condition for the PCR product of the designed primers pair is shown in Figure 3-8. The results appear the best annealing temperature are 63 C° which be utilized for *ATP1A2* gene polymorphism. While the result of the optimized PCR product of the designed primers pair which be used for rs373796693 genotyping is 380 bp as seen in Figure 3-9.

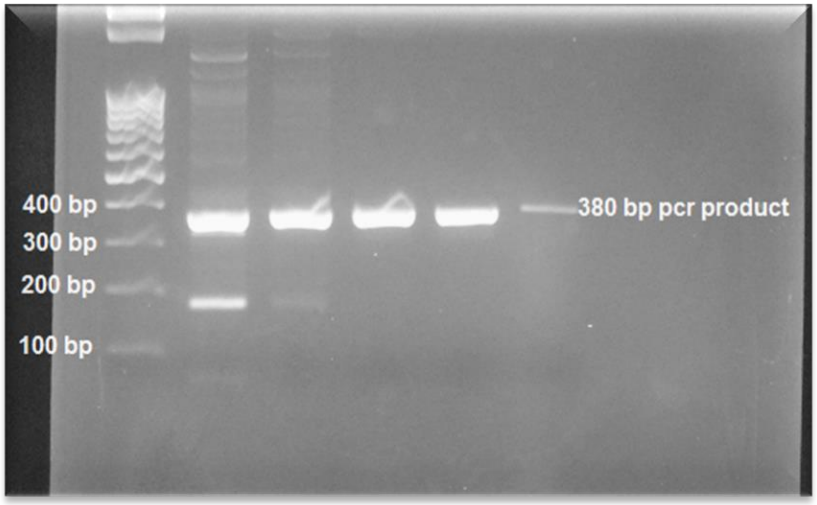


Figure 3-8: Starting Thermo-Cycling Condition for rs373796693, the Electrophoresis Carried PCR Product Out on Agarose Gel (2%), Lane L = 100 bp, Other Lanes= 380 bp for

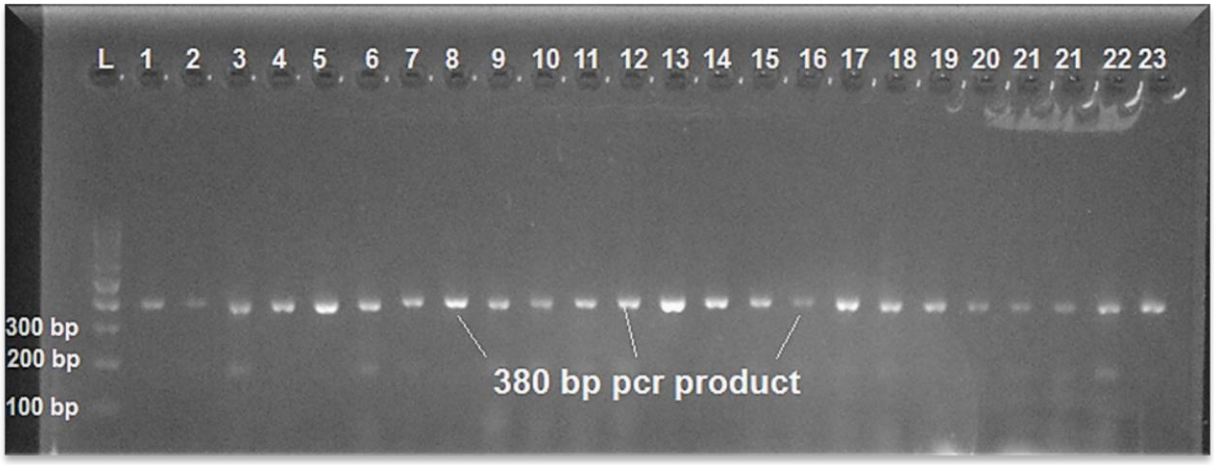


Figure 3-9: Optimized for PCR Product for rs373796693, the Electrophoresis Carried Out on Agarose Gel (2%), Lane L = 100 bp, Other Lanes= 380 bp for PCR Product

3-3-2-2: Single Stand Conformation Polymorphism of rs373796693 Genotyping

The amplification product of rs373796693 of *ATP1A2* gene polymorphism was analyzed by PCR –SSCP technique as shown in Figure 3-10 which contain three patterns of polymorphism obtained by sequencing technique.

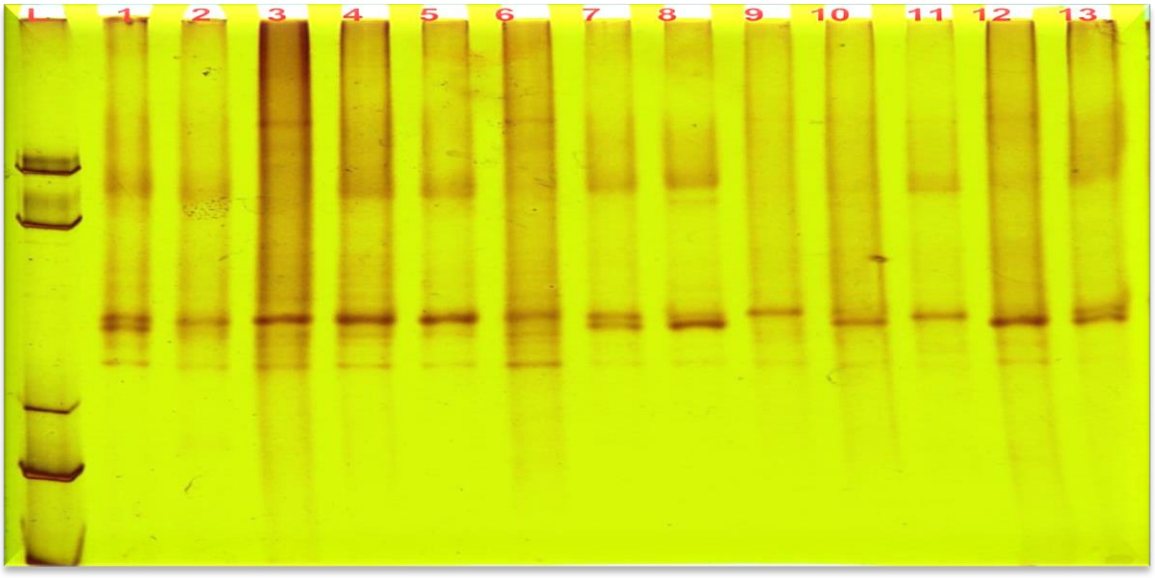


Figure 3-10: SSCP Genotyping of the Selected Fragment of *ATP1A2* Gene, Lane L DNA Ladder, Lanes 2,3,4,5,6,9,11 II Genotype; Lanes 8,10,12 DD Genotype; Lanes 1,7,13 ID Genotype

3-3-2-3: Allelic and Genotypic Association of rs373796693 with Pre-eclampsia

Alleles frequency for patients and control are listed in the Table (3-11). The results showed that there was no significant allele frequency difference between patients and control groups.

