1. Introduction

1.1 Hypertensive disorders of pregnancy:

Every year it is estimated that worldwide, more than 500 000 women die of complications of pregnancy and childbirth. At least 7 million women who survive childbirth suffer serious health problems and a further 50 million women suffer adverse health consequences after childbirth. The overwhelming majority of these deaths and complications occur in developing countries (WHO, 2008).

Hypertensive disorders of pregnancy are an important cause of severe morbidity, long term disability and death among both mothers and their babies. In Africa and Asia, nearly one tenth of all maternal deaths are associated with hypertensive disorders of pregnancy, where as one quarter of maternal deaths in Latin America have been associated with those complications (WHO, 2011).

The hypertensive disorders carry an increased risk of preterm delivery, placental abruption, disseminated intravascular coagulation, cerebral hemorrhage, hepatic failure, and acute renal failure (Nijdam, 2010).

Among the hypertensive disorders that complicate pregnancy, preeclampsia and eclampsia stand out as major causes of maternal and prenatal mortality and morbidity (WHO, 2011).

Preeclampsia is the second leading cause of maternal mortality in the United States, affecting 7–10% of all pregnancies, and contributes significantly to neonatal mortality and morbidity. Several recent studies have suggested that the renin angiotensin system (RAS) may be playing a role in the development of preeclampsia. During normal pregnancy, the RAS is activated due to increased estrogen levels, which consequently cause levels of angiotensinogen and renin to rise. The activation of the RAS ultimately leads to increases in angiotensin II (Ang II) levels however, previous studies illustrated that pregnant women are resistant to

the presser effects of Ang II resulting in normal or decreased blood pressure levels. The role of the RAS in normal pregnancy is incompletely understood; however, dysregulation of the RAS has been hypothesized to play a role in the Pathophysiology of preeclampsia (Lauren *et al.*, 2009).

The etiology of preeclampsia is still unknown, but it is accepted that susceptibility for its development is given by the presence of complex gene–gene and gene–environment interactions between the mother and the foetus (Norma, 2006).

The majority of deaths due to pre-eclampsia and eclampsia are avoidable through the provision of timely and effective care to the women presenting with these complications (WHO, 2011).

1.2. Rationale:

Preeclampsia is heterogeneous disorders, which complicates 5-7% of all pregnancies and remain a leading cause of maternal and fetal morbidity and mortality.

Most reports have focused on Caucasian, Japanese, Romanian or Chinese individuals while no large studies have yet, to our knowledge been reported on African women, a population which has a high and unexplained prevalence of pre-eclampsia.

In Sudan, The incidence and prevalence of preeclampsia continues to rise but no published data concerning RAS and genetic basis of preeclampsia so the study want to insight the light about this point mutation and its effect on plasma renin activity among Sudanese pregnant women.

1.3.Objectives:

1.3.1. General objective:

To assess plasma renin activity and to detect the Angiotensinogen gene *M235T* polymorphism among Sudanese pregnant women with preeclampsia.

1.3.2. Specific objectives:

1. To measure plasma renin activity in Sudanese pregnant women with preeclampsia (patients) compared to apparently healthy Sudanese pregnant women (controls).
2. To detect the polymorphism *(M235T)* Angiotensinogen gene frequency among the study group using PCR and RFLP.
3. To compare between the genotypes of Angiotensinogen gene and plasma renin activity.
4. To correlate plasma renin activity to mean arterial blood pressure.

2. Literature Review

2.1 Pregnancy:

Pregnancy is the carrying of one or more embryos by female mammals, including human inside their bodies. Human pregnancy is the most studied of all mammalian pregnancies. In many societies the medical and legal definition of human pregnancy is somewhat arbitrary and divided into three trimester periods to simplify reference to different stages of fetal development. The first trimester period carries the highest risk of miscarriage, during the second trimester period the development of fetus can start and monitored. The third trimester makes the beginning of viability or the ability of the fetus to survive with or without medical help (Zilva and Panel, 1994).

2.1.1 Fertilization:

The sperms take 0.5-1hour to reach the ovum (in stage of 2ary oocyte) at the uterine tube. The ovum remains viable for about 3days: however, it is fertilizable for shorter period. On the other hand; sperms are viable for about 3days (sometimes up to 5days).That is why the possibility of fertilization is highest with coitus at one to two days before ovulation. Fertilization occurs at outer portion of the uterine tubes" the ampulla". Before fertilization, sperm complete their maturation at the isthmus of the uterine tubes Capacitation (Triage, 2011).

About 50-100 sperm reach the ovum; however one of these fertilizes the ovum as flow: Chemo attraction of sperm to the ovum (by chemicals released by the ovum), adherence of zona pellucid (by the sperm protein fertilin), penetration of the zona pellucida and acrosomal reaction (release of enzymes in the acrosome to facilitate penetration through the zona pellucida) and adherence of the sperm head to the cell membrane of the ovum then release of nucleus into the cytoplasm of the ovum. After

fertilization, the fertilize ovum (blastocyst) takes about 3days to reach the uterus (Triage, 2011).

2.1.2 Implantation:

When the blastocyst reaches the uterus, it becomes surrounded by two types of trophoblasts: syncytiotrophoblast, cytorophoblasts. The usual site of implantation is the dorsal wall of the uterus; however there are abnormal sites of implantation. These include: the uterine tube, other sites within the uterus and rarely the abdomen (extra-uterine pregnancy) (Triage, 2011).

2.1.3. Duration of pregnancy:

In case in which pregnancy has followed single coitus, the average duration of pregnancy from date of intercourse is 255 days (around 36weeks). If the calculation made from the first day of last menstrual period (LMP) the average duration is 280days (around 40weeks) because ovulation most frequently occurs on the14^{th} day of 28 day menstrual cycle. However there is considerable variation in duration of normal pregnancy even in cases which the menstrual cycle were previously regular and had normal length and also in cases in which the date of coitus is known (Zilva and Panel, 1994).

2.2. Hypertension disorders of pregnancy:

Hypertension is a diastolic blood pressure of 90 mmHg or more. Hypertensive disorders of pregnancy affect about 10% of all pregnant women around the world (Duleyl, 2009; Eric *et al.*, 2010). Hypertensive disorders of pregnancy are an important cause of severe acute morbidity, long term disability and death among mothers and babies (Khan *et al.*, 2006; Duleyl, 2009).

2.2.1. Classification of the hypertension disorders of pregnancy:

To classify these disorders in the past have been confusing and sometimes misleading .More recently their classification has been rationalized and simplified to reflect different situation encountered in clinical practice. The hypertensive disorders of pregnancy include ; pregnancy-induced hypertension(PIH), or gestational hypertension, refer to raised blood pressure occurring for the first time in the second half of pregnancy, but without proteinuria (<300 mg/24 h). Pre-eclampsia (PE) is reserved for the new occurrence of hypertension and proteinuria in the second half of pregnancy. Eclampsia is the occurrence of convulsions superimposed on pre-eclampsia.Pre-eclampsia superimposed on chronic hypertension is when a woman with chronic hypertension develops new signs or symptoms of pre-eclampsia in the second half of pregnancy (Duleyl, 2003)

2.2.1.1. Pregnancy induced hypertension (PIH):

Pregnancy-induced hypertension (PIH) may progress from a mild hypertension disease to a life-threatening condition, as follows: Hypertension without proteinuria or edema, mild pre-eclampsia, severe pre-eclampsia and eclampsia. Proteinuria changes the diagnosis from pregnancy-induced hypertension to the more serious condition of pre-eclampsia (WHO, 2008).

Women with pregnancy-induced hypertension generally have a good outcome. The risk to them and their baby increases only if they progress to pre-eclampsia, or has very high blood pressure (Duleyl, 2003)

2.2.1.2. Preeclampsia (PE):

Preeclampsia (PE) is an idiopathic multisystem disorder specific to human pregnancy and puerperium and is associated with significant fetal and maternal morbidity (Velloso *et al.,* 2007).

Pre-eclampsia is generally defined as new hypertension (diastolic blood pressure of ≥90 mm Hg) and substantial proteinuria (≥300 mg in 24 h) at or after 20 weeks gestation (Lindheimer *et al.*, 2008; Eric *et al.*, 2010).

2.2.1.2.1. Classification of preeclampsia:

Preeclampsia is classified into mild or sever according to the following finding: (WHO, 2008).

Finding	Mild pre-eclampsia	Severe pre-eclampsia
Diastolic blood Pressure	Raised to 90–110 mmHg on two occasions1–4 hours apart after 20 weeks gestation	Raised to 110 mmHg or more after 20 weeks gestation
Proteinuria	Up to 2+	3+ or more

2.2.1.2.2Sings of sever preeclampsia:

Headache, visual disturbances, upper abdominal pain (Epigastric region), oligouria(less than 400 ml in 24 hours) hyper-reflexes and pulmonary edema (WHO, 2008).

2.2.1.3 Eclampsia:

Eclampsia is the onset of fits in a woman whose pregnancy is usually complicated by pre-eclampsia. The fits may occur in pregnancy after 20 weeks gestation, in labour, or during the first 48 hours of the postpartum period. There is a high incidence of maternal death in women with eclampsia. Perinatal mortality is also high (WHO, 2008).

As with pre-eclampsia, the pathogenesis of eclampsia remains largely unknown and 5%–8% of women with pre-eclampsia present this condition in developing countries (Eric *et al.*, 2010; WHO, 2010; WHO, 2011).

Pre-eclampsia and eclampsia are part of the same disorder with eclampsia being the severe form of the disease. Pre-eclampsia almost

always precedes eclampsia. However, not all cases follow an orderly progression from mild to severe disease and some women develop severe pre-eclampsia or eclampsia very suddenly (WHO, 2008).

2.2.2. Epidemiology of preeclampsia/eclampsia

Pre-eclampsia is a multisystem disorder of unknown etiology, unique to pregnancy. It complicates an estimated 2–8% of pregnancies and is a major cause of maternal morbidity, prenatal death and premature delivery, although outcome for most women is good. Eclampsia, the occurrence of one or more convulsions superimposed on the syndrome of preeclampsia, occurs less frequently, complicating between 1 in 100–1700pregnancies in the developing world and about 1 in 2000 pregnancies in Europe and other developed countries. Eclampsia is often a serious and life-threatening condition. Compared to pre-eclampsia it carries a much higher risk of death and serious morbidity for the woman and her baby. In the UK, for example, 1 in 50 of the women who have eclampsia dies (Duleyl, 2003).

Worldwide, over half a million women die each year of pregnancy related causes and 99% of these deaths occur in the developing world. Put another way, women in industrialized countries have an average life time risk (calculated as the average number of pregnancies multiplied by the risk associated with each pregnancy) of dying from pregnancy related causes of between 1 in 4000 and 1 in 10,000, whereas women in low income countries have a risk that is between 1 in 15 and 1 in 50. In poor countries, maternal mortality is 100–200 times higher than in Europe and North America (Duleyl, 2003).

There is no other public health statistic for which the disparity between rich and poor countries is so wide. Although rare, eclampsia probably accounts for 50,000 maternal deaths a year. In areas where maternal mortality is very high, infection and hemorrhage are the main

causes of death, but as deaths from these causes become less common, those associated with pre-eclampsia and eclampsia assume greater importance (Duleyl, 2003).