Table 3-11: Alleles Frequency and Allelic Association of rs373796693

Allele	Control		Case		OR (95% CI)	P-Value
	Count	Proportion	Count	Proportion		
D	65	0.65	34	0.53	0.610 (0.322-1.158)	0.120
I	35	0.35	30	0.47	1.639 (0.864-3.108)	

The findings of our study revealed that the allele frequencies of the Babylon province population are within the global range and do not exhibit extreme values, as shown in Figure 3-11.

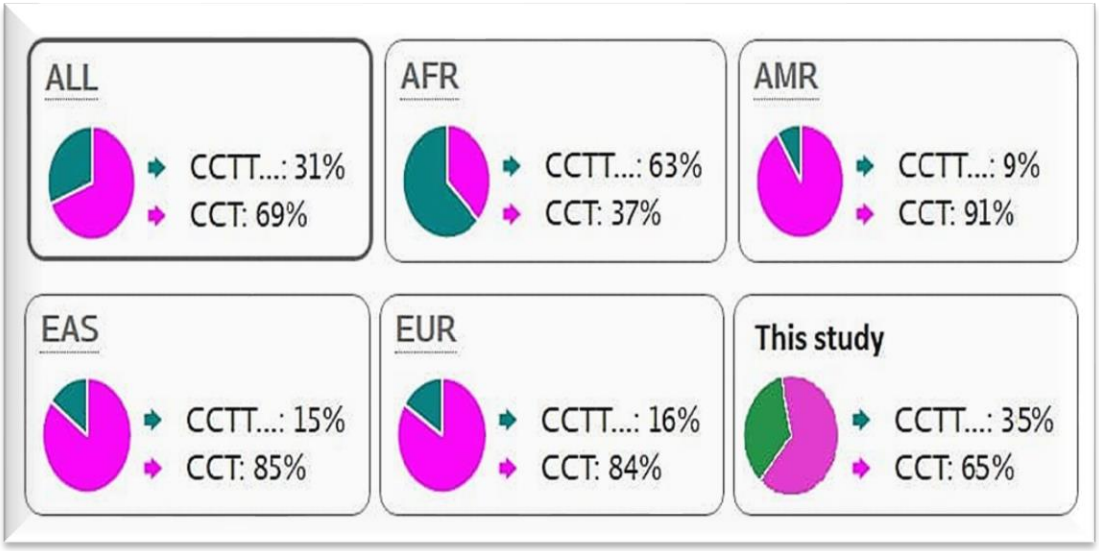


Figure 3-11: Alleles Frequency of rs373796693 in Babylon Province Population Compared to Several Populations. The Data and Figures Other Than Studied Population are Adapted From 1000 Genomes Project Phase (ALL= All Phase, AFR= Africans, AMR= Americans EAS= East Asians, EUR=Europeans)

The result from Hardy-Weinberg equilibrium exact test revealed that the both control and patient groups genotype frequency flow the Hardy-Weinberg equilibrium as seen in the Table 3-12.

Table 3-12: Genotypes Frequency of rs373796693 and the Deviation from Hardy- Weinberg Equilibrium Represented by the Exact Test P-Value

Group	Genotype					P-value
	DD	DI	II	D	I	
Control	23	19	8	65	35	0.23
Case	10	14	8	34	30	0.49

The association of each genotype with preeclamptic was further tested under different models of inheritance. The result seen that there was no significant association of DD, II (homozygote) and DI (heterozygote) genotype with pre-eclampsia, as seen in the Table 3-13.

Table 3-13: Association of rs373796693 Genotypes with Preeclamptic Under Different Models of Inheritance

Model	Genotype	control	Case	OR (95% CI)	P-value
Codominant	D/D	23 (46%)	10 (31.2%)	1.00	0.36
	I/D	19 (38%)	14 (43.8%)	1.69 (0.61-4.67)	
	I/I	8 (16%)	8 (25%)	2.30 (0.67-7.86)	
Dominant	D/D	23 (46%)	10 (31.2%)	1.00	0.18
	I/D-I/I	27 (54%)	22 (68.8%)	1.87 (0.74-4.76)	
Recessive	D/D-I/D	42 (84%)	24 (75%)	1.00	0.32
	I/I	8 (16%)	8 (25%)	1.75 (0.58-5.26)	
Overdominant	D/D-I/I	31 (62%)	18 (56.2%)	1.00	0.6
	I/D	19 (38%)	14 (43.8%)	1.27 (0.51-3.13)	

3-3-2-4: Sequence for *ATP1A2* Gene Polymorphism

The alignment results of the 380 bp samples revealed the presence of an interesting deletion in some of the analyzed samples in comparison with the referring reference DNA sequences were shown in the Figure 3-12.

The results of sequence observed that TCCT deletion was detected in some of the investigated samples, in which three polymorphic patterns were identified in the investigated samples as shown in Figure 3-13.

Also, the sequencing chromatogram of the identified variation region, as well as its detailed annotations, were documented, and the chromatogram of this sequence was shown according to its position in the PCR amplicon. However, this deletion was detected in heterozygous form in both S1 and S2 as shown in Figure 3-13 A.

Meanwhile, both S3 and S4 exhibited a particular homozygous form of the same observed deletion as seen in Figure 3-13 B, and both S5 and S6 exhibited a non-deleted homozygous form for the same investigated polymorphic locus as appeared in Figure 3-13 C.