Where overall maternal mortality is high, most deaths are associated with eclampsia. In places where mortality is low, a greater proportion of deaths are related to pre-eclampsia. There are few reliable data on the maternal morbidity associated with pre-eclampsia and eclampsia, but it is likely that this is also substantial. In the UK, for example, preeclampsia accounts for an estimated one-fifth of antenatal admissions, two thirds of referrals to day care assessment units, and a quarter of obstetric admissions to intensive care units. Although maternal mortality in the UK is low, pre-eclampsia/eclampsia accounts for 10–15% of direct obstetric deaths1as it does in many developing countries. Reducing the morbidity and mortality associated with these conditions is an important priority (Duleyl, 2003).

Most studies indicate that preeclampsia and eclampsia are widely distributed in Sudan; there is an extremely high maternal mortality in Sudan with pre-eclampsia/eclampsia accounting for 4.2% of the obstetric complications and 18.1% of maternal deaths (Ali and Adam, 2011; Ali *et al.*, 2012)

2.2.3 Pathophysiology of preeclampsia/eclampsia

The exact mechanisms which lead to pre-eclampsia are not clear, several factors are known to play a part in determining who will develop this disease .Some women have predisposing factors . These include family history, age and parity. Current thinking is that the primary Pathophysiology in preeclampsia is placental (Duleyl, 2003).

Preeclampsia occurs in women who have an abdominal pregnancy and in those who have hydatiform mole. In addition, it is more common amongst women who have conditions associated with large placenta (such

as multiple pregnancies) and in women who have microvascular disease (such as chronic hypertension and diabetes) (Duleyl, 2003; Eric *et al.*, 2010; WHO, 2011).

The secondary pathology in preeclampsia appears to be endothelial cell injury. The proposed model is that reduced blood supply to the placenta result in production of unknown factors which are released in the maternal circulation and act on endothelial cells, leading to endothelial dysfunction. This result in; vasospasm with consequent reduction in plasma volume and activation of coagulation of cascade. The changes antedate other clinical finding (Duleyl, 2003).

Although the risk factors for preeclampsia are both environmental and genetic. Environmental factors such as seasonal factors and humidity are said to influence the incidence of hypertensive disorders of pregnancy. In Ghana, more cases of eclampsia have been noted in the rainy season, but there are some studies, however, which do not show any significant correlation between seasonal change and preeclampsia. Neela *et al* supported the speculated relationship between increasing humidity and a lower temperature range with the increased incidence of eclampsia (Soroori *et al.*, 2007).

Wacker *et al* found an increased incidence of preeclampsia at the end of dry season and in the first months of rainy seasons. Magnus *et al* reported a systematic seasonal variability in occurrence of preeclampsia with a peak in the winter months and a minimum in the summer. Magann *et al* indicated no statistical correlation between preeclampsia and meteorological changes. This lack of correlation between meteorological factors and hypertensive disorder of pregnancy were also reported in Makhseed study (Soroori *et al.*, 2007)

Phillips *et al* identified a seasonal variation in preeclampsia that appears to be more strongly related to timing of conception than to the

timing of delivery. Conception during the summer months had the highest risk compared with the spring. Winter conceptions were associated with intermediate rates of preeclampsia (Soroori *et al*., 2007).

Jamelle in Karachi found an increase in eclampsia cases from April to June and in September; otherwise the incidence remained stable. In Peshawar and Quette, with more severe cold and dry winter, the incidence peaked in winter and summer months. However, his statistical analysis revealed no significant relationship of incidence of eclampsia with temperature. The results of Soroori et al study revealed no statistical difference between incidence of preeclampsia and seasonal changes (Soroori *et al*., 2007).

In Sudan study showed a significant increase in the incidence of eclampsia in the monsoon season, with no such change in the incidence of preeclampsia. It is widely understood that preeclampsia and eclampsia are progressive manifestations of the same patho-physiological spectrum. This study showed that the meteorological factors had no influence on the incidence of preeclampsia. In contrast, lower temperature, higher rainfall and humidity and lower barometric pressure were related to the triggering of seizures in patients primed with preeclampsia. This is similar to other studies associating eclampsia with lower temperatures and increased humidity and lower barometric (Ali and Adam, 2011).

Regarding genetic factors, the presence of preeclampsia in first degree relatives increases a woman's risk of preeclampsia by 2 to 4 fold. Genetic factors may play an important role in the angiogenic imbalance found in patients. For instance, reported that compared to the offspring or relatives of normotensive pregnancies, daughters of preeclamptic mothers suffered from much higher incidence of risk for the disease ranging from 20 to 40 percent, 11 to 37 percent for sisters of preeclamptic women and 22 to 47 percent in twin studies. There is some evidence to suggest that in

addition to maternal genotype, paternal (or fetal) genotype may also contribute to risk of preeclampsia. The risk of fathering a preeclamptic pregnancy is increased among males who fathered a preeclamptic pregnancy with a different partner. Also, men who are born from a pregnancy complicated by preeclampsia are at a higher risk of fathering a preeclamptic pregnancy (Mustfa *et al.*, 2012; Jie *et al.,* 2013).

2.2.4. Therapies of preeclampsia:

Currently the main therapy for preeclampsia is to deliver the baby as soon as possible to enhance maternal and fetal wellbeing (Wang *et al.*, 2012; Dasgupta *et al.,* 2012).

Aspirin use when inflammation appears to play a significant role in the pathogenesis of preeclampsia, some investigators has studied the role of aspirin in prevention and therapy of preeclampsia (Maraco *et al.,* 2011).

2.3. The renin-angiotensin system (RAS):

The definition the renin angiotensin system is peptidergic system with endocrine characteristics (Martin *et al*., 2006).

Renin is an aspartyle protease secreted by the juxta glomerular apparatus, a cellular complex, adjacent to the renal glomeruli, lying between afferent arteriole and the distal convoluted tubule. Secretion increase in response to a reduction in renal artery blood flow, possibly mediated by change in mean pressure in the afferent arterioles, and beta – adrenergic stimulation (Costanzo, 2011).

Renin spilt a decapeptide (angiotensin I) from circulating α_2 globulin known as renin substrate (angiotensinogen). Another proteolytic enzyme, angiotensin-converting enzyme (ACE), which is located predominantly in the lungs but is also present in other tissues such as the kidney, split off further two amino acid residues (Crook and Zilva, 2009 ;Costanzo,2011).

ACE is a key RAS component and play important role in the blood pressure homeostasis by generating the vasoconstrictor angiotensin II and by in activated the vasodilator bradykinin and angiotensin I (Merrill *et al.*, 2002; Robson *et al.*, 2000).

2.3.1. Important actions Angiotensinogen II:

It acts directly in capillary walls, causing vasoconstriction, and so probably helps to maintain blood pressure. Stimulates the cell of the zona glomerulosa to synthesize and secrete aldosterone. Also it stimulates the thirst center and so promotes oral fluid intake (Crook and Zilva, 2009).

2.3.2 Results of renin secretion:

Poor renal blood flow is often associated with an in adequate systemic blood pressure. The release of renin results in the production of angiotensin II, which tends to correct this by causing aldosterone release, which stimulates sodium and subsequently water retention and hence restores the circulating volume (Crook and Zilva, 2009).

2.3.3 The circulating RAS in normal pregnancy

The role of the RAS in normal pregnancy is incompletely understood; however, dysregulation of the RAS has been hypothesized to play a role in the pathophysiology of preeclampsia (Lauren *et al.*, 2009).

In recent years the studies showed that he RAS system is a major determinant of sodium balance in pregnancy, and there is clear increase in renin activity in pregnant women which might be expected to result in higher level of Angiotensin in the plasma .Since angiotensin II most potent of the circulating vasoconstrictors in the human placenta (Glenn *et al.*, 2000).

Normal pregnancy is characterized by an early increase in the circulating levels of renin. The origin of this increase has been ascribed to both ovarian secretion of renin and the decidual production of renin. Angiotensinogen levels also increase in pregnancy, but the exact timing of

this change is not very well defined. Aldosterone levels are increased, and this may contribute to sodium retention and resultant obligatory water retention, which is one of the mechanisms of volume expansion in pregnancy. The effect of ANG II on the systemic vasculature would be expected to increase vasomotor tone; however, pregnancy is characterized by a lack of such a response. The systemic vasculature is thus refractory to ANG II in normal pregnancy, i.e., requires a higher ANG II infusion rate (almost twice as high compared with non-gravid at the peak refractory state) for the same degree of vascular response. This vascular refractoriness to ANG II in pregnancy has been ascribed, in part, to progesterone and prostacycline (Dinesh ,2005).

So that renin angiotensin systems plays the key role in blood pressure regulation and many investigators have postulated that alteration in the RA system plays a significant role in the pathophysiology of PIH, and recently the association between the angiotensinogen locus and PE/ eclampsia has been found (Nałogowska *et al.*,2000).

2.3.4 Angiotensinogen and preeclampsia:

To clarify the genetic nature of PE, a range of studies have been conducted to investigate the linkage between genes (e.g. angiotensinogen) and PE. Angiotensinogen (AGT) is the precursor of angiotensin II that plays crucial roles in the regulation of blood pressure. The AGT gene, located on exon2 chromosome 1, has been reported to be related to the development of PE and essential hypertension. A common polymorphism AGT M235T (the substitution of threonine [Thr] for methionine [Met] at codon 235(a T- to –C transition at nucleotide 704,704 T/C) was first identified in 1992, and the Thr 235 variant has been reported to be associated with higher AGT levels and abnormal remodeling of the uterine spiral arteries, which is an early cause of PE (Pocipciucil, 2002;Aggarwal *et al.*,2010 , Aggarwal *et al.*,2011 ; Procopciuc *et al.* ,2011).

To date, large numbers of epidemiological studies have been performed to examine the relationship between the AGT M235T polymorphism and risk of PE. (Benedetto *et al.*, 2006; Aggarwal *et al.*, 2010; Aggarwal et *al.*, 2011).

The results, however, were controversial in different studies. Some investigators reported that the AGT M235T polymorphism was associated with the risk of PE in Caucasian (Pocipciucil, 2002) and Japanese patients (Kobashi *et al.*, 1999). In contrast, other investigators have reported that the AGT M235T polymorphism was not a risk factor for the development of PE in Africans (Roberts *etal* ,2004) , Asians,- and Caucasians.(Shansha *et al.*, 2012) .

Despite all of these conflicting evidences, in 2011, Procopciuc *et al.* , provied that the risk of preeclampsia increased significantly for women homozygous for Met235Thr AGT, polymorphism. More than that, their results suggested that there was a relationship between mutated Met235Thr AGT, genotypes and lower gestational age at delivery and/or birth weight. This study confirmed the hypothesis that mutated newborn RAS genes (Met235Thr AGT) and the interaction of RAS maternal/newborn genotypes make an important contribution to the pathogenesis of preeclampsia in mothers, as well as to intrauterine growth retardation (Jie *et al*., 2013).

3. Materials and Methods

3.1. Study design:

This is a cross sectional, case control hospital based study, done in Sudanese pregnant women with preeclampsia to assess the *M235T* polymorphism as a risk factor and its affect on plasma renin activity using PCR-RFLP.

3.2. Study area:

This study was carried out at Khartoum state. Samples were collected from Omdurman Maternity Hospital, Omdurman Sudan.

3.3. Study period:

This study was carried out during the period of April 2012 to April 2014

3.4. Experimental design:

3.4.1. Study populations:

Fifty Sudanese pregnant women who diagnosed to have preeclampsia were reported to the above mentioned hospital was enrolled in the study.