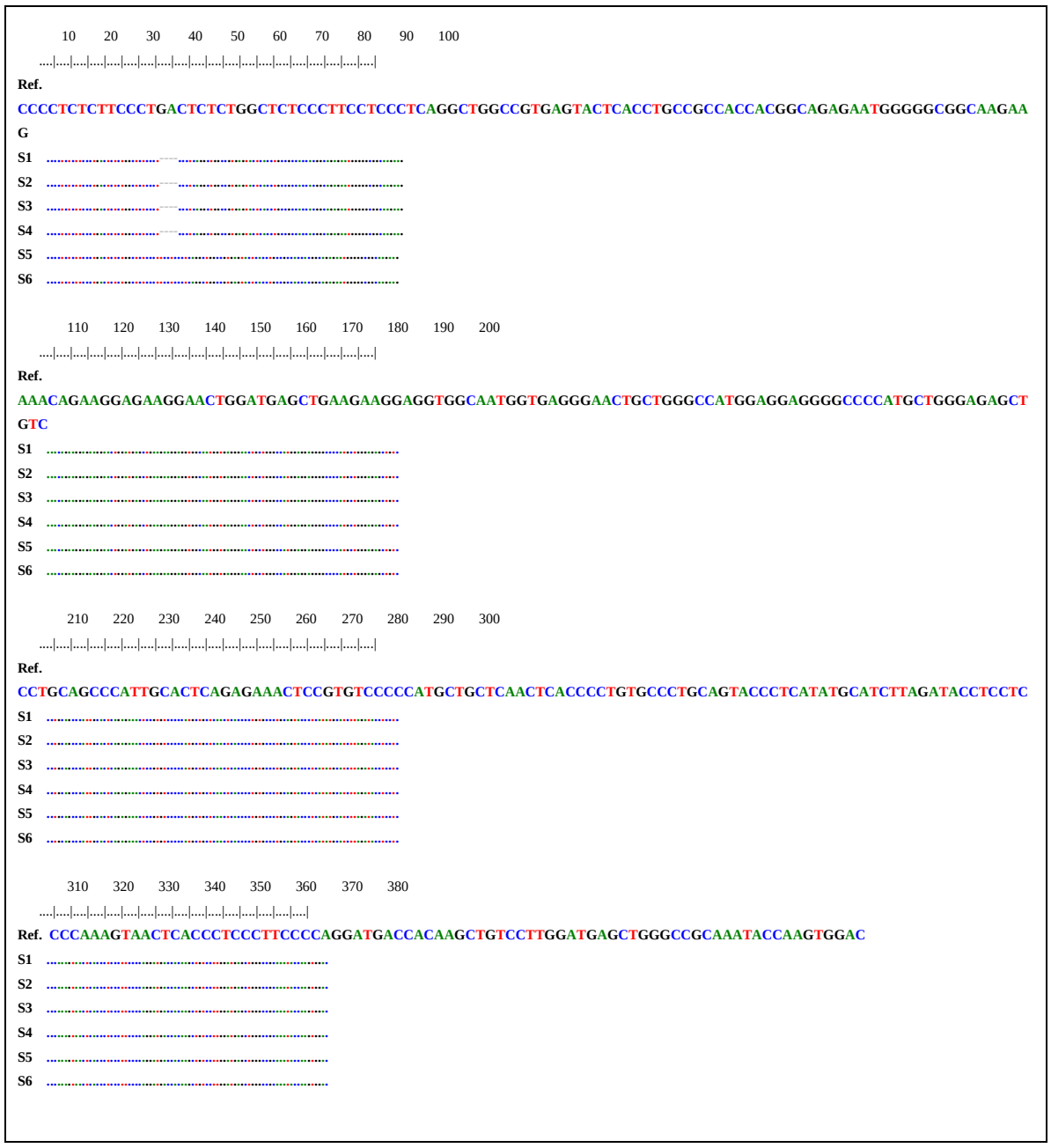


Figure 3-12: DNA sequences alignment of the 380 bp amplicons of the *ATP1A2* gene. The symbol "Ref." refers to the NCBI referring sequence, "S1-S6" refer to the samples 1 to 6, respectively.

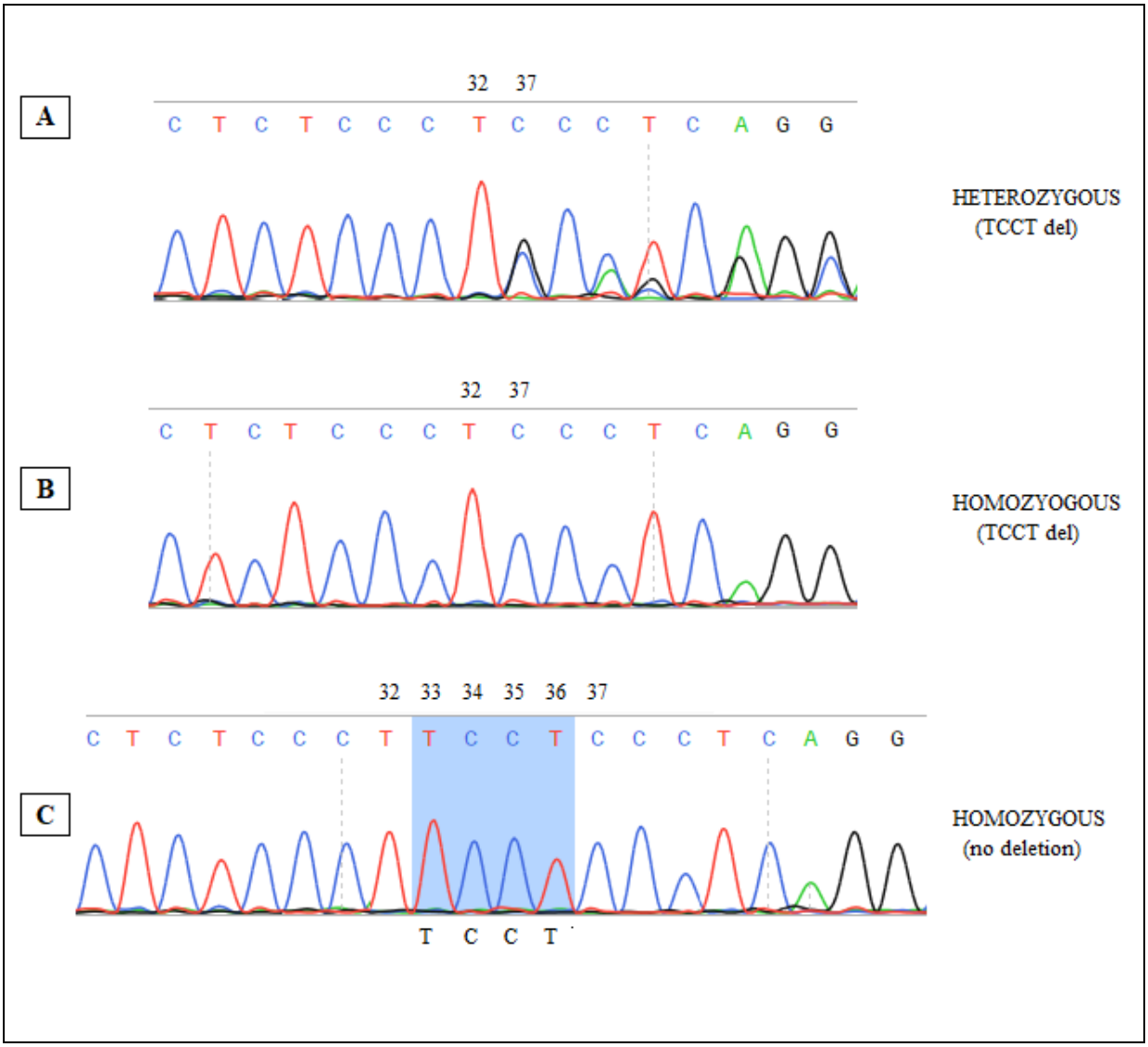


Figure 3-13: The pattern of the detected SNP within the DNA chromatogram of the targeted 380 bp amplicons of the *ATP1A2* gene. The identified SNP was highlighted according to its position in the PCR amplicons

To elucidate the position of the targeted SNP concerning their deposited SNP database of the sequenced 380 bp fragment, the corresponding position of the *ATP1A2* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/).

To find out the nature of this SNP, a graphical representation was performed concerning the ATP1A2 dbSNP database within chromosome no. 1 (GenBank Acc. No. NG_008014.1). By reviewing the dbSNP engine, it was found that this detected

SNP was found to be previously known as rs373796693, and it was deposited with several patterns of allelic polymorphisms as seen in Figure 3-14. However, this SNP was located in the intron-2 of the targeted *ATP1A2* gene.

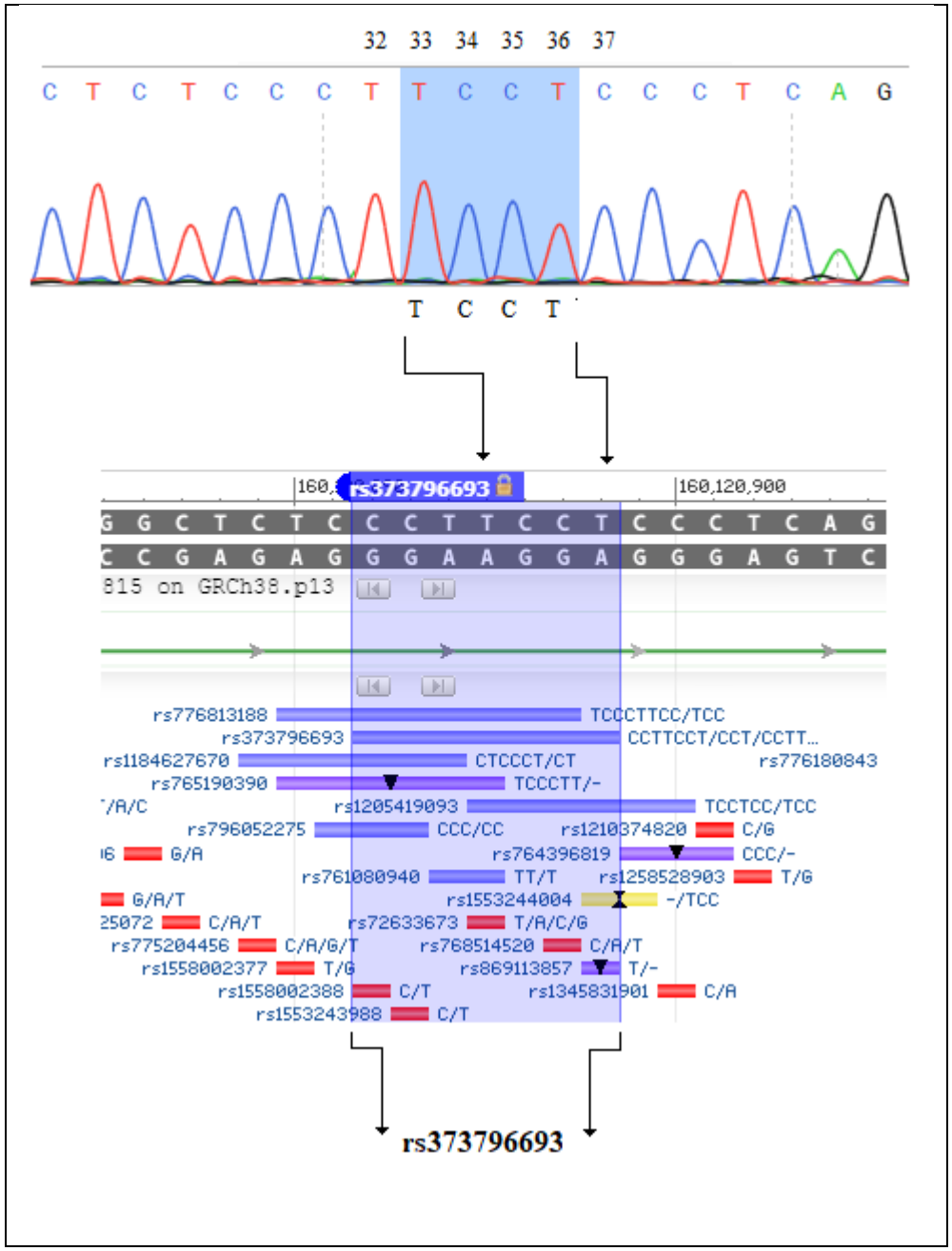


Figure 3-14: The SNP's Novelty Checking of *ATP1A2* Genetic Single Nucleotides Polymorphisms Using the dbSNP Server. The Identified rs37396693 SNP was Marked with Blue Color. The GenBank acc. no. NG_008014.1 was Used in the Positioning of the Highlighted Deletion SNP. The Position of the Targeted Sequences was Found in the Positive Strand

3-4: Histologic and Immunohistochemically Staining Results

3-4-1: Histologic Results

Concerning the results of the histomorphometric in placental tissue are shown in Table 3-14. The results showed syncytial knots significantly higher in preeclamptic patients compared with control group. Also, the results of stromal fibrosis showed significantly higher in preeclamptic patient compared with control group. As well as the results of the number of capillaries in terminal villi appeared significantly higher in preeclamptic patients compared with control group.