Enrollment was done after the patients examinations by physician. Blood samples from fifty age-matched apparently healthy pregnant controls that had no evidence of preeclampsia also included for comparison.

3.5. Selection criteria:

3.5.1. Inclusion criteria:

Test group: Sudanese patients with preeclampsia and no history of pervious hypertension.

Control group: healthy pregnant volunteers matched for age.

3.5.2. Exclusion criteria:

Patients with clinical history of diabetes, renal disease, cardiac disease, proteinuria, and chronic hypertension were excluded from this study.

3.6 Ethical consideration

- Permission of this study was obtained from authorities of Omdurman Maternity Hospital; Omdurman Sudan.
- The objectives of the study were explained to all individual in this study.
- Inform consent was obtained from all participants in this study.

3.7. Data collection and Analysis:

3.7.1. Interview with a questionnaire:

An interview with a questionnaire to obtain the clinical data was done for each participant in this study. A questionnaire was specifically designed to obtain information which help in either including or excluding certain individuals in or from the study respectively (Appendix I).

3.7.2. Clinical examination of Patients

Clinical history and examination of the test group and the controls were done by physicians to help in excluded or included of study group.

3.8. Sampling:

3.8.1. Urine sample:

A random urine sample (mid stream) was collected in a dry clean urine container from the entire participant to detect protein.

3.8.2. Blood samples:

In sterile condition by using a local antiseptic for skin, 5 ml of venous blood was collected; of which 2.5 mL was drown in EDTA container for DNA extraction, and 2.5ml in other EDTA container, for measurement of plasma renin activity. Plasma was obtained by

centrifugation at 3000 rpm for 5 minutes, collected and stored in tubes at -20 C° till used

3.9. Laboratory investigations:

3.9.1. Urine protein:

Urine protein was detected using deep stick method (Appendix II).

3.9.1.1. Principle:

The protein present in the urine sample react with a dye bromphenol blue to produce color range from light yellow to blue color (Sood, 2006).

3.9.1.2. Procedure:

The test strip was immersed into a fresh urine sample, the excess urine was swept out and the color obtained was matched with chart on the strip container within 30-60 second (appendix II).

3.9.2. Serum Creatinine:

Serum creatinine was measured by using kinetic Jaffe method.

3.9.2.1 Principle:

Picric acid in an alkaline medium reacts with creatinine to form orange colored complex with alkaline picreate. Intensity of the colored formed during the fixed time is directly proportional to the amount of creatinine in the sample (Sood, 2006).

3.9.2.2 Procedure:

Pipette into a cuvette:

Working reagent	1.0 ml
STD or Sample	0.1 ml

Mixed well, and read the initial absorbance A1 for the standard (STD), and test after exactly 30 seconds. Read another absorbance A2for the standard, test after exactly 60 seconds.

3.9.2.3. Calculation:

The concentration of creatinine in the sample was calculated using this formula

$_{(}A_2 - A_{1)}$ Test/ $_{(}A_2 - A_{1)\ STD}$ x Con of STD = Con of sample (appendix III).

3.9.3. Plasma Rennin activity:

Plasma rennin activity was measure using DRG Renin ELISA Kit, a solid phase Enzyme- linked immunosorbant assay (ELISA) based on sandwich principle.

3.9.3.1Principle:

The microtiter wells are coated with a monoclonal [mouse] antibody directed towards a unique antigenic site of the human active Renin molecule. An aliquot of specimen sample containing endogenous Renin is incubated in the coated well together with Assay Buffer. After incubation, unbound components were washed off. Finally, Enzyme Conjugate, which is a monoclonal anti-Renin antibody conjugated with horseradish peroxidase, is added, and after incubation, unbound enzyme conjugate is washed off. The amount of bound peroxidase is proportional to the concentration of Renin in the sample. Having added the substrate solution, the intensity of color developed is proportional to the concentration of active Renin in the specimen sample (Drg-diagnostics, 2013).

3.9.3.2 Procedure:

The reagents and samples were allowed to reach room temperature. The desired numbers of microtiter wells were secured in the frame holder and 150µl of the assay buffer were dispensed in all wells, then 50 µl of each standards, controls and samples were added into appropriate wells and incubated for 90 minutes at room temperature on a plate shaker with – 700rpm.After that the microtiter plate was washed 3times with 300 µl diluted wash buffer. 100 µl of enzyme conjugate was added into each well

and incubated for 90 minutes at room temperature on a plate shaker with 700rpm. After the incubation time was completed the microtiter plate was washed with 300 µl diluted wash buffer, then100 µl of substrate solution was added to each well. In order to stop the enzymatic reaction 100 µl of stop solution was added to each well.Finally the optical density of each well was measured at 450nm, using microtiter plate reader (appendix IV).

3.9.3.3. Calculation:

The average absorbance values of each set of standards, controls and patient samples were calculated. Standard curve was constructed using a linear graph paper by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis .The concentration of the samples were obtain directly from this standard curve.

3.9.4. DNA Extraction:

Genomic DNA was isolated using salting out method (Jawdat *et al.*, 2011)

3.9.4.1. Procedure:

2.5ml of EDTA blood was thawed at room temperature and then transferred to sterile polypropylene tube, then diluted with 5ml of phosphate buffer saline and mixing by inverting the tube and centrifuged at 3000rpm for 10 minutes. The supernatant was powered off .The pellet (reddish) was resuspended with 12.5 ml of sucrose Triton X 100 lysing buffer. The mixture was vortex and placed on ice for 5 minutes, then was spuned for 5minutes at 3000 rpm in TH4 rotor and the supernatant was powered off. The pellet (pinkish or white) is resuspened in 1.5 ml of T20E5, 100µl of 10% SDS is add to final concentration of 1% then 20 µl of 10mg/dl proteinase K is add the mixture was mixed by inversion after adding each solution then the samples are incubated at 45 ċ over night.0.5 ml of saturated Nacl was added to each sample and mixed vigorously for

15 seconds then it was spun for 30 minutes at 3000 rpm. A white pellet was formed which was consisting of protein preceipted by salt, the supernatant that contained the DNA was transferred to a new tube. Precipitation of DNA was achieved by adding 4ml of absolute alcohol, the solution was agitated gently and DNA was spooled off and transferred to eppendorf tube. The DNA was washed in1ml 70% ice cold alcohol, air dried and was dissolved in 100µl of TE and stored over night to dissolve (Jawdat *et al*., 2011).

3.9.5 Gel Electrophoresis:

3.9.5.1 Principle:

DNA has a negative charge in solution; therefore it migrates to the positive electrode in an electric field. In agarose gel electrophoresis DNA is forced to move through a sieve of molecular proportions resulting in a slower migration of larger molecules. The ethidium bromide dye in the gel intercalates between the base pairs of the dsDNA resulting in an intense orange fluorescence. This fluorescence may be visualized as bands, under UV light; at the point the DNA has migrated (Arti and Ayyagari, 2007).

3.9.5.2 Procedure:

Three micro litters of extracted DNA was mixed with 2µL (6X) loading dye and loaded on 1.5% Agarose gel. The gel was prepared by dissolved 1.5 gram of agarose (promega, USA) in 100ml (1X) tris borate EDTA (TEB) and heated until boiling using a microwave oven. Electrophoresis was performed using (1X) TBE buffer at 100 volte at constant voltage, for 20 minutes (Appendix VI). The gel was stained with 4 µL ethidium bromide (0.5 µg/ mL) and visualized by UV transluminar JY-025 (Beijing, China). The gel was photographed by gel documentation system. The size of the DNA bands was determined by comparing with 1 Kb DNA ladder (Arti and Ayyagari, 2007).

3.9.6. PCR amplification:

Polymerase chain reaction (PCR) was used to amplify a 303 base pair fragment in AGT gene (exon 2) in chromosome (1) using synthetic oligonucleotide primers complementary to the DNA sequence of these regions. The forward primer strand5′-GAT GCG CAC AAG GTC CTG TC-3′ 3', and-reverse-strand 5′-CAG GGT GCT GTC CAC ACT GGA CCC C-3′ (Laura *et al.*, 2008) were synthesized by the Trilink Biotechnology (San Diego California, USA). d NTPs was obtained from Intron Biotechnology (South Korea), PCR amplifications were conducted a thermocycler Alpha laboratories. (UK).

Amplification was performed in 20µL a total reaction volume. Each reaction 20µLPCR reaction contained 2.5mM dNTPs, 2.5 U Taq DNA polymerase, 1µL genomic DNA template (200ng), 1µL of each primer (10pmol/µL), and 17µL nuclease free water. PCR was carried out under the following conditions: denaturing at 94ºC for 2min, followed by 30 cycles at 94ºC for 20 s, annealing at 57for 30 s, extension at 72ºC for 30 s, and a final extension at 72º C for 5 min.

The amplified fragments of 303bp electrophoresis on 1.5% Agrose gel, and visualized under UV transelminater JY-025 (Beijing, China). (Appendix VI)

3.9.7. Restriction fragment length polymorphism (RFLP):

A standard restriction enzyme analysis consist of the following component to a final volume of 50 µL; 10 µL of PCR product, 1µL (4U) restriction enzyme TthIII I (New England Biolabs, UK), 5 µL of 1X buffer and 34 µL from DW. The mixture was incubated at 65C°for 1 hour (Appendix VII).

3.9.8. RFLP product gel electrophoresis:

After incubation was completed the RFLP product was carried out in 2 % Agarose gel electrophoresis with1XTBE buffer and 5 µl ethidium

bromide staining. 20 µL of RFLP products was mixed with 4 µl of blue dye (6X) and disposed in the holes. 5 µL molecular weight markers 1 kb also was disposed in one well. A voltage of 120 volts was applied during 30 min of the run. Gel was observed under UV and the size of RFLP product was compared with the molecular marker and photographed.

3.10. Quality control:

The precision and accuracy of all methods used in this study were checked each time a batch was analyzed by including commercially prepared control sera.

3.11. PCR reaction QC:

For all PCR reaction positive and negative control were run parallel to each test sample. Positive control was performed by applying all reaction mixture except for the template DNA, and used other known DNA template. Negative control was performed by applying all reaction mixture except for the template DNA to ensure the amplification quality Figure (4.1)

3.12. Statistical analysis

SPSS software (version 16) was used for analysis of clinical variables. Descriptive statistics were used to analyze all variable studies such as the demographic characteristics data were summarized as mean ± SD or present. Variables were compared between preeclampsia and control group by Student's t test and ANOVAs method, p value of < 0.05 is considered to be significant. Liner regression analysis was used to assess correlation between the plasma renin activity and MAP, maternal age respectively. Genotype and allele frequencies in control and preeclampsia groups were compared by Chi-square ($\chi 2$) analysis. Statistical significance was accepted at $p<0.05$.Odds ratio was used for measurement of association.

4. Results

4.1. Demographic data of the study group:

The study was done in Khartoum state during the period of April 2012 to April 2014. A total of 100 subjects recruited in the study (50 with preeclampsia as study group and 50 normotensive pregnant women as control group). All patients were primarily from Khartoum. Blood samples from100 age, sex, gestational age, and race matched, for comparison as in (Table 4.1).