Table 3-14: Histomorphometric Finding in Placental Tissue for Preeclamptic Patients Compared with Normal Pregnancies

No.	Histological Lesion	Control Mean ± SD	Pre-eclampsia Mean ± SD	P-value
1.	Calcifications	0.23 ± 0.61	0.21 ± 0.67	0.96
2.	Fibrinoid Necrosis	3.80 ±1.40	4.1 ± 2.90	0.073
3.	Number of Capillaries in Terminal Villi	2.30 ±1.22	4.90 ±1.54	< 0.001*
4.	Placental Infarction	0.13 ± 0.34	0.23 ± 0.64	0.427
5.	Stromal Fibrosis	0.42 ± 0.53	2.02 ± 0.71	0.002*
6.	Syncytial Knots	21.60 ± 3.10	26.25 ± 5.1	0.013*

**p* value < 0.05 was significant

While the results of fibrinoid necrosis showed no significance in the present study between preeclamptic patients compared with control group. Also, the results of placental infarction showed no significance in preeclamptic patient compared with control group. As well as the results of the calcifications appeared no significance in preeclamptic patients compared with control group.

3-4-2: Immunohistochemically Staining Results

Immunohistochemistry is an invaluable validation tool in biomarker discovery. However, considering the excessive number of existing studies proposing novel IHC biomarkers, markers validated in several clinical cohorts are extremely few, stressing the need to raise quality standards for clinical biomarker studies. Even if results can be reproduced, the transition towards a routinely used marker is complex. For a new factor to become of potential value in the clinic, it has to add an important value compared with other already used factors.

The results of immunohistochemical staining of antibody Na-K ATPase of placental tissue as shown in Tables 3-15, 3-16 and Figures 3-15, 3-16, 3-17, 3-18 and 3-19 for control and preeclamptic patient showed higher percent significantly for cytotrophoblasts at >75% and <50% in preeclamptic patient compared with the control group. Also, the results appeared syncytial knots higher present significantly at >50% in preeclamptic patients compared with the control group. As well as the results showed decidual cells higher present significantly at 30-50% in preeclamptic patients compared with the control group.

While the results of spiral arteriolar endothelial cells and villous arteriolar endothelial cells showed no significance in the present study between preeclamptic patients compared with control group.

Table 3-15: Expersion of Na-K ATPase in Cytotrophoblats Cell and Syncitial knots of Placenta by Immunohistochemical

	Cytotrophoblats			Syncitial knots		
	<50%	**50-75%**	**>75%**	**<30%**	**30-50%**	**>50%**
Control	1 (2%)	42 (84%)	4 (8%)	14 (28%)	32 (64%)	4 (4%)
Pre-eclampsia	2 (6.06%)	6 (18.18%)	25 (75.75%)	10 (30.3%)	17 (51.5%)	6 (18.18%)
P-value	<0.001*	0.03*	<0.001*	0.75	0.53	<0.001*

Table 3-16: Expersion of Na-K ATPase in Spiral Arteriolar Endothelial Cells, Villous Arteriolar Endothelial Cells and Decidual Cells of Placenta by Immunohistochemical

	Spiral Arteriolar Endothelial Cells			Villous Arteriolar Endothelial Cells			Decidual Cells		
	<10%	**10-30%**	**>30%**	**<10%**	**10-30%**	**>30%**	**<30%**	**30-50%**	**>50%**
Control	13 (26%)	14 (28%)	23 (47%)	16 (32%)	20 (40%)	14 (28%)	31 (62%)	10 (20%)	9 (18%)
Pre-eclampsia	9 (27.27%)	12 (36.36%)	12 (36.36%)	8 (24.24%)	13 (39.39%)	12 (36.36%)	4 (12.12%)	11 (33.33%)	8 (24.24%)
P-value	0.45	0.702	0.38	0.34	0.64	0.81	0.14	0.087*	0.946