4.2. Maternal age, blood pressures, serum creatinine and proteinuria in preeclampsia and normotensive pregnant women:

In Preeclampsia group (n = 50) the mean±SD maternal age ;(28.50 ± 6.382years) versus (25.26 ± 6.127 years) for the control group (n = 50), (P = 0.509). Mean of systolic blood pressures (SBP); (141.48 ± 20.721 mmHg) versus (115.20 ± 2.483mmHg), (p = 0.00). Diastolic blood pressure (DBP) (100.62 ± 17.012 mmHg); (74.86 ± 2.603 mmHg), (p = 0.00). Mean arterial blood pressure (MAP); (141.48 ± 20.72mmHg) versus (100.62 ± 17.01mmHg) for the control group, (P= 0.00).Serum creatinine ;(0.7740 ± 0.02962 mg/dL), versus (0.8332 ± 0.08970 mg/dL) for the control group, (P = 0.00) table (4.2). Proteinuria was more than one cross in all patients, while it was not detected among the control group as in table (4.1).

4.3. Genotypes and allele frequency of AGT M235T:

Tth111I restrictions enzyme digested the fragment into 2 parts, the longer fragment, 279 bp and the shorter 24 bp. However, the 2% agarose gel was unable to retain the shorter fragment and it was suspected to have migrated out of the gel. Therefore, a band at 303 bp indicates homozygous wild-type (MM), a band at 279 bp indicates homozygous mutated (TT)

and two bands at 303 bp and 279 bp indicates heterozygous mutation (MT).figure (4.2)

The frequency AGT M235T missense mutation in all the subjects was 48 for homozygous mutation (37preeclampsia and 11 control group), 26 for heterozygous mutation (9 preeclampsia and 17 for control group), and 26 for homozygous wild-type (4 preeclampsia and 22 control). In preeclampsia group 92% had M235T (n=46) (9 were heterozygous and 37were homozygous), compared with 56% (n=28) in control group (17 heterozygous, 11 homozygous).

In preeclampsia group the frequency of M allele; 26% (n=13) versus 78% (n=39) for the control group, while the frequency of T allele in preeclampsia group; 92% (n=47) versus 56% (n=28) for the control group as in table (4.3) fig (4, 3).In preeclampsia group the frequency of the TT genotype; 74%(n=37) versus 22 (n=11). The risk for preeclampsia was X^2 = 16.39 odd ratio =2.597 P value= 0.000 for TT compared to MM genotype as in table (4. 3).

4.4. Plasma renin activity in preeclampsia and normotensive pregnant women:

Serum renin activity in patients whom had TT genotype; (51.63 ± 5.214 pg/ml) versus (82.00 ± 14.022 pg/ml) for the control group (p=0.00), MM genotype; (30.37±4.205pg/mL) versus (62.92 ± 2.300pg/mL) for control group (p=0.00), and MT genotype; (45.99 ± 1.779pg /mL) versus for (67.78 ± 0.972 pg/mL) control group (p= 0.000) as in table (4. 4).

There was significant positive correlation between plasma renin activity and MAP (r = 0.9, P= 0.000) as in fig (4.4), while there was no statistical significant between plasma renin activity and age in preeclampsia group (r = 0.044, P = 0.759) as in fig (4.5).

Table (4.1): Demographic and clinical data of the study group

Characteristic	Normotensive pregnant	Preeclampsia pregnant
Maternal Age/(years)	25.26 ± 6.127	28.50 ±6.382
Gestational age/(weeks)	27.08±4.68	32.04 ± 2.66
Race: Afro-asiatic Nilo-Saharan Niger-Kordofanian	 40(80%) 5(10%) 5(10%)	 27(54%) 3(6%) 20 (40%)
Parity: Primigraved Multparity	 19(38.0%) 31(62.0%)	 19(38.0%) (31)62.0%
Proteinuria (±)	Negative(100% -)	Positive (72%++,28%+++)

The table shows the mean ± SD and percent (%)

Table (4.2): Comparison of the mean of age, diastolic blood pressure (DBP), systolic blood pressure (SPB), mean arterial pressure (MAP), plasma creatinine (S.cr), and plasma renin activity in preeclampsia compared to control.

Variable		**Means ±SD**	**P value**
Age (Years)	Cases	28.50 ±6.382	0.509
	Control	25.26 ± 6.127	
DBP (mmHg)	Cases	100.62±17.012	0.000*
	Control	74.86±2.603	
SBP (mmHg)	Cases	141.48±20.721	0.000*
	Control	115.20±2.483	
MAP (mmHg)	Cases	114.350±16.878	0.000*
	Control	88.270±2.1781	
S.cr(mg/dL)	Cases	0.8332±.08970	0.000*
	Control	0.7740±.02962	
Renin(pg/ml)	Cases	40.3560±9.92332	0.000*
	Control	77.9140±13.95512	

The table shows the mean± SD and probability (P).

t-test was used for comparison.

* P value ≤ 0.05 is considered significant

Table (4.3):

Genotype and alleles distribution of AGT M235Tin Sudanese women with preeclampsia and control group.

Group	No	Genotypes			Alleles	
		MM	MT	TT	M	T
Control	50	44% (n=22)	34% (n=17)	22% (n=11)	78% (n=39)	56% (n=28)
Cases	50	8% (n=4)	18% (n=9)	74% (n=37)	26% (n=13)	92% (n=46)
Total	100	26	26	48		
X^2 = 16.39 odd ratio =2.597 df=1 P value= 0.000 *						

Chi squire test was be used

*P value ≤ 0.05 is considered significant

Table (4.4):

Comparison between Plasma renin activity levels according to genotype of AGT gene in Sudanese patients with preeclampsia and control

Genotype	Number	Means ±SD (pg/ml)	P value
MM			
cases	4	30.37±4.205	0.00*
control	22	62.92±2.300	
MT			
cases	9	45.99±1.779	0.00*
control	17	67.78±0.972	
TT			
cases	37	51.63±5.214	0.00*
control	11	82.00±14.022	

The table shows the mean ± SD and probability (P).

t-test was used for comparison.

* P value ≤ 0.05 is considered significant

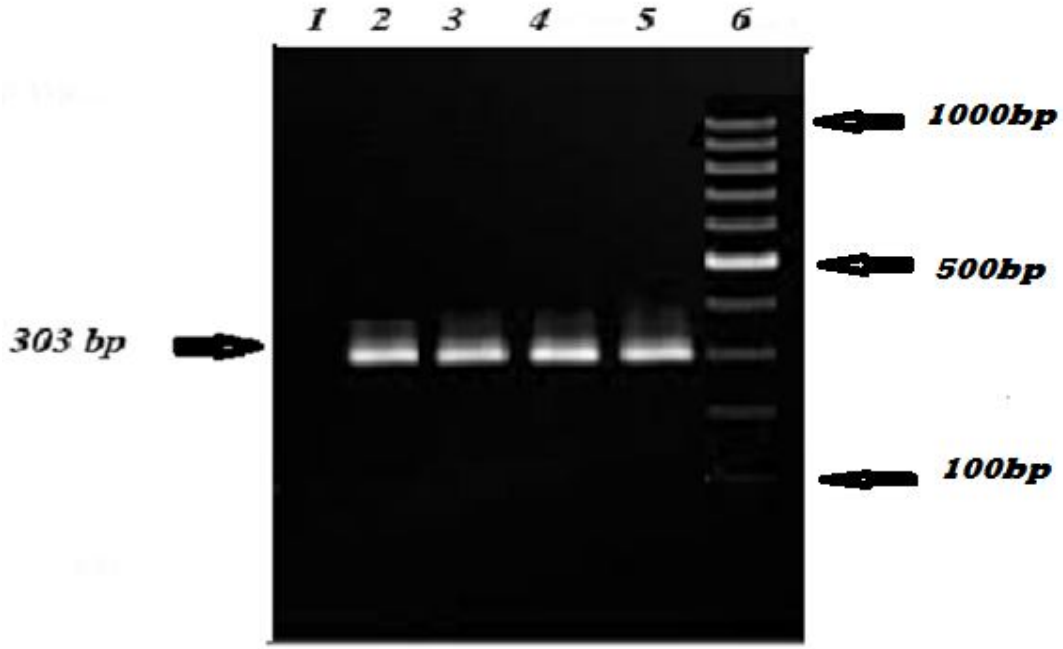

Figure (4.1):1.5% Agarose gel electrophoresis illustrating the amplification of 303 bp PCR product of AGT ,**Lane**(1) Negative Control, **Lane**(**2,3,4**) Samples PCR product ,**Lane(5)** Positive Control, **Lane (6) a 100 bp DNA ladder (Solis BioDyne)**

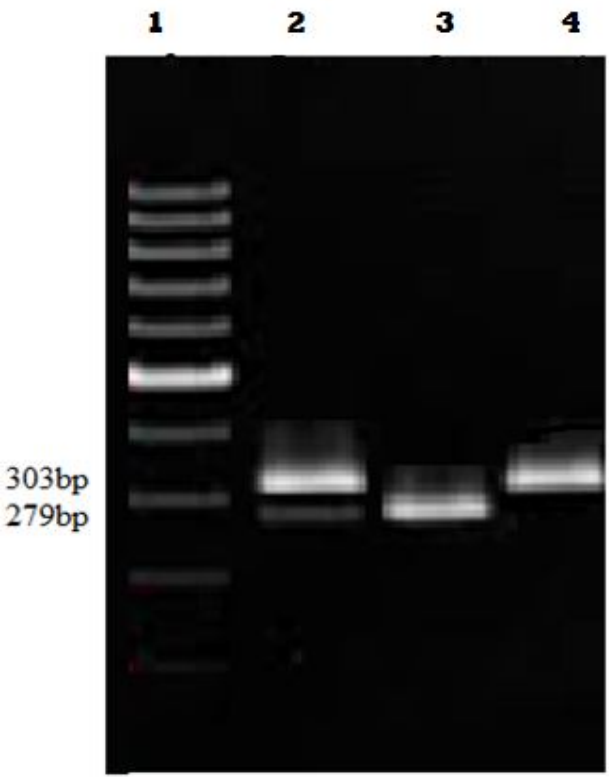

Figure (4.2): 2% Agarose gel electrophoresis illustrating the amplification of 303 bp in the angiotensinogen gene and enzymatic digestion of this fragment with Tth111I restrictions endonuclease enzyme. Lanes (2, 3, 4) correspond to RFLP pattern ,

Lane:

1-100 bp DNA ladder (Solis BioDyne), (Appendix V)

2-heterozygous (MT), (fragments of 303bp and 279bp)

3-homozygous mutant (TT), (fragments of 279bp)

4-homozygous wild-type (MM), (undigested fragment of 303bp)

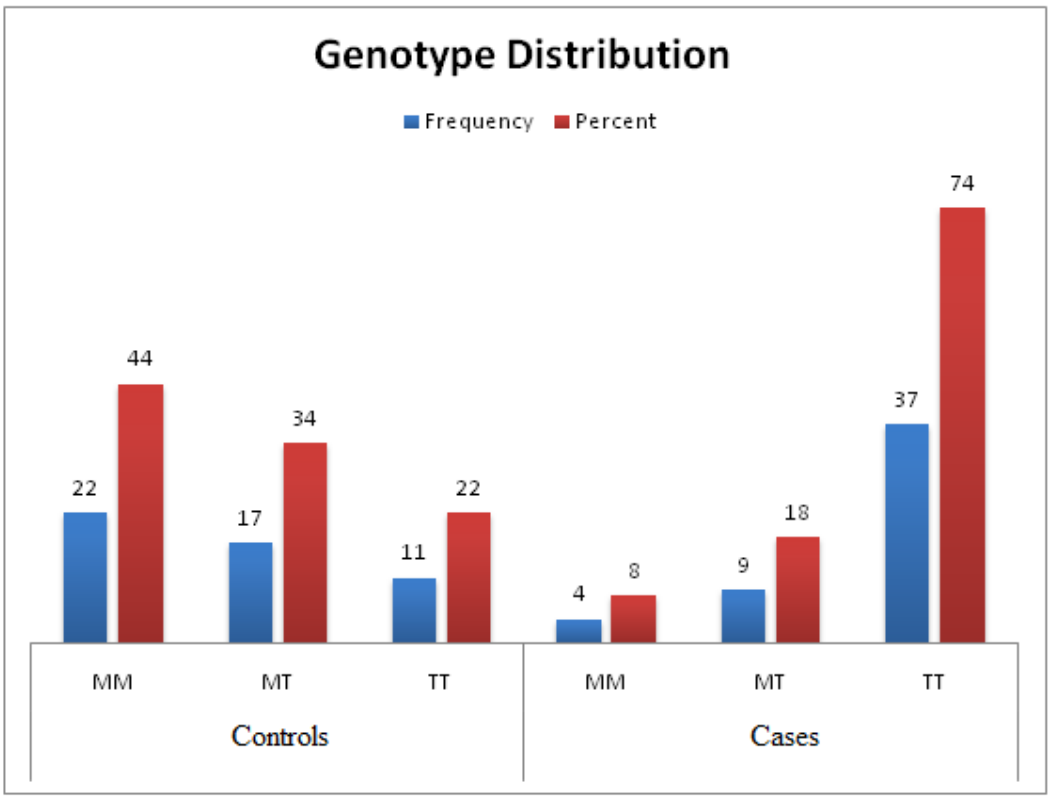

Figure (4.3): shows distribution of AGT gene genotypes in Sudanese patients with preeclampsia and control group.

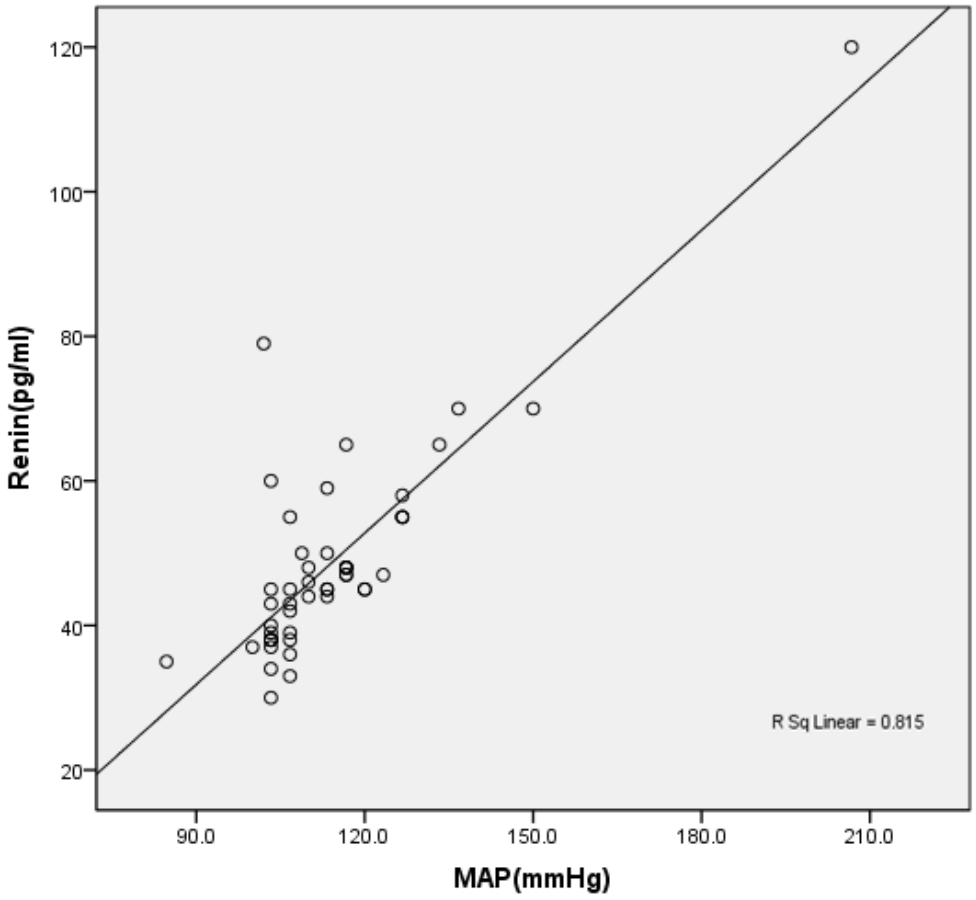

Figure (4.4): Scattered plot shows significant positive correlation between mean arterial pressure mmHg (as a measure of PE severity) and plasma rennin activity Pg/ml (P=0.00, r= 0.902).

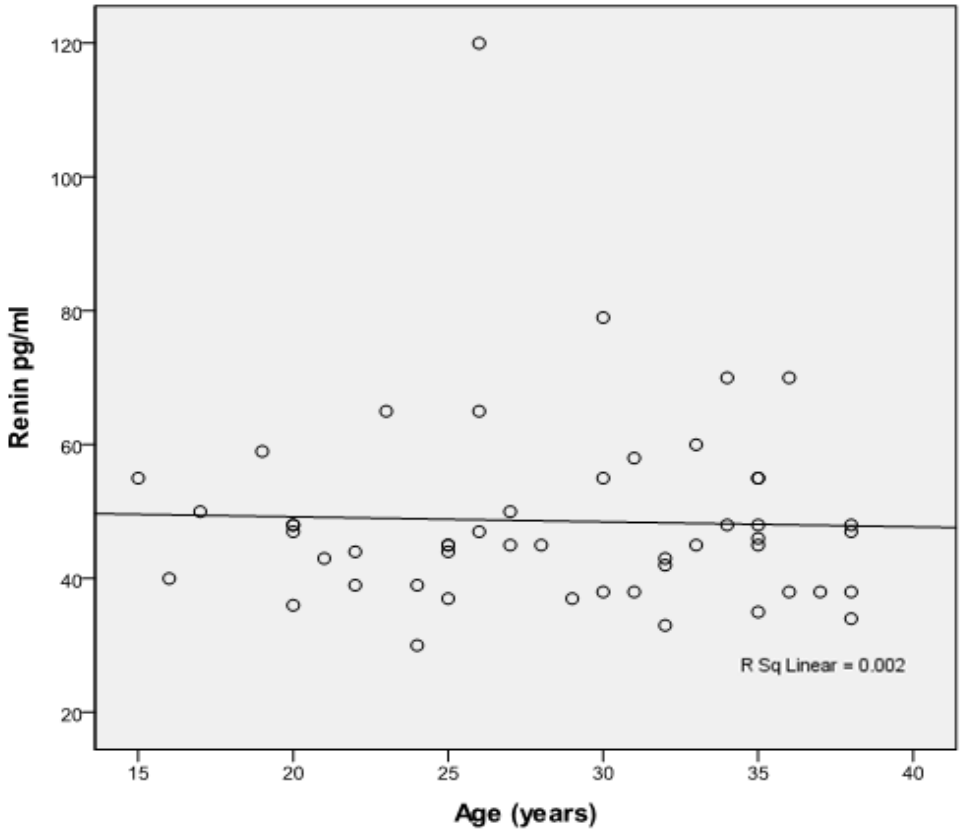

Figure (4.5): Scattered plot shows insignificant correlation between mean of age (years) and plasma rennin activity (Pg/ml) (P= 0.759, r = 0.044)**.**

5. Discussion

5.1 Discussion

Pre-eclampsia (PE) affects 5–7% of all pregnancies and is one of the leading causes of maternal mortality (Shagun, 2011). It is believed to be a multifactorial disorder with a strong genetic component;. It contributes to 16% of maternal and 23% of perinatal deaths worldwide. In developing countries like India, where other causes of maternal mortality predominate, PE is believed to be responsible for 13–15% of maternal deaths (Khan *et al.*, 2006).

PE is a significant obstetric problem in Sudan, however, the contribution of genetic polymorphisms to PE have not been well studied in this country. The present study showed that preeclampsia occurs at maternal age range from 15 to 38 years with mean (28.50 ±6.382years) this result was in agreement with veslloso *et al.*, 2007 who observed that preeclampsia appears almost at same maternal age (veslloso *et al.*, 2007), maternal age was considered to be risk factor for preeclampsia especially in young teenagers and women over 35 years (WHO, 2008). Nationwide US data suggest that the risk of pre-eclampsia increases by 30% for every additional year of age past 34(Duckitt *et al.*, 2005).

Our study showed 62% preeclampsia patients had multiparty. Epidemiological and family based studies in several geographically and ethnically distinct populations indicate that preeclampsia is a multifactorial disorder with a familial tendency and it is influenced by race, parity, health status of placenta, diet and body size. First degree relatives of women with preeclampsia have a fivefold increased risk while second degree relatives have twofold increased risk of having the disease compared with women who have no family history (Bouba *et al.*, 2003)

The study showed significant increase in SBP, DBP and MAP of test group compared to control group this agrees with other studies done by Bouba *et al., 2003* and Chio *et al.*, 2004.

In the current study the mean of plasma renin activity (PRA) value among preeclampsia was significant lower than that of control group this result was inagrement with Laskowska *et al.*,2004 and Irani and Xia,2011; in addition there was a positive significant correlation between PRA and MAP. This finding has also been detected in previous studies. The absence of stimulation of the RAS in women developing hypertension during pregnancy or in women with preeclampsia has been confirmed by many studies (Bouba *et al.,* 2003 and August *et al.*, 1990).

The entire renin–angiotensin axis is affected in established preeclampsia, in particular, plasma renin concentrations are markedly decreased and there has been considerable recent interest in the possible role of alteration in the RAS in the pathophysiology of preeclampsia. There is extensive evidence that plasma renin activity, plasma renin concentration, angiotensin II, angiotensinogen and plasma urinary aldosterone levels are all lower in preeclampsia compared with normotensive pregnant women (Bouba *et al.*, 2003). Distribution of allele and genotypes of AGT *M235T* gene revealed increased significant differences in frequency of T allele and TT genotype in pregnant women with preeclampsia compared with control group. This result was in agreement with (Song *et al.*, 2013) who studied the AGT polymorphism in Chinese women and state; compared to the controls, the AGT homozygous of TT genotype in PE occurred significantly more frequently and the T allele was observed to occur more frequently than the M allele ($p < 0.05$). In contrast, (Choi *et al.*, 2004) found there was no significant difference in the genotype and allele of angiotensinogen *M235T* in the tow group.

In our study there was a positive risk of developing preeclampsia when having TT genotype, and the results were highly statistically significant for TT genotype compared to MM genotype, these results was agree with the results of Radkov *et al*., 2013 who found that the TT genotype or T allele of angiotensinogen *M235T* gene polymorphism was associated with the risk of preeclampsia.

In our study maximum renin activity in the preeclampsia was noted in carriers of TT genotype of AGT gene *M235T* polymorphism as compared to other genotypes. Despite the fact that plasma renin activity decreased in preeclampsia, an increase of its gene expression in the placental tissue and reciprocal inhibition of circulating RAAS activity were noted (Herse *et al*., 2007; Anton and Brosnihan, 2008).