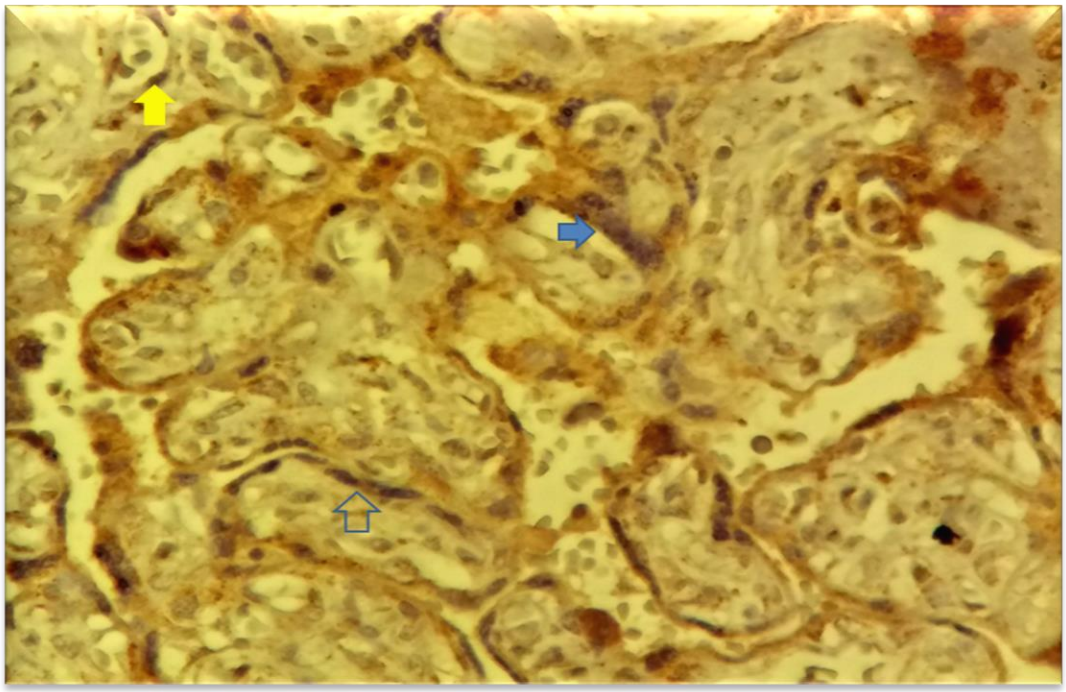


Figure 3-15: Immunohistochemical Staining of Placental Tissue for Control Showing Positive Staining of Syncytial Knots (Solid Blue Arrow) and Cytotrophoblats (Empty Blue Arrow), with Staining of Endothelial Cells (Yellow Arrow)

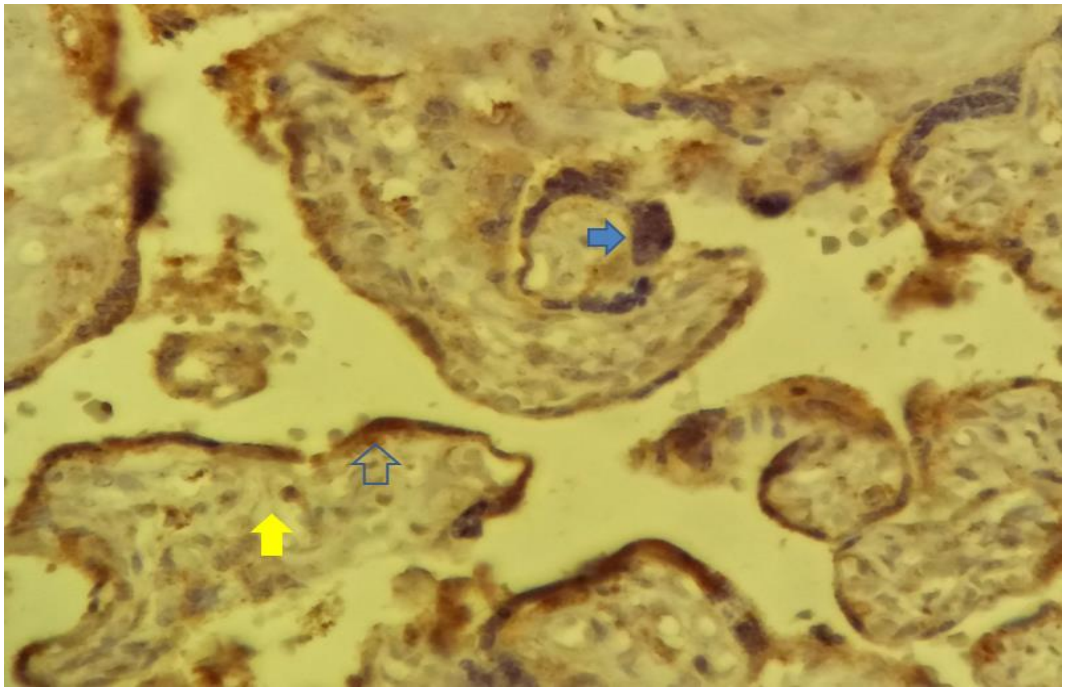


Figure 3-16: Immunohistochemical Staining of Placental Tissue for PreeclampticShowing Positive Staining of Syncytial Knots (Solid Blue Arrow) and Cytotrophoblats (Empty Blue Arrow), with Staining of Endothelial Cells (Yellow Arrow)

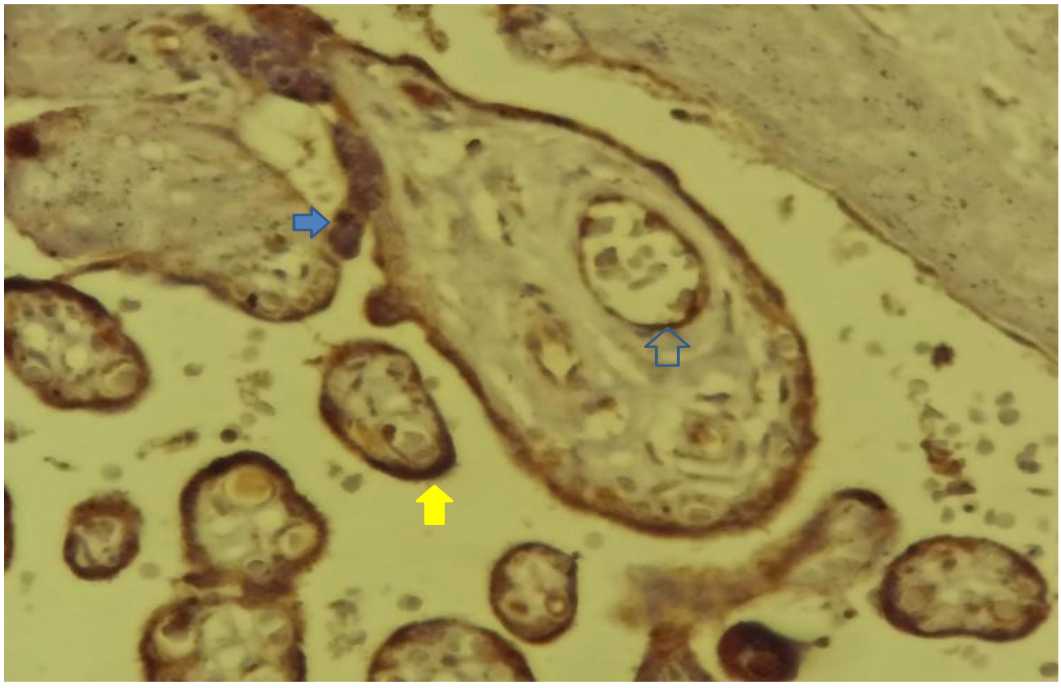


Figure 3-17: Immunohistochemical Staining of Placental Tissue for Preeclamptic Showing Positive Staining of Syncytial Knots (Solid Blue Arrow) and Cytotrophoblats (Empty Blue Arrow), with Staining of Endothelial Cells (Yellow Arrow)