The development of preeclampsia in TT genotype carriers was associated with maximum activity of plasma renin. Heterozygous genotype of the AGT gene *M235T* polymorphism in pregnant women with hypertension was associated with a 1.13-fold decrease in renin activity, and MM genotype with a1.7-fold decrease (P=0.019) as compared to the TT genotype, this are agree with study done by Radkov *et al*.,2013. This specific feature distinguishes preeclampsia from arterial hypertension in non-pregnant individuals, when TT genotype is associated with maximum angiotensinogen concentration and minimum plasma renin activity (Radkov *et al.,* 2013).

5.2. Conclusion:

In conclusion, plasma renin activity was significantly decreased in preeclampsia. An increased risk for preeclampsia in women carrying the TT genotype of the AGT gene was observed.

5.3. Recommendations:

1. In all pregnant ladies arterial blood pressure should be monitored regularly throughout the pregnancy to achieve control in order to minimize development of preeclampsia.
2. The AGT TT genotype may be used as a marker for susceptibility of preeclampsia and further studies with large number of patients are needed to confirm this hypothesis.
3. Plasma renin activity can be measured in pregnant woman to give an idea about the dysregulation of RAS and may help in the diagnosis of preeclampsia.
4. Further studies on the components of RAAS are required in order to identify alteration which might contribute to clarification of understanding of preeclampsia
5. Most molecular genetic studies of preeclampsia to date have focused on maternal susceptibility genes, maternal-fetal interactions have attracted ever increasing attention and will be the direction of future studies

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Appendix I

Sudan University of Science & Technology
College of Graduate Studies
Questionnaire
Assessment of Plasma Renin and Angiotensinogen*M235T* gene polymorphism among Sudanese Pregnant Women with Preeclampsia

General information

Name…….No……..

Sex; female

Maternal age…….

Residence …….

Race……

Mobile No……..

Clinical information

Blood pressure …….

Gestational age…….

Gestational type

Parity; yes ( ) NO ( )

Single gestation yes ( ) NO ( )

Multiple gestation yes ( ) NO ( )

Family history of preeclampsia yes ( ) NO ( )

Investigations:

Urine protein……

S.creatinine……. (mg/dl)

Plasma renin activity …….. (pg/ml)

Angiotensinogen genotype……

Appendix II

A-Series

Urinalysis Reagent Strips User's Guide

Rev:02/2012

This User's Guide is to be used with the following products:
DIRUI A10/ 9 Items/8 Items/7 Items/5 Items/4 Items(glucose, protein, pH, blood)/4 Items (glucose, protein, pH, SG)/ 3 Items/ 2 Items (Protein-pH)/ 2 Items (bilirubin-Urobilinogen)/2 Items (Glucose-Ketone) /2 Items (Glucose-Protein) / Glucose/ Ketone/ Bilirubin/ Protein/ Blood/ Urobilinogen

General Summary:
This guide instructs the methods, reaction principles and points for attention for the use of DIRUI A Series of Reagent Strips.
DIRUI A Series of Reagent Strips are made for urinalysis of both qualitative and semi-quantitative, which are in vitro reagent for diagnostics. It tests Leukocytes, Nitrite, Urobilinogen, Protein, pH, Blood, Specific Gravity, Ketone (acetoacetic acid), Bilirubin, and Glucose in urine. Please refer to the out-side box carton and bottle label for the specific test parameters of the product you are using.
The strips are for professional use only.
The results on the strips can be read visually and instrumentally.
You are required to read the User's Guide before taking use of the strips.

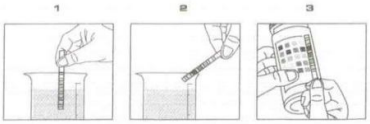
Collecting and Preparing Specimen
Collect fresh urine in a clean and dry container. Don't centrifuge the urine. Mix the sample well before taking the test. The urine test must be taken within two hours. All specimens must always be taken and kept under sanitary conditions.

Note: Water should not be used as negative control liquid. The preservatives will not prevent the deterioration of ketones, bilirubin or urobilinogen. The growth of bacteria in the long-term storage specimen may affect the test results on glucose, pH, nitrite and blood.

Visual Reading Technique
1. Immerse the reagent area of the strip in the urine specimen and take it up quickly and immediately.
2. Run the edge of the strip against the rim of the container to remove the excess urine.
3. Hold the strip up horizontally and compare the result on the strip with the colour chart on the bottle label closely. Make note of the result. For a semi-quantitative result, take the result according to the time specified on the colour chart. The pH and Protein can be read at any time within 60 seconds after dipping. For a qualitative result, the strip should be read between 1-2 minute after dipping. If a positive result is obtained, repeat the test and compare with the colour chart at the specified time . Colour changes beyond 2 minutes are of no diagnostic value.

Instrumental Reading Technique
Follow the directions given in appropriate instrument-operating manual.

STORAGE
The strips must be kept in the original bottle. Never use the products after the expiration date. Every strip can be used only once. Do not remove the desiccant(s). Don't take the strips from the bottle if it is for immediate use. Cap the bottle immediately and tightly after taking out the strips. The strips should be stored in dry place at the temperature between 2℃-30℃. Don't store the strips in refrigerator and keep them away from direct sunlight. Do not touch the reagent area of the strip. PROTECTION AGAINST AMBIENT MOISTURE, LIGHT AND HEAT IS ESSENTIAL TO GUARD AGAINST ALTERED



REAGENT REACTIVITY. Deterioration may result in discoloration or darkening of the reagent area of the strip. If all these happen, or the test results are questionable or inconsistent with the expected results, check and make sure the strips are within the expiration date and also compare with the control urine. Please dispose the used strips as wastes according to Treatment Regulations Of Lab Biohazard Materials .

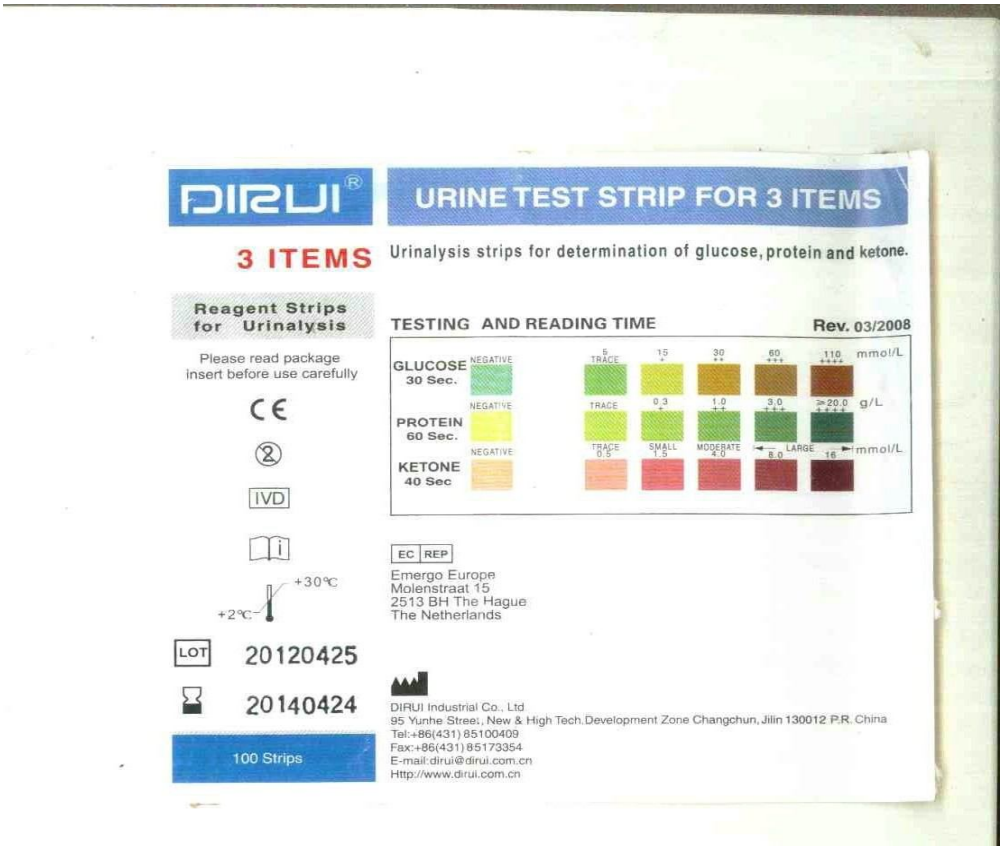
Limitation of Procedures
Like all the other laboratory tests, definitive diagnostic or therapeutic decisions should not be made or based on any single result or method.

Reaction Principles
Glucose: The glucose oxidized by glucose oxidase catalyzes the formation of glucuronic acid and peroxide hydrogen. Peroxide hydrogen releases neo-ecotypes oxide [O] under the function of peroxidase. [O] oxidizes iodide potassium , which makes the colour changes.
Bilirubin: The direct bilirubin and dichlorobenzene diazonium produce azo dyes in a strongly acid medium.
Ketone: The acetoacetate and sodium nitroprusside cause reaction in alkaline medium, which produces violet colour.
Specific Gravity: Electrolyte (M^+X^-) in the form of salt in urine reacts with poly methyl vinyl ether and maleic acid(-COOH),which are weak acid ionic exchanger. The reaction produces hydrogenous ionogen, which reacts with pH indicator that causes the colour change.
Blood: Hemoglobin acts as peroxidase. It can cause peroxidase release neo-ecotypes oxide(O). (O) oxidizes the indicator and make the colour change subsequently.
pH: The method of pH indicator is applied.
Protein: This is based on the protein-error-of-indicator principle. Anion in the specific pH indicator attracted by cation on protein molecule makes the indicator further ionized, which changes its colour.
Urobilinogen: This test is based on the Ehrlich reaction in which p-diethylamino benzaldehyde in conjunction with a colour enhancer reacts with urobilinogen in a Strongly acid medium to produce a pink-red colour.
Nitrite: Nitrite in the urine and aromatic amino sulphanilamide are diazotized to form a diazonium compound. The diazonium compound reacting with tetrahydro benzo(h) quinolin 3-phenol causes the colour change.
Leukocytes: Granulocyte leukocytes in urine contains esterases that catalyze the hydrolysis of the pyrrole amino acid ester to liberate 3-hydroxy 5-pheny pyrrole. This pyrrole reacting with diazonium forms a purple colour.

Points for attention
Glucose
The test is for specificity of glucose. There is no false positive result occurred in reagent strip, caused by any substance in urine.
When the ascorbic acid concentration≥2.8mmol/L or acetoacetic acid concentration≥1.0mmol/L, the sample of glucose concentration is 3~7mmol/L may occur false negative result.
Bilirubin
Normally, even the most sensitive method can't detect bilirubin in urine. It is abnormal to have little bilirubin in urine, which requires further inspection. Medicines that dyes urine red and anything that shows red itself in an acid medium e.g., phenazopyridine may affect the test result. High concentration of the ascorbic acid may cause false negative result.
Ketone
The reagent strip reacts with acetoacetic acid in urine. It doesn't do with acetone or β-hydro butyric acid. Normal urine specimens usually conduct negative results in the test. False positive results may occur in highly pigmented urine or those containing a large amount of levodopa metabolites.
Specific Gravity
The reagent strip for Specific Gravity allows the urine specimens specific gravity between 1.000 and 1.030.In general, the mean error between the

DIRUI® DIRUI INDUSTRIAL CO.,LTD.
EC REP Emergo Europe Molenstraat 15 2513 BH The Hague The Netherlands

Appendix II


DIRUI®
URINE TEST STRIP FOR 3 ITEMS
3 ITEMS
Urinalysis strips for determination of glucose, protein and ketone.
Reagent Strips for Urinalysis
TESTING AND READING TIME
Rev. 03/2008
Please read package insert before use carefully
GLUCOSE 30 Sec.
NEGATIVE
TRACE
PROTEIN 60 Sec.
NEGATIVE
TRACE
KETONE 40 Sec
NEGATIVE
TRACE
SMALL
MODERATE
LARGE
mmol/L
g/L
mmol/L
IVD
+30℃
+2℃
EC REP
Emergo Europe
Molenstraat 15
2513 BH The Hague
The Netherlands
LOT 20120425
20140424
100 Strips
DIRUI Industrial Co., Ltd
95 Yunhe Street, New & High Tech.Development Zone Changchun, Jilin 130012 P.R. China
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E-mail:dirui@dirui.com.cn
Http://www.dirui.com.cn

Appendix III

SPINREACT

Creatinine

Jaffé Colorimetric - kinetic

Quantitative determination of creatinine
IVD

Store at 2-8ºC

PRINCIPLE OF THE METHOD

The assay is based on the reaction of creatinine with sodium picrate as described by Jaffé.
Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents.
The intensity of the color formed is proportional to the creatinine concentration in the sample[1].

CLINICAL SIGNIFICANCE

Creatinine is the result of the degradation of the creatine, component of muscles, it can be transformed into ATP, that is a source of high energy for the cells. The creatinine production depends on the modification of the muscular mass, and it varies little and the levels usually are very stable.
Is excreted by the kidneys. With progressive renal insufficiency there is retention in blood of urea, creatinine and uric acid.
Elevate creatinine level may be indicative of renal insufficiency[1,4,5].
Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

REAGENTS

R 1 Picric Reagent	Picric acid	17.5 mmol/L
R 2 Alkaline Reagent	Sodium hydroxide	0.29 mol/L
CREATININE CAL	Creatinine aqueous primary standard 2 mg/dL	

PRECAUTIONS

Sodium hydroxide: Irritant (Xi): R36/38: Irritating to eyes and skin. S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. S37/39: Wear suitable gloves and eye/face protection. S45: In case of accident or if you feel unwell, seek medical advices immediately.

PREPARATION

Working reagent (WR):
Mix equal volumes of R 1 Picric Reagent and R 2 Alkaline reagent.
The working reagent is stable for 10 days at 15-25ºC.

STORAGE AND STABILITY

All the components of the kit are stable until the expiration date on the label when stored tightly closed at 2-8ºC, protected from light and contaminations prevented during their use.
Do not use reagents over the expiration date.
CREATININE CAL Once open is stable up to 1 month when stored tightly closed at 2-8ºC, protected from light and contaminations prevented during their use.
Signs of reagent deterioration:
- Presence of particles and turbidity.
- Blank absorbance (A) at 492 nm ≥ 1.80.

ADDITIONAL EQUIPMENT

- Spectrophotometer or colorimeter measuring at 492 nm (490-510).
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

SAMPLES

- Serum or heparinized plasma[1].
  Creatinine stability: 24 hours at 2-8ºC.
- Urine[1]. Dilute sample 1/50 with distilled water. Mix. Multiply results by 50 (dilution factor):
  Creatinine stability: 7 days at 2-8ºC.

PROCEDURE

1. Assay conditions:
   Wavelength: ..... 492 nm (490-510)
   Cuvette: ..... 1 cm. light path
   Temperature ..... 37ºC / 15-25ºC
2. Adjust the instrument to zero with reagent blank.
3. Pipette into a cuvette:

	Blank	Standard	Sample
WR (mL)	1.0	1.0	1.0
Standard[(Note 1,2)] (µL)	--	100	--
Sample (µL)	--	--	100

4. Mix and start stopwatch.
5. Read the absorbance (A_1) after 30 seconds and after 90 seconds (A_2) of the sample addition.
6. Calculate: $\Delta A = A_2 - A_1$.

CALCULATIONS

$$\frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times 2 \text{ (Standard conc.)} = \text{mg/dL of creatinine in the sample}$$

Conversion factor: mg/dL x 88.4 = µmol/L.

QUALITY CONTROL

Control sera are recommended to monitor the performance of assay procedures: SPINTROL H Normal and Pathologic (Ref. 1002120 and 1002210).
If control values are found outside the defined range, check the instrument, reagents and calibrator for problems.
Each laboratory should establish its own Quality Control scheme and corrective actions if controls do not meet the acceptable tolerances.

REFERENCE VALUES[1]

Serum or plasma:

Male	0,7 - 1,4 mg/dL	≅ 61.8 – 123.7 µmol/L
Female	0,6 - 1,1 mg/dL	≅ 53.0 – 97.2 µmol/L

Urine: 15-25 mg/Kg/24 h

Male	10 - 20 mg/Kg/24 h	≅ 88– 177 µmol/Kg/24 h
Female	8 – 18 mg/Kg/24 h	≅ 71– 177 µmol/Kg/24 h

These values are for orientation purpose; each laboratory should establish its own reference range.

PERFORMANCE CHARACTERISTICS

Measuring range: From detection limit of 0.09 mg/dL to linearity limit of 15 mg/dL.
If the results obtained were greater than linearity limit, dilute the sample 1/2 with NaCl 9 g/L and multiply the result by 2.
Precision:

	Intra-assay (n=20)		Inter-assay (n=20)	
Mean (mg/dL)	1.06	3.58	1.03	3.31
SD	0.22	0.06	0.04	0.06
CV (%)	2.07	1.54	3.97	1.75

Sensitivity: 1 mg/dL = ΔA 0,03 A/min . mg/dL
Accuracy: Results obtained using SPINREACT reagents (y) did not show systematic differences when compared with other commercial reagents (x).
The results obtained using 50 samples were the following:
Correlation coefficient (r): 0.986
Regression equation: y= 0.975x + 0.047
The results of the performance characteristics depend on the analyzer used.

INTERFERENCES

Hemoglobin (1 g/L), Bilirrubin (55 mg/dL), interfere[1].
A list of drugs and other interfering substances with creatinine determination has been reported by Young et. al[2,3].

NOTES

1. Calibration with the aqueous Standard may cause a systematic error in automatic procedures. In these cases, it is recommended to use a serum Calibrator.
2. Use clean disposable pipette tips for its dispensation.
3. **SPINREACT has instruction sheets for several automatic analyzers. Instructions for many of them are available on request.**

BIBLIOGRAPHY

1. Murray R.L. Creatinine. Kaplan A et al. Clin Chem The C.V. Mosby Co. St Louis. Toronto. Princeton 1984; 1261-1266 and 419.
2. Young DS. Effects of drugs on Clinical Lab. Tests, 4th ed AACC Press, 1995.
3. Young DS. Effects of disease on Clinical Lab. Tests, 4th ed AACC 2001.
4. Burtis A et al. Tietz Textbook of Clinical Chemistry, 3rd ed AACC 1999.
5. Tietz N W et al. Clinical Guide to Laboratory Tests, 3rd ed AACC 1995.

PACKAGING

Ref: 1001111	Cont.	2 x 150 mL

Appendix IV

6 ASSAY PROCEDURE

6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipette tips for each standard, control or sample in order to avoid cross contamination.
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents are ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- As a general rule the enzymatic reaction is linearly proportional to time and temperature.

6.2 Test Procedure

Each run must include a standard curve.

1. Secure the desired number of Microtiter wells in the frame holder.
2. Dispense **150 µL** of Assay Buffer in all wells.
3. Dispense **50 µL** of each ***Standard, Control*** and **samples** with new disposable tips into appropriate wells.
4. Incubate for **90 minutes** at room temperature on a plate shaker with ~ 700 rpm.
5. Briskly shake out the contents of the wells.
   Rinse the wells **4 times** with **300 µL** diluted ***Wash Solution***. Strike the wells sharply on absorbent paper to remove residual droplets.
   Important note:
   The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Dispense **100 µL** ***Enzyme Conjugate*** in all wells.
7. Incubate for **90 minutes** at room temperature on a plate shaker with ~ 700 rpm.
8. Briskly shake out the contents of the wells.
   Rinse the wells **4 times** with **300 µL** diluted ***Wash Solution***. Strike the wells sharply on absorbent paper to remove residual droplets.
9. Add **100 µL** of ***Substrate Solution*** to each well.
10. Incubate for **15 minutes** at room temperature.
11. Stop the enzymatic reaction by adding **100 µL** of ***Stop Solution*** to each well.
12. Determine the absorbance (OD) of each well at **450 ± 10 nm** with a microtiter plate reader.
    It is recommended that the wells be read **within 10 minutes** after adding the *Stop Solution*.

6.3 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted or reported as > 128 pg/mL. For the calculation of the concentrations this dilution factor has to be taken into account.

Appendix V

Solis BioDyne
Data Sheet

100 bp DNA Ladder

Ready to Load

Cat. No.	Pack Size	Conc.
07-11-0000S	1.5 µg SAMPLE	0.1 µg/µl
07-11-00050	50 µg	0.1 µg/µl

For *in vitro* use only

Description:

The 100 bp DNA Ladder is a ready-to-use molecular weight marker suitable for DNA fragment size determination on gel electrophoresis. The 100 bp DNA Ladder is formulated to run accurately and to provide crisp band patterns. It contains bromophenol blue dye which serves as visual aid to monitor the progress of migration during agarose gel electrophoresis. The 100 bp DNA Ladder contains 12 discrete DNA fragments ranging from 100 bp to 3,000 bp.

bp	ng/10µl
— 3,000	108
— 2,000	147
— 1,500	116
— 1,000	77
— 800	62
— 700	54
— 600	46
— 500	150
— 400	60
— 300	56
— 200	60
— 100	60

Concentration:

0.1 µg/µl

Size range:

100 – 3,000 bp

No of Bands:

12

Recommendations:

For best results, please load 5-10 µl of the 100 bp DNA Ladder per well.

Storage solution:

10 mM EDTA, 10% glycerol, 0.015% bromophenol blue and 0.17% SDS.

Shipping and Storage conditions:

Shipping and storage for up to 9 months at room temperature has no detrimental effects on the quality of this reagent. -20°C is recommended for long term storage.

Safety warnings and precautions:

This product and its components should be handled only by persons trained in laboratory techniques. It is advisable to wear suitable protective clothing, such as laboratory overalls, gloves and safety glasses. Care should be taken to avoid contact with skin or eyes. In case of contact with skin or eyes, wash immediately with water.