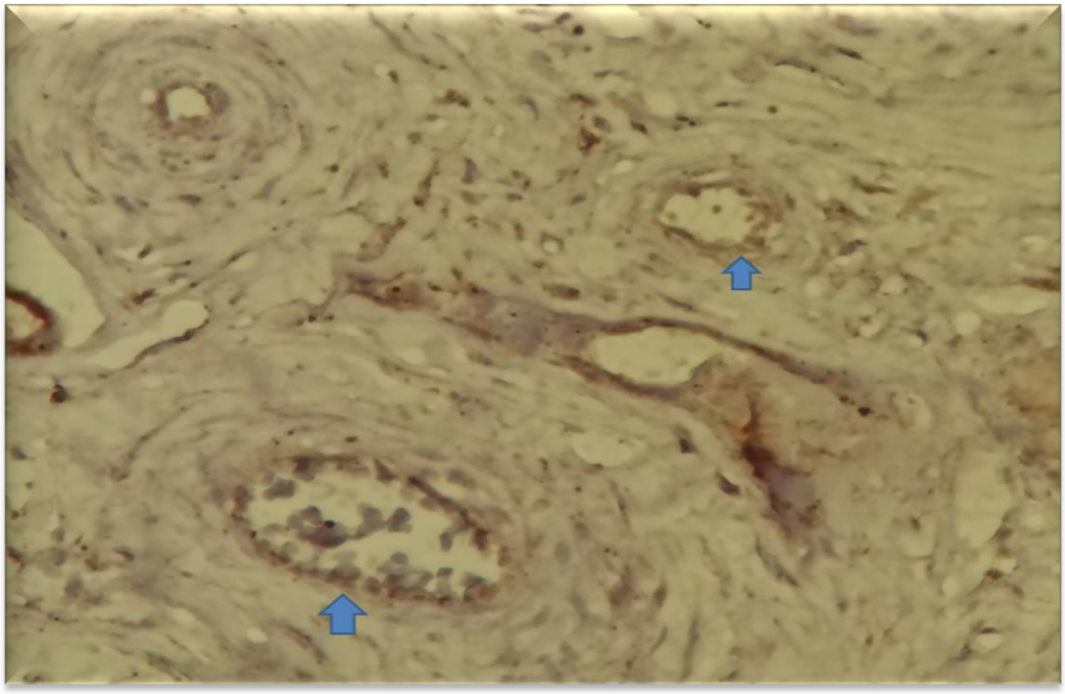


Figure 3-18: Immunohistochemical Staining of Placental Tissue for Control with Anti Na-K ATPase of Spiral Arterioles Showing Thin Muscular Wall and Positive Endothelial Cell

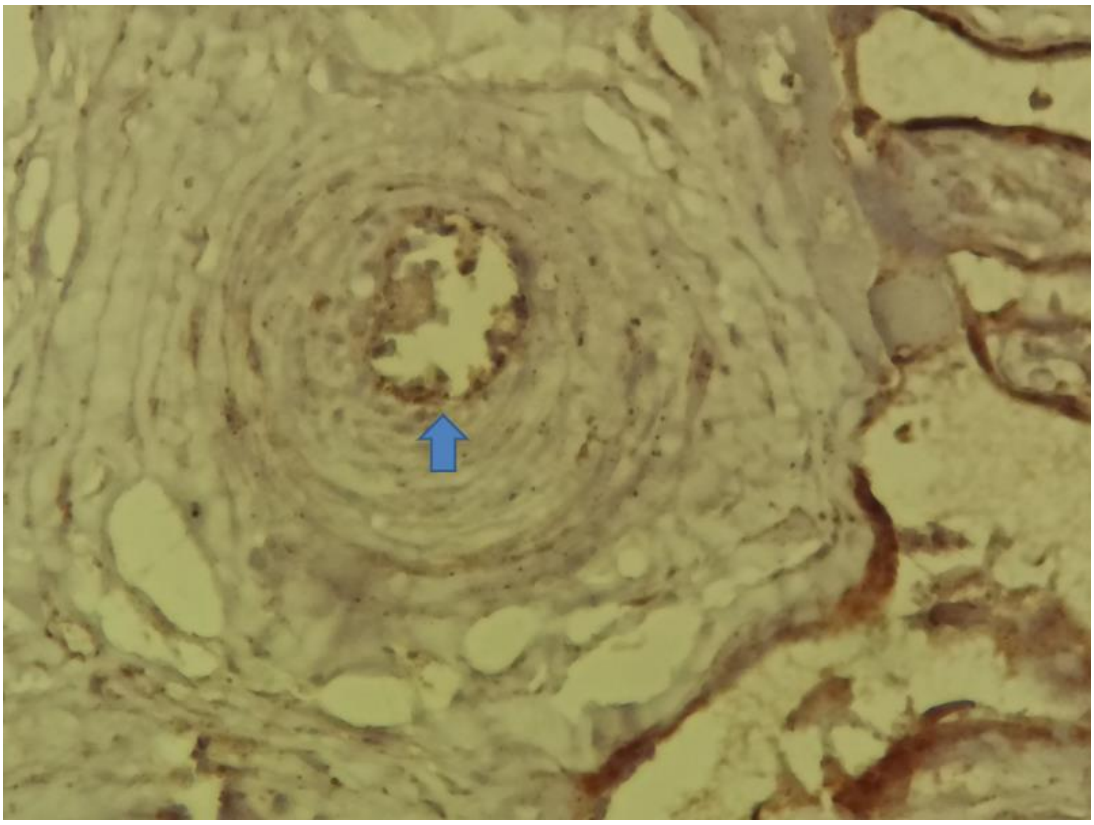


Figure 3-19: Immunohistochemical staining with anti-Na-K ATPase of spiral arterioles showing thick arteriosclerosis and positive endothelial staining for Preeclamptic

4: Conclusions and Recommendations

4-1: Conclusions

The conclusions of this study indicate that:

1. Pre-eclampsia is associated with significantly lower erythrocyte sodium pump activity than normotensive pregnancy.
2. The results of inhibition percentage of endogenous digitalis shown elevated in patients with pre-eclampsia compared with normal pregnancy, and this correlates well with reduced enzyme activity in this group.
3. There was no significant alteration in the polymorphism of both studied genes (*ATP1A1*, rs10924081, and *ATP1A2*, rs373796693) for patient and control groups.
4. The sequence data for gene *ATP1A2* (rs373796693) observed that TCCT deletion in the investigated samples.
5. Histomorphometric changes revealed significant difference for the following parameters: syncytial knots, stromal fibrosis, and number of capillaries in terminal villi, while there was no significant difference for the following parameters: placental calcification, fibrinoid necrosis and placental infarcts. These changes may be attributed to early maturation of placental tissue in pre-eclamptic patients.
6. Immunohistochemical expression of placental Na-K ATPase revealed significant difference in expression only of cytotrophoblasts in pre-eclamptic placental tissue, while other cellular components (syncytial knots, spiral arteriolar endothelial cells, villous arteriolar endothelial cells and decidual cells) showed no significant difference.

4-2: Recommendations

The present study is the first one held in Iraq for detecting a correlation between Na-K ATPase and pre-eclampsia. Consequently, other factors to be sought for a better understanding of this problem such as:

1. It is recommended to increase the sample size for the best results.
2. It is recommended to investigate other candidate genes that may influence pre-eclampsia like β1 and β2 genes.

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APPENDICES

Appendices

Appendix (A)

No.: **Date: / /**

Name:

Age:

Blood Pressure:

Albumin in Urine: **Edema:**

Platelet Count:

PCV:

Blood Group:

History of Hypertension:

Gestation of Delivery (Weeks):

Weight of Baby:

Type of Delivery: **Normal Labor** **Cesarean Section**

Other Disease: **Diabetes Mellitus** **Hypertension**

Note:

Scheme (A-1): The Questionnaire Form Used in This Study

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

Na-K ATPase Activity in Preeclamptic Women in Labour

A Thesis

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

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Summary

Preeclamptic is a human pregnancy-specific disorder defined by the appearance of proteinuria-related hypertension after the 20th week of gestation in a previously normotensive woman and entirely cured by the 6th postpartum week. It is the main cause of morbidity and mortality during pregnancy. It is a multisystem sickness, but its etiology is unknown. Preeclamptic has been associated with system anomalies, such as ion transport deficits in neonatal, maternal, and placental cell lines.

The main aim of this research is to study the Na-K ATPase from biochemical parameters, then the genetic study of Na-K ATPase by selecting two types of isoforms of gene alpha and estimate the expression of placental Na-K ATPase in preeclamptic patients compared with normal pregnancies by immunohistochemistry, as well as other histological parameters.

This was a case-control study involved 130-woman participants ranging in age from 16-40 years. Samples included blood and placental tissue. The blood samples divided into three groups: negative control (30), positive control (55), and patient group (45). While the placenta samples divided into two groups: control (50) and patient (40).

The current study included three parts: the first part is the biochemical study of blood samples. The blood samples were collected in the EDTA tube as an anticoagulant and centrifuged to isolate red blood cells from plasma. A modified approach was utilized to measure the specific activity of Na-K ATPase in the red blood cell membranes. While a new method was used to determine the inhibition percentage of endogenous digitalis in the plasma.

The second part included the genetic study of Na-K ATPase enzyme of the placenta tissue by selected two genes Alpha 1 (*ATP1A1*, rs10924081), and Alpha 2

(*ATP1A2*, rs373796693). The genetic variants that would be studied in this project were carefully selected according to the recent findings in this field, and by employed several specialized databases. The genotyping designed and optimized for a gene Alpha 1 by the polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method. While the genotyping designed for gene Alpha 2 by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method then sequencing.

The third part of current study included histologic examination of placental tissue along with immunohistochemical expression of Na-K ATPase was carried out in all samples.

The results of the biochemical tests shown the specific activity of Na-K ATPase in preeclamptic patients was significantly low compared to the control group. While the results of inhibition percentage of endogenous digitalis showed significantly higher in the preeclamptic patient compared to the control group. Also, this study found the inverse correlation between the specific enzyme activity and inhibition percentage of endogenous digitalis.

Concerning the results of the genetic test showed that there was no significant allelic or genotypic association recorded between patient and control groups for the two genes studied. While the sequence data for gene *ATP1A2* (rs373796693) showed TCCT deletion in the investigated samples.

Concerning the results of the histomorphometric for placental tissue of normal pregnancies compared to preeclamptic showed that were syncytial knots, stromal fibrosis and number of capillaries significantly higher in preeclamptic patients compared with control group. While the results of fibrinoid necrosis, placental infarction and calcifications showed no significance difference between preeclamptic patients compared with control group.

Finally, the results of immunohistochemical staining of antibody Na-K ATPase of placental tissue for control and preeclamptic patient showed higher percent significantly for cytotrophoblasts at <50% and >75% in preeclamptic patient compared with the control group. Also, the results appeared syncytial knots higher present significantly at >50% in preeclamptic patients compared with the control group. As well as the results showed decidual cells higher present significantly at 30-50% in preeclamptic patients compared with the control group. While the results of spiral arteriolar endothelial cells and villous arteriolar endothelial cells showed no significance difference between preeclamptic patients compared with control group.

The conclusions of this study indicate that: preeclamptic is associated with significantly lower erythrocyte sodium pump activity than normotensive pregnancy. While the results of inhibition percentage of endogenous digitalis show elevated in patients with preeclamptic compared with normal pregnancy, and this correlates well with reduced enzyme activity in this group.

There was no significant alteration in the polymorphism of both studied genes (*ATP1A1*, rs10924081, and *ATP1A2*, rs373796693) for patient and control groups.

Histomorphometric changes revealed significant difference in the following parameters: syncytial knots, stromal fibrosis, and number of capillaries in terminal villi, while there was no significant difference for the following parameters: placental calcification, fibrinoid necrosis and placental infarcts. These changes may be attributed to early maturation of placental tissue in preeclamptic patients.

Finally, Immunohistochemical expression of placental Na-K ATPase revealed significant difference in the expression of cytotrophoblasts in preeclamptic placental tissue, while other cellular components (syncytial knots, spiral arteriolar endothelial cells, villous arteriolar endothelial cells and decidual cells) showed no significant difference.