Some applications this product is used in may require a license which is not provided by the purchase of this product. Users should obtain the license if required

Related products:

Product name	Pack size	Cat. No.
FIREPol® DNA Polymerase	500 U	01-01-00500
FIREPol® DNA Polymerase	1000 U	01-01-01000
FIREPol® DNA Polymerase	2000 U	01-01-02000
HOT FIREPol® DNA Polymerase	500 U	01-02-00500
HOT FIREPol® DNA Polymerase	1000 U	01-02-01000
dNTP SET *(100 mM)*	4 x 25 µmol	02-21-00100
dNTP SET *(100 mM)*	4 x 100 µmol	02-21-00400
dNTP MIX *(20 mM of each)*	20 µmol	02-31-00020
dNTP MIX *(20 mM of each)*	100 µmol	02-31-00100

Solis BioDyne, Riia 185a, 51014 Tartu, Estonia, tel: +372 740 9960, fax: +372 740 2079, e-mail: solis@sbd.ee, www.sbd.ee

Appendix VI

Maxime PCR PreMix Series

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) Cat. No. 25026(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

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

STORAGE

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

CHARACTERISTICS

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

CONTENTS

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

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

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

PROTOCOL

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

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

Example Total 20μl or 50μl reaction volume

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

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

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

SUGGESTED CYCLING PARAMETERS

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

EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

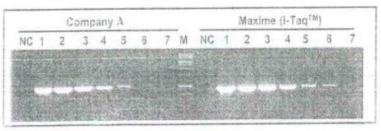


Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the cDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SIZer-1000 DNA Marker; lane 1, undiluted cDNA; lane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

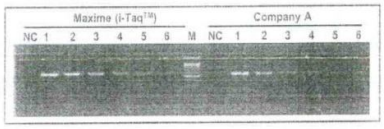
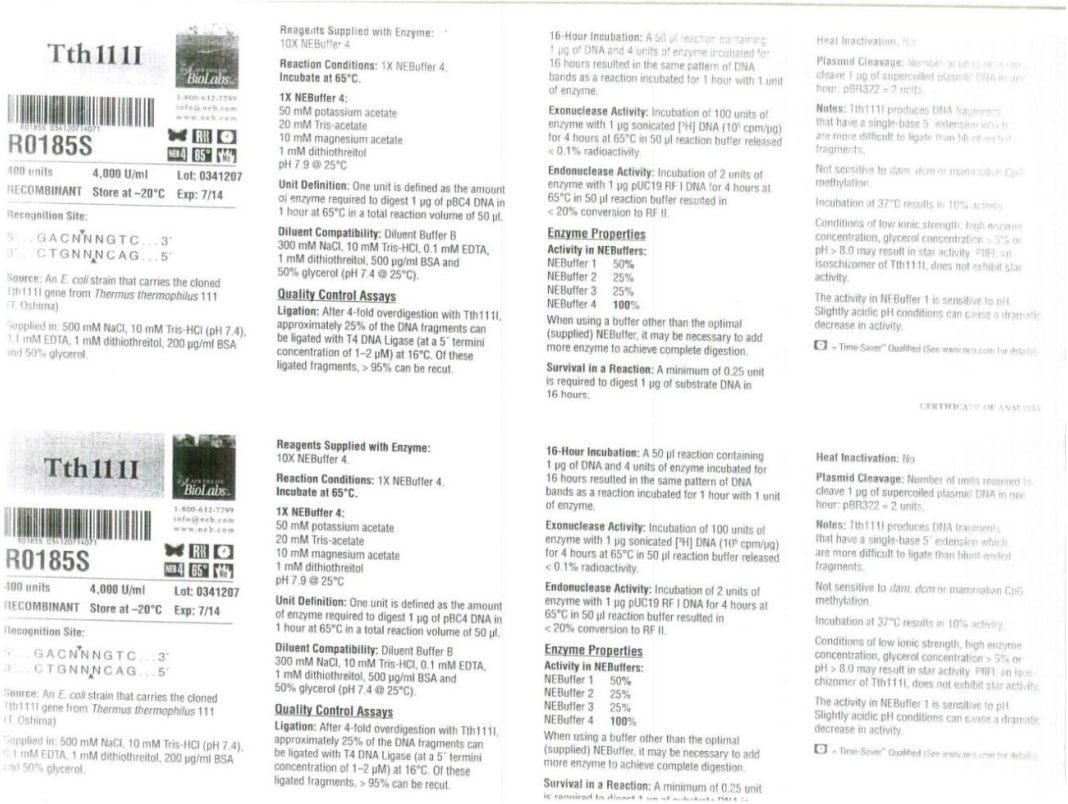


Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 579 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
Lane M, SIZer-100 DNA Marker; lane 1, undiluted cDNA; lane 2, 1/2 diluted cDNA; lane 3, 1/4 diluted cDNA; lane 4, 1/8 diluted cDNA; lane 5, 1/16 diluted cDNA; lane 6, 1/32 diluted cDNA; lane NC, Negative control

iNtRON BIOTECHNOLOGY

Appendix VII



Tth111I

BioLabs

1-800-612-7799
info@neb.com
www.neb.com

R0185S

RR
NEB4 65° ½t

400 units 4,000 U/ml Lot: 0341207
RECOMBINANT Store at –20°C Exp: 7/14

Recognition Site:

5′...GACNNNGTC...3′
3′...CTGNNNCAG...5′

Source: An *E. coli* strain that carries the cloned Tth111I gene from *Thermus thermophilus* 111 (T. Oshima)

Supplied in: 500 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 200 µg/ml BSA and 50% glycerol.

Reagents Supplied with Enzyme:
10X NEBuffer 4

Reaction Conditions: 1X NEBuffer 4.
Incubate at 65°C.

1X NEBuffer 4:
50 mM potassium acetate
20 mM Tris-acetate
10 mM magnesium acetate
1 mM dithiothreitol
pH 7.9 @ 25°C

Unit Definition: One unit is defined as the amount of enzyme required to digest 1 µg of pBC4 DNA in 1 hour at 65°C in a total reaction volume of 50 µl.

Diluent Compatibility: Diluent Buffer B
300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 µg/ml BSA and 50% glycerol (pH 7.4 @ 25°C).

Quality Control Assays

Ligation: After 4-fold overdigestion with Tth111I, approximately 25% of the DNA fragments can be ligated with T4 DNA Ligase (at a 5′ termini concentration of 1–2 µM) at 16°C. Of these ligated fragments, > 95% can be recut.

16-Hour Incubation: A 50 µl reaction containing 1 µg of DNA and 4 units of enzyme incubated for 16 hours resulted in the same pattern of DNA bands as a reaction incubated for 1 hour with 1 unit of enzyme.

Exonuclease Activity: Incubation of 100 units of enzyme with 1 µg sonicated [^{3}H] DNA (10^5 cpm/µg) for 4 hours at 65°C in 50 µl reaction buffer released < 0.1% radioactivity.

Endonuclease Activity: Incubation of 2 units of enzyme with 1 µg pUC19 RF I DNA for 4 hours at 65°C in 50 µl reaction buffer resulted in < 20% conversion to RF II.

Enzyme Properties

Activity in NEBuffers:
NEBuffer 1 50%
NEBuffer 2 25%
NEBuffer 3 25%
NEBuffer 4 **100%**

When using a buffer other than the optimal (supplied) NEBuffer, it may be necessary to add more enzyme to achieve complete digestion.

Survival in a Reaction: A minimum of 0.25 unit is required to digest 1 µg of substrate DNA in 16 hours.

Heat Inactivation: No

Plasmid Cleavage: Number of units required to cleave 1 µg of supercoiled plasmid DNA in one hour: pBR322 = 2 units.

Notes: Tth111I produces DNA fragments that have a single-base 5′ extension which are more difficult to ligate than blunt-ended fragments.

Not sensitive to *dam*, *dcm* or mammalian CpG methylation.

Incubation at 37°C results in 10% activity.

Conditions of low ionic strength, high enzyme concentration, glycerol concentration > 5% or pH > 8.0 may result in star activity. PflFI, an isoschizomer of Tth111I, does not exhibit star activity.

The activity in NEBuffer 1 is sensitive to pH. Slightly acidic pH conditions can cause a dramatic decrease in activity.

= Time-Saver™ Qualified (See www.neb.com for details)

CERTIFICATE OF ANALYSIS

Tth111I

BioLabs

1-800-612-7799
info@neb.com
www.neb.com

R0185S

RR
NEB4 65° ½t

400 units 4,000 U/ml Lot: 0341207
RECOMBINANT Store at –20°C Exp: 7/14

Recognition Site:

5′...GACNNNGTC...3′
3′...CTGNNNCAG...5′

Source: An *E. coli* strain that carries the cloned Tth111I gene from *Thermus thermophilus* 111 (T. Oshima)

Supplied in: 500 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, 200 µg/ml BSA and 50% glycerol.

Reagents Supplied with Enzyme:
10X NEBuffer 4.

Reaction Conditions: 1X NEBuffer 4.
Incubate at 65°C.

1X NEBuffer 4:
50 mM potassium acetate
20 mM Tris-acetate
10 mM magnesium acetate
1 mM dithiothreitol
pH 7.9 @ 25°C

Unit Definition: One unit is defined as the amount of enzyme required to digest 1 µg of pBC4 DNA in 1 hour at 65°C in a total reaction volume of 50 µl.

Diluent Compatibility: Diluent Buffer B
300 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 500 µg/ml BSA and 50% glycerol (pH 7.4 @ 25°C).

Quality Control Assays

Ligation: After 4-fold overdigestion with Tth111I, approximately 25% of the DNA fragments can be ligated with T4 DNA Ligase (at a 5′ termini concentration of 1–2 µM) at 16°C. Of these ligated fragments, > 95% can be recut.

16-Hour Incubation: A 50 µl reaction containing 1 µg of DNA and 4 units of enzyme incubated for 16 hours resulted in the same pattern of DNA bands as a reaction incubated for 1 hour with 1 unit of enzyme.

Exonuclease Activity: Incubation of 100 units of enzyme with 1 µg sonicated [^{3}H] DNA (10^5 cpm/µg) for 4 hours at 65°C in 50 µl reaction buffer released < 0.1% radioactivity.

Endonuclease Activity: Incubation of 2 units of enzyme with 1 µg pUC19 RF I DNA for 4 hours at 65°C in 50 µl reaction buffer resulted in < 20% conversion to RF II.

Enzyme Properties

Activity in NEBuffers:
NEBuffer 1 50%
NEBuffer 2 25%
NEBuffer 3 25%
NEBuffer 4 **100%**

When using a buffer other than the optimal (supplied) NEBuffer, it may be necessary to add more enzyme to achieve complete digestion.

Survival in a Reaction: A minimum of 0.25 unit

Heat Inactivation: No

Plasmid Cleavage: Number of units required to cleave 1 µg of supercoiled plasmid DNA in one hour: pBR322 = 2 units.

Notes: Tth111I produces DNA fragments that have a single-base 5′ extension which are more difficult to ligate than blunt-ended fragments.

Not sensitive to *dam*, *dcm* or mammalian CpG methylation.

Incubation at 37°C results in 10% activity.

Conditions of low ionic strength, high enzyme concentration, glycerol concentration > 5% or pH > 8.0 may result in star activity. PflFI, an isoschizomer of Tth111I, does not exhibit star activity.

The activity in NEBuffer 1 is sensitive to pH. Slightly acidic pH conditions can cause a dramatic decrease in activity.

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Appendix VIII

جامعة السودان للعلوم والتكنولوجيا
كلية الدراسات العليا
بحث لنيل درجة الماجستير في الكيمياءالسريرية
عنوان البحث

Assessment of Plasma Renin and Angiotensinogen *M235T*gene polymorphism among Sudanese Pregnant Women with Preeclampsia

اقرار المشاركين

لقد تم اختياري انا .....للمشاركة في الدراسة التي تقوم بها الدارسة/ **داليا ابراهيم وقيع الله** عن القابلية الوراثيه لحدوث تسمم الحمل عند النساء السودانيات.لقد تم توضيح اهداف الدراسة وخطوتها ، وعليه اقر بان مشاركتي طوعية وان المعلومات عن نتائج تحليل العينات خاصة وسريةوتستخدم فقط بغرض البحث. بناء علي ذلك وافقت علي المشاركة في الدراسة.

الاسم :.....

التوقيع : .....

التاريخ: .....

رقم الهاتف : .